Fungal Pathogens of Insects: Cuticle Degrading Enzymes and Toxins

A. KEITH CHARNLEY

Department of Biology and Biochemistry, University of Bath, Bath BA2 7AY, UK

Advances in Botanical Research Vol. 40
incorporating Advances in Plant Pathology
ISBN 0-12-005940-1

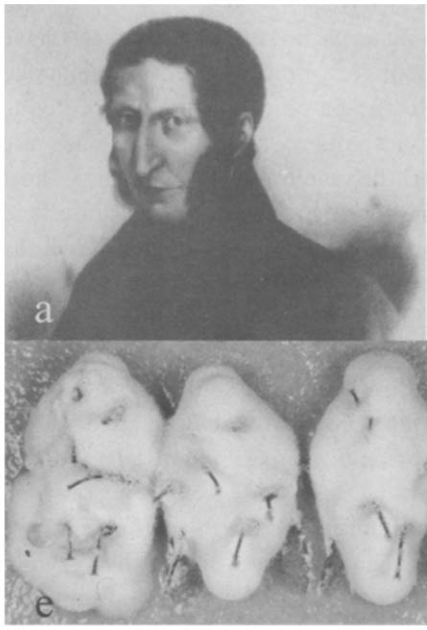
ABSTRACT

Pathogenic fungi are important natural regulators of insect populations. However, many attempts to harness their potential for pest control have met with comparatively minor commercial success. Studies on mechanisms of pathogenesis have yet to contribute to the development of more efficient mycoinsecticides, but new insights into the pathogenic process are laying the groundwork. Significant progress has been made in particular in understanding enzymes involved with the penetration of host cuticle and the role of insecticidal toxins. Insect cuticle comprises up to 70% protein and it is not surprising that extracellular fungal proteases appear to be particularly important in the penetration process. Subtilisins, chymotrypsins, trypsins and metalloproteases, usually with multiple isoforms of each, provide an impressive backed-up arsenal. Pathogenic fungi produce a wide variety of toxic metabolites, which vary from low molecular weight products of secondary metabolism to complex cyclic peptides and proteolytic enzymes. Comparatively few compounds have been found in diseased insects, in quantities sufficient to account for symptoms of mycosis. An exception, a family of cyclic peptides called the destruxins, are dealt with in detail. The potential for synergy between toxins is explored also.

I. INTRODUCTION

Insect fungal pathogens hold a special place in the study of microbial pathogenesis. Agostino Bassi's monograph in 1835 established for the first time that a microorganism (the fungus later to be called *Beauveria bassiana* in Bassi's honour) could cause an infectious disease in an animal (the silkworm, *Bombyx mori*) (see Fig. 1). Prophetically Bassi suggested further that microbes could be used to control pest insects (Bassi, 1835).

Natural epizootics of insect fungal diseases are comparatively common, and their impact on insect populations further demonstrates the potential of microbial pest control (Carruthers and Soper, 1987). This fact was recognised in the latter part of the 19th century and culminated in the seminal attempts by Metchnikoff and Paliokov to use the Deuteromycotina fungal pathogen *Metarhizium anisopliae* for insect control (Gillespie, 1988). Despite these and other early successes, synthetic chemical pesticides have been the mainstay of insect pest control for the last 50 years. However, the advent of insecticide resistance and concern over the environmental impact of agricultural inputs focus attention on biologically based forms of pest control. Mycoinsecticides have a toe-hold in a biological crop protection market (see e.g. Fig. 1c) dominated by the toxins from *Bacillus thuringiensis* (Bt) and crop plants transformed with Bt delta endotoxin genes (Charnley, 1997).



DEL MAL DEL SEGNO

CALCINACCIO o MOSCARDINO

Malattia che affligge

I BACHI DA SETA

E SUL MODO

DI LIBERARNE LE BIGATTAJE

ANCHE LE PIÙ INVETERATE

Opera

DEL DOTTORE AGOSTINO BASSI

DI LODI

la quale oltre a contenere molti utili precetti intorno al miglior governo dei Filugelli, tratta altresì delle Malattie

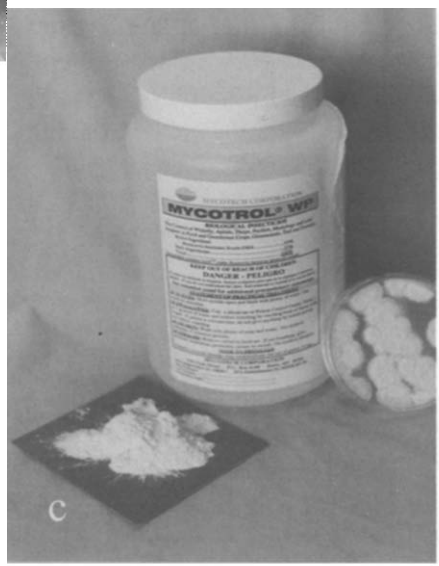
DEL NEGRONE E DEL GIALLUME

b

LODI

DALLA TIPOGRAFIA ORCESI

1835



ON THE MARK DISEASE,

CALCINACCIO OR MUSCARDINE,

A disease that affects

SILK WORMS

AND ON THE MEANS
OF FREEING THEREFROM
EVEN THE MOST DEVASTATED
BREEDING ESTABLISHMENTS

A Work

BY DR. AGOSTINO BASSI

OF LODI

Which, besides containing many useful precepts for the better management of silk worms, also treats of

THE BLACK AND YELLOW DISEASES

LODI

TIPOGRAFIA ORCESI

1835

d

Fig. 1. (a) Agostino Bassi*, (b) Frontise piece to Bassi's 1835 paper in Italian*, (c) A formulation of *Beauveria bassiana* developed by Myotech and now produced by Emerald BioAgriculture Corporation for control of whitefly, aphids and thrips on vegetables and ornamentals (with permission from S. Jaronski), (d). Frontise piece to Bassi's 1835 paper in English*, (e) *Beauveria bassiana* sporulating on cadavers of house flies (with permission from D. Steinkraus) *taken from Bassi (1835) (with permission from the American Phytopathological Society)

Studies on the mechanisms of fungal pathogenesis in insects have yet to contribute to the development of more efficient commercial mycoinsecticides, but new insights into the pathogenic process are laying the groundwork. Comparative genomics and post-genomic studies are providing exciting new insights into the evolution of virulence, host adaptation and gene function in pathogenic bacteria (e.g. Waterfield *et al.*, 2002). A similar revolution in the study of fungal pathogens is on the horizon with the publication or pending publications of the genome sequences of the plant pathogen *Magnaporthe grisea* and the human pathogens *Candida albicans* and *Cryptococcus neoformans* (e.g. Tunlid and Talbot, 2002). A further 14 fungal pathogens are the subject of either full sequencing or expressed sequence tag projects including two insect pathogens, *Metarhizium anisopliae* and *Conidiobolus coronatus* (Tunlid and Talbot, 2002; Freimoser *et al.*, 2003a,b).

In the last 20 years the study of mechanisms of fungal pathogenesis in insects has progressed particularly with regard to the study of the enzymes involved with invasion of host cuticle and toxins. This contribution reviews current knowledge in these areas. Previous significant reviews include: (Roberts, 1980; Charnley and St. Leger, 1991; Khachatourians, 1991, 1996; St. Leger, 1993, 1995; Clarkson and Charnley, 1996; Vey *et al.*, 2001; Anke and Sterner, 2002; Soledade *et al.*, 2002).

II. TAXONOMY

Relationships between fungi and insects may be mutualistic, through commensal to obligately pathogenic. The term entomogenous is often used to encompass all types of association between insects and fungi, with disease-causing fungi being referred to as entomopathogenic. A further distinction can be made between fungi which are aggressively pathogenic like *Metarhizium anisopliae* and opportunists like the wound pathogen *Mucor haemalis* (McCoy *et al.*, 1988; Samson *et al.*, 1988; Tanada and Kaya, 1993).

Entomopathogenic fungi are found in most taxonomic groupings in the fungal kingdom, apart from the higher Basidiomycetes. The primitive water fungi, Mastigomycotina, have representatives with complex life cycles e.g. *Coelomomyces psorophorae* a mosquito pathogen with an obligate copepod secondary host. Among the Ascomycotina, *Cordyceps* spp. have fruiting structures or perithecia which can dwarf the cadavers of their insect victims. Entomophthorales are widespread members of the Zygomycotina. Mummified aphids stricken by fungi of this group are familiar features of cereal crops in temperate regions. The most widespread insect pathogenic

fungi are found in the Hyphomycetous Deuteromycotina of the genera *Metarhizium* and *Beauveria.*

The fungi described above are all destructively pathogenic. Many species of Entomophthorales are host-specific pathogens. *Beauveria bassiana* and *Metarhizium anisopliae*, facultative pathogens, have broad host ranges though considerable specificity occurs among isolates. Laboulbeniomycetes (Ascomycotina) on the other hand are biotrophic and specific to a single host or even a specific location on the host. They remain largely external gaining nutrition via a penetrant haustoria while apparently causing little harm. Most Trichomycetes (Zygomycotina) have a commensal existence in the guts of their Dipteran hosts, though some are pathogenic. Fast growing opportunists like *Mucor haemalis* may invade through wounds and it is important to note in the context of this review that many fungi are pathogenic if their spores are injected. This experimental strategy by-passes the exoskeleton and highlights the importance of the cuticle as a barrier to microbial pathogens.

III. OVERVIEW OF THE INFECTION PROCESS

Unique among entomopathogenic microorganisms, fungi do not have to be ingested and can invade their hosts directly through the exoskeleton or cuticle. Therefore they can infect non-feeding stages such as eggs and pupae. The site of invasion is often between the mouthparts, at intersegmental folds or through spiracles, where locally high humidity promotes germination and the cuticle is non-sclerotised and more easily penetrated (Charnley, 1989; Hajek and St. Leger, 1994).

M. anisopliae and *B. bassiana* have hydrophobic spores that appear to bind to insect cuticle by non-specific interactions though failure to adhere to particular insect species may help to define isolate host range. Zoospores of *Lagenidium giganteum* are host selective. Cuticle-degrading enzymes are present on the surface of conidia of *M. anisopliae* and therefore there is the potential for the fungus to modify the cuticle surface to aid attachment. Host and fungal lectins have been implicated also in the process of attachment. Germination *in vitro* of nutrient-dependent spores of *M. anisopliae* and *B. bassiana* is consequent upon a non-specific accessible source of carbon and nitrogen though *in vivo* isolate specificity may depend on response to qualitative and quantitative differences in available nutrients on host cuticle. More selective pathogens appear to have more specific requirements. Ability to withstand antifungal compounds in the cuticle such as short chain fatty acids is a prerequisite for successful invasion (see Boucias and Pendland, 1991). The importance of signal exchange between

host and pathogen is becoming increasingly clear and is first seen in the cues which cause the fungus to stop horizontal growth on the surface of the cuticle and initiate penetration. Differentiation of the germ tube to produce the holdfast structure, or appressorium, is most completely understood for *M. anisopliae*. Isolate 2575 (formerly ME1) requires low concentrations of a complex carbon and nitrogen source and a hard surface. *Metarhizium* isolates which have come from Homoptera, form appressoria in media (high concentrations of simple sugars) which are repressive for isolates from Coleoptera. This is probably an adaptation to parasitism as the cuticle of plant-sucking bugs (Homoptera) is contaminated with sugars from their copious liquid excreta (St. Leger *et al.*, 1992b).

Once the fungus breaks through the cuticle and underlying epidermis then it may grow profusely in the haemolymph, in which case death is probably the result of starvation or physiological/biochemical disruption brought about by the fungus. Alternatively insecticidal secondary metabolites may contribute to the demise of the insect and in this case extensive growth of the fungus may only occur on the cadaver of the host (Roberts, 1980; Gillespie and Claydon, 1989). For many fungi the reality is probably somewhere between these two extremes. Few studies have looked at the effect of fungal infection on host physiology/behaviour. This is unfortunate because sub-lethal or prelethal effects of mycosis may be just as useful as the death of the host from the point of view of crop protection. Detrimental effects of mycosis on food consumption, egg laying and flight behaviour have been recorded (Nnakumusana, 1985; Seyoum *et al.*, 1995).

The life cycle is completed when the fungus sporulates on the cadaver of the host. Under the right conditions, particularly high RH, the fungus will break out through the body wall of the insect producing aerial spores (Fig. 2). This may allow horizontal or vertical transmission of the disease within the insect population. Resting spores produced within the dead insect will enable the fungus to survive for long periods under adverse conditions (Samson *et al.*, 1988).

IV. INVASION OF HOST CUTICLE

The many light and electron microscope studies on cuticular penetration by entomopathogenic fungi have led to the conclusion that both physical force and enzymic degradation are involved (see Fig. 3 for an overview of the structure of insect cuticle). Ingression of cuticle around a fungal penetration peg suggests mechanical penetration, but this does not necessarily preclude a role for enzymes also (Fig. 4). Physical penetration is prominent in host invasion by some entomophthoralean pathogens where characteristic

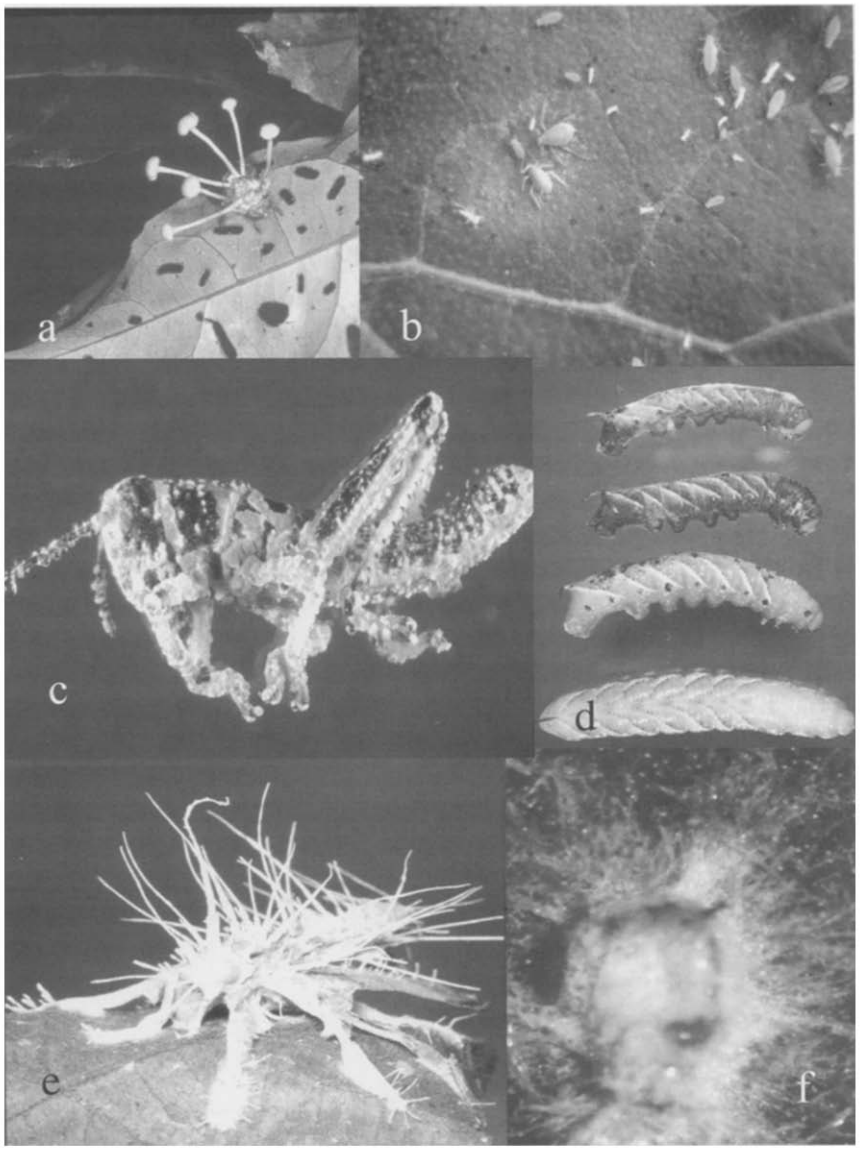


Fig. 2. (a) Stromata bearing perithecia of a *Cordyceps* spp. on a fly cadaver (with permission from H. Evans), (b) *Erynia neoaphidis* on *Macrosiphum euphorbiae*, note white halo of spores on the leaf around dead aphids (centre-left), (c) *Metarhizium anisopliae* sf. *acridum* sporulating on a cadaver of the desert locust, *Schistocerca gregaria*, (d) symptoms of mycosis caused by *Metarhizium anisopliae* sf. *anisopliae* 2575 on 5th instar caterpillars of the tobacco hornworm *Manduca sexta*, note the black melanic pigment, a host defensive response. (e). *Akanthomyces aculeatus*, a Deuteromycotina on a leaf mimicking moth from Papua New Guinea (with permission from C. Prior), (f). *Verticillium lecanii* sporulating on a dead aphid.

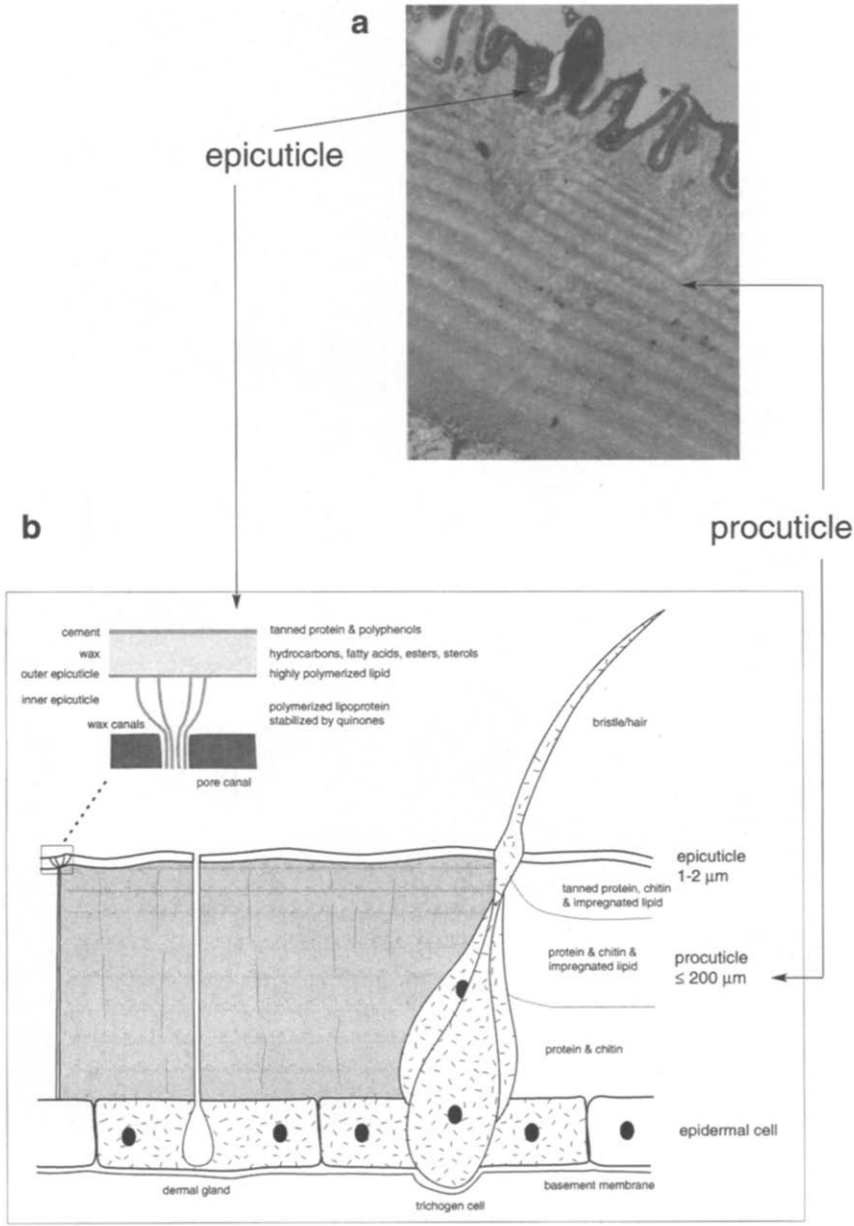


Fig. 3. Structure and composition of insect cuticle, (a) a transmission electron micrograph of cuticle from a newly moulted 5th instar caterpillar of the tobacco hornworm moth *Manduca sexta*, note the folds of the epicuticle and the procuticle laid down in lamellae (appears striated) (from Hassan and Charnley, 1987) with permission from Elsevier); (b) a diagram showing the main components of the cuticle and associated epidermis.

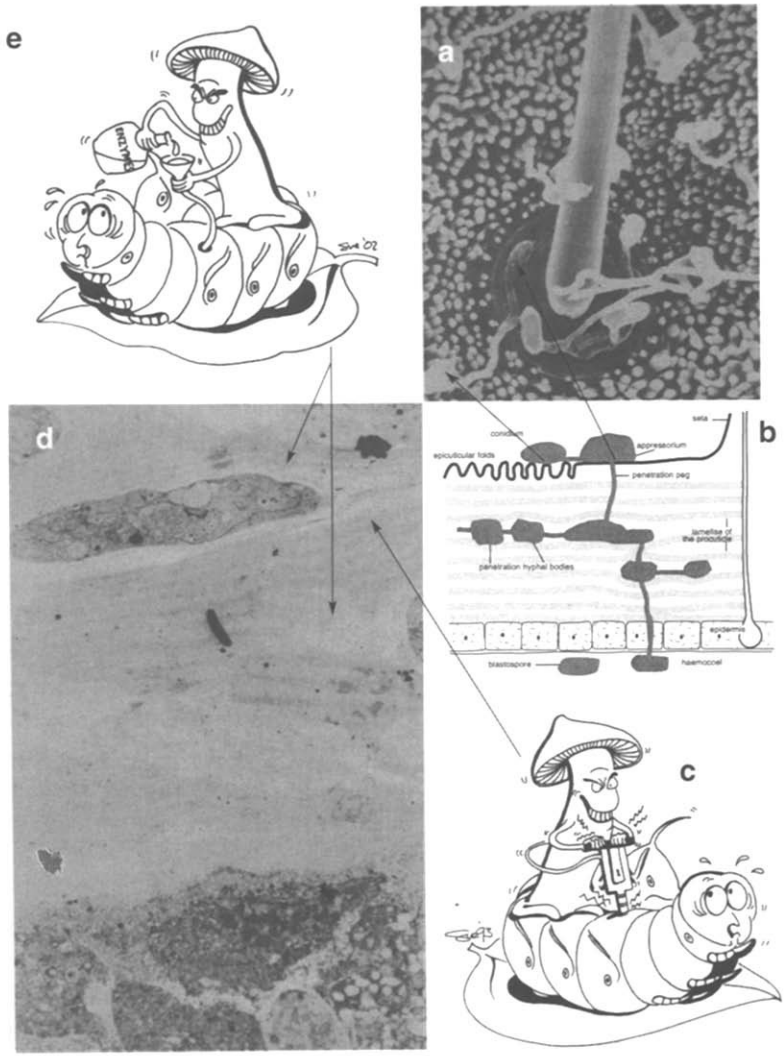


Fig. 4. (a) Shows a scanning electron micrograph of *Metarhizium anisopliae* on the surface of the cuticle of a newly moulted 5th instar *Manduca sexta* caterpillar, note the folded epicuticle ("pimples"), appressoria form preferentially on the flat surface at the base of the seta; (b) a diagram depicting the invasion of cuticle by the fungus, note the surface features and the displacement of the lamellae around the hyphal bodies in the cuticle, suggesting a mechanical component; (c) cartoon (with permission from S. Fairhurst) emphasising the physical side of the penetration process; (d) transmission electron micrograph of *Metarhizium anisopliae* in cuticle of 5th instar *Manduca sexta* caterpillars. Note the lamellae (striations) of the procuticle, which are disturbed around the hyphal bodies, indicating mechanical ingress, and also the partial clearing of the lamellar structure around the fungus suggestive of partial enzymic hydrolysis, probably the action of proteases (from Hassan and Charnley (1987) with permission from Elsevier); (e) A cartoon (with permission from S. Fairhurst) to emphasise the role of the cuticle-degrading enzymes in the penetration process.

irradiate and tetraradiate fissures appear in the epicuticle (Brobyn and Wilding, 1983; Butt, 1987). Initial fungal growth within the outer layers of the procuticle often occurs laterally. These subepicuticular expansions can cause fractures, which favour penetration (Brey *et al.*, 1986). Vertical penetrant hyphae may appear thin and constricted within the outer layers of the procuticle (exocuticle) presumably due to resistance to mechanical penetration engendered by the sclerotisation of the proteins, whereas the growing tip swells in the more pliant unsclerotised inner layers (endocuticle) (Robinson, 1966). Indentation or displacement of lamellae by lateral penetrant hyphae in both exo- and endocuticles is another clear sign of a mechanical component to the penetration process (see Charnley, 1984, and Fig. 4).

Disappearance of the wax layer beneath appressoria of *Metarhizium anisopliae* on wireworm (Elaterid) cuticle indicates enzyme activity (Zacharuk, 1970b) as does the presence of circular holes around germ tubes of *Beauveria bassiana* at the point of entry into larvae of *Heliothis zea* (Pekrul and Grula, 1979). Early studies (reviewed by Charnley, 1984) showed changes in the histochemistry of insect cuticle around penetrant fungal hyphae consistent with enzymic hydrolysis. Brey *et al.* (1986) reported wide zones of complete histolysis in cuticle beneath penetrant hyphae of *Conidiobolus obscurus* in *Acyrthosiphon pisum*. In a transmission electron microscope study Askary *et al.* (1999) also found significant degradation of cuticle around hyphae of *Verticillium lecanii* in the aphid *Macrosiphum euphorbiae*.

However, fine structural studies of many other insect–fungus interactions suggest that significant enzymolysis is not the norm. Absence of mechanical damage or displacement of lamellae suggests the action of enzymes, but without obvious zones of histolysis it must be assumed that fungal enzymes are usually limited to the vicinity of fungal structures in the initial stages of infection (Charnley, 1984, and Fig. 4). However, Goettel *et al.* (1989) and Hassan and Charnley (1989) noted clearing of the lamellar pattern but not complete histolysis around hyphae of *Metarhizium anisopliae* in the cuticle of *Manduca sexta* (Fig. 4). A consideration of the chemistry and physical properties of the cuticle *a priori* provides a way of assessing the likely impact of hydrolases. The tanned proteins of the cement layer may resist the proteases secreted profusely by the appressoria. The outer epicuticle is resistant to chemical and enzymic degradation (Hepburn, 1985), but in most insects it is probably fragile and thus susceptible to mechanical force (see St. Leger, 1991). In contrast, the inner epicuticle may yield to a combination of endoprotease and lipoprotein lipases. The procuticle constitutes the bulk of the cuticle and must provide a significant barrier to the invading fungus. This layer comprises chitin fibrils embedded in a protein matrix, together with lipids and quinones (Neville, 1984) (Fig. 3). The mechanical properties

of different cuticles depend on the proportions of the two main constituents, the nature and extent of hydration of the proteins and the degree of sclerotisation also called tanning (because of homology with the curing of leather) viz. cross-linking of the proteins by quinones (Hillerton, 1984). In soft-bodied juvenile endopterygote insects like Lepidopteran caterpillars, exocuticle is essentially restricted to the head, while in the majority of adult insects and juvenile exopterygote insects, non-sclerotised cuticle is present only at joints and arthrodial membranes. Pliant cuticles have a higher chitin (~46%) content than rigid cuticles (as little as 17%) (Hillerton, 1984). Clearly chitinases and proteases would be particularly useful for the fungus in facilitating passage through and gaining nutrient from the procuticle. Passage of the fungus across the procuticle may be more or less direct or involve a degree of lateral proliferation between cuticular lamellae before or during vertical penetration (Charnley, 1984). Thickness of the procuticle correlates with disease resistance in that young larvae (with thin cuticles) are more susceptible than old larvae (with thick cuticles). The degree of cuticle sclerotisation appears also to have a strong influence on penetrability. Although there are reports that sclerotised cuticle can be traversed, in the main it seems that insects with heavily sclerotised body segments are invaded via arthrodial membranes or spiracles (see Charnley, 1984; St. Leger, 1991). There is good reason for this behaviour. The resistance to compressive force of sclerotised cuticle (exocuticle see Fig. 3) and enzymic hydrolysis (from endogenous moulting fluid enzymes (Hepburn, 1985) and purified fungal enzymes (St. Leger *et al.*, 1986d) suggests that it presents a stronger barrier than pliant, non-sclerotised endo and mesocuticle.

While a considerable amount of work has been done on cuticle-degrading enzymes, hydrolysis of cuticle polymers could be facilitated by weak organic acids. Oxalic acid is produced both on the surface of some mycosed insects and in the haemolymph of infected insects and can hydrolyse cuticle proteins *in vitro* (Bidochka and Khachatourians, 1991). However, alkalisation of the cuticle during infection, which promotes protease production and activity (St. Leger *et al.*, 1999), militates against a direct role in pathogenicity. Furthermore, hyperproductive oxalic acid mutants showed no difference in pathogenicity from wild-type (Bidochka and Khachatourians, 1993a).

V. CUTICLE-DEGRADING ENZYMES

A. OVERVIEW

Cuticle-degrading enzymes have been studied in detail in *Metarhizium anisopliae* (see Table I for a compendium of *Metarhizium anisopliae* protease

TABLE I
Proteases produced by Metarhizium anisopliae

Enzyme		pH optimum	pI	Mr (kDa)	Substrate	Specificity
Subtilisin	Pr1A	8–9	10.2	30.2	Ala-Ala-X	X = amino acid branched at second carbon atom, phenylalanine preferred
	Pr1B	8–9	9	31.5	Phe-Leu-X	
Chymotrypsin			8.3	18.5		Similar to Pr1A
Metalloprotease	Mp	7	7.3		Broad, large hydrophobic residues preferred	
Trypsin	Pr2A	9	4.4	30	Val-Leu-Arg > Lys	
	Pr2B	9	4.9	27	Val-Leu-Arg > > Lys	
Aminopeptidase M(multiple isoforms)		7	4.4–5.4	31–105	Broad, alanine preferred	Inhibited by 1,10-phenylanthroline
Post proline dipeptidyl peptidase IV (2 isoforms)		8	4 and 4.3	74	Y-Pro-X	Inhibited by DFP
Post alanine dipeptidyl peptidase (3 isozymes)			~5.8		Lys-Ala-X	
Serine carboxypeptidase		6.8	9.8	30	Broad, phenylalanine preferred	Specifically complements Pr1
Zinc carboxypeptidase	MeCPA	7.5		35		Preference for branched aliphatic and aromatic COOH-terminal aa, complements Pr1

Based on St. Leger and Bidochka (1996).

activities). While it is useful to build up a body of knowledge on a single organism, generalisations cannot be made because of the huge diversity in biology and biochemistry between groups of entomopathogenic fungi. Initial studies showed that when *M. anisopliae* is grown on comminuted locust cuticle in liquid medium, a range of extracellular cuticle-degrading enzymes are produced corresponding to the major components of insect cuticles, viz. protein, chitin, and lipid (St. Leger *et al.*, 1986c). Enzymes appeared sequentially. Esterase and proteolytic enzymes (endoprotease, aminopeptidase, and carboxypeptidase) were produced first (~24 h) followed by N-acetylglucosaminidase (NAGase). Chitinase and lipase were produced 3–5 days later. The order of appearance of the enzymes is supported by the sequence of cuticle constituents solubilised into the culture medium, where a rapid increase in amino sugars followed early release of amino acids. Since chitinase is an inducible enzyme (Smith and Grula, 1983; St. Leger *et al.*, 1986e) (see later), and cuticular chitin is masked by protein (St. Leger *et al.*, 1986d), the late appearance of chitinase is presumably a result of induction as chitin eventually becomes available after degradation of encasing cuticle proteins. The late detection of lipase appears to be due to the fact that the enzyme is largely cell bound in young cultures (St. Leger, Charnley, and Cooper, unpublished). By testing purified enzymes against locust cuticle *in vitro*, St. Leger *et al.* (1986d) showed that pretreatment or combined treatment with endoprotease (Prl; see later) was necessary for high chitinase activity. Samsinakova *et al.* (1971) and Smith *et al.* (1981) also concluded that cuticular chitin is shielded by protein from studies using semipure commercial enzyme preparations against cuticles from *Galleria mellonella* larvae and *Heliothis zea* larvae, respectively. When locust exuviae (non-digested remains of old cuticle shed at ecdysis; exocuticle only) were used as substrate for purified pathogen enzymes instead of cuticle from larval sclerites (exo- and endocuticle), comparatively little hydrolysis occurred (St. Leger *et al.*, 1986d). Similarly, while unsclerotised cuticle from fledgling locusts was rapidly degraded by fungal proteases, the cross-linking of cuticle proteins with glutaraldehyde (as a model for sclerotisation) substantially reduced their susceptibility to proteolytic attack (St. Leger, Charnley, and Cooper, unpublished).

B. PROTEOLYTIC ENZYMES

1. Endoproteases

The most prominent endoprotease in cuticle cultures of *M. anisopliae* 2575, termed Pr1, is an alkaline, serine enzyme with an essential histidine residue in the active site (St. Leger *et al.*, 1987a). Prl possesses a broad primary

specificity for amino acids with a hydrophobic side group at the second carbon atom (e.g., phenylalanine, methionine, and alanine) but also possesses a secondary specificity for extended hydrophobic peptide chains with the active site recognising at least five subsite residues. This comparative non-specificity accounts for it being a good general protease with activity against a range of proteins (casein, elastin, bovine serum albumin, collagen) and insect cuticle (St. Leger *et al.*, 1987a). Essential binding of Prl to negatively charged cuticle groups is dictated by its basic nature (St. Leger *et al.*, 1986b). Only following adsorption does the active site come into contact with susceptible peptide bonds; solubilised peptides are further degraded until a chain length of about 5 is obtained (St. Leger *et al.*, 1986d). The *Pr1* cDNA has been cloned, revealing that Pr1 is synthesised as a large precursor containing a signal peptide, a propeptide and a mature 29 kDa protein. Originally Pr1 was termed a 'chymoelastase' in line with its substrate specificity, however, the predicted amino acid sequence places Pr1 in the subtilisin subclass of serine proteases, which are the predominant class of microbial extracellular proteases.

St. Leger *et al.* (1994b) resolved the Pr1 activity of *M. anisopliae* 2575 produced on cockroach cuticle into four isoforms. Three of the purified isoforms (pI 10.2, 9.8, 9.3) had similar primary specificities viz. phenylalanine at P1 was more reactive than leucine. With regard to secondary subsite specificity, pI 10.2 isoform differed from the others in preferring alanine over bulky hydrophobic groups at S1 or S2. They appeared to be equally effective in degrading proteins from insect cuticle. Multiple isoforms of metalloprotease were also found. They were inhibited by 1,10-phenanthroline and phosphoramidon, a specific inhibitor of thermolysin-like metalloproteases. The metalloproteases were not produced to the same degree as the subtilisins although they had a similar amino acid specificity. A further *Metarhizium* subtilisin gene *Pr1B* has been cloned and sequenced (Joshi *et al.*, 1997). Application of contour-clamped homogeneous electric field electrophoresis (CHEF) separated the *M. anisopliae* genome into seven chromosomes. Hybridisation of *Pr1B* occurred strongly to one chromosome and weakly to two others, which, along side evidence from Southern analysis, suggested single copies of both the *Pr1B* gene and the 1st *Pr1* gene cloned (*Pr1A*) and the existence of a third subtilisin *Pr1C*. It is not yet clear whether the four Pr1 isoforms so far purified are products of the three ORFs. Bidochka and Melzer (2000) showed that *Pr1* genes from *M. anisopliae* make up a multigene family and suggested that they may be derived from a common ancestral subtilisin. The RFLP pattern for *Pr1A* is more complex than that for *Pr1B* or *Pr1C*. Thus *Pr1A* could be the original gene which duplicated. However, the situation is further complicated by

the revelation from an EST project that *M. anisopliae* genome has at least 11 subtilisin genes (*Pr1* A-K) (Freimoser *et al.*, 2003a). Bagga *et al.* (2003) have divided the genes into three subfamilies. Further phylogenetic analysis comparing subtilisins from *Metarhizium* with those from fungi and other organisms confirms the authenticity of these subdivisions and suggests that gene duplication occurred before speciation of major fungal lineages.

Recently Screen and St. Leger (2000) identified a chymotrypsin (CHY1) by express sequence tag analysis of cuticle cultures. CHY1 belongs to the S2 group of chymotrypsins. It is most closely related to *S. griseus* protease C. Both have only 15% identity to mammalian S1 chymotrypsin and have Ala190 and Thr213 in the S1 specificity pocket. These residues define a specificity for hydrophobic amino acids, so that like Pr1, CHY1 is most effective against Suc $(Ala)_2$ Pro Phe. Substituting Leu or Meth for the Phe reduced catalytic efficiency, replacing Phe with Ala or Val left only trace activity. The latter two substitutions produce elastase substrates which are effectively hydrolysed by Pr1a. Interestingly plant and insect Ascomycotina and actinomycete bacteria are the only known microbial sources of S1 trypsins. So *M. anisopliae* may have acquired CHY1 by lateral gene transfer from an actinomycete.

Pr2, a third class of serine protease produced by *M. anisopliae* (2575) occurs as four isozymes (*ca.* pI 4–4.9) with little activity against insect cuticle, insoluble insect cuticular proteins or elastin but high activity against casein and basic solubilised cuticular proteins. It has a primary specificity for arginine and lysine residues comparable to that of bovine trypsin and is sensitive also to trypsin inhibitors (e.g. leupeptin, tosyl-lysine-chloroketone, soybean trypsin inhibitor). Maximum activity was against Val Leu Arg AFC. The catalytic efficiency of Pr2 can be influenced by subsite residues at a distance from the cleaved site (St. Leger *et al.*, 1987a,b, 1996a). N termini of the two main isoforms (pI 4.4 and 4.9) resemble those of other trypsins. Cole *et al.* (1993) purified a pI 4.6 enzyme with trypsin-like specificity, which they termed a cysteine protease on the basis of its susceptibility to inhibition by sulphydryl reagents. St. Leger *et al.* (1996a) have suggested, however, that the studied by Cole *et al.* (1993) is in fact equivalent to their pI 4.9 isoform (the same isolate was used in both studies) that may have free sulphydryl groups close to the active site.

Why *M. anisopliae* should have such a complex protease arsenal with subtilisin, chymotrypsin, metalloproteases and trypsins, in most cases with multiple isoforms, is not clear. Comparative analysis of the 10 *Pr1* genes that code for exocellular enzymes, expressed on cuticle by *M. anisopliae* 2575, showed differences in the S4, S3 and S2 subsites consistent with variation in catalytic efficiency and secondary specificity noted in biochemical analyses

(Bagga *et al.*, 2003). Other differences in sequence will affect environmental stability and adsorption as well as substrate activity between isoforms. Pr1 D-J all have small non-polar or hydrophilic residues at 189 (Gly, Thr, His, Asp) which will preclude an interaction with certain types of proteases inhibitor e.g. chymotrypsin inhibitor 2. Though Pr1A is the predominant form produced during growth on cuticle, ESTs for Pr1A are 10 times more abundant than Pr1J, the next most abundant transcripts, the minor isoforms could play a key role in pathogenesis. Multiple Pr1 isoforms with subtle differences in specificity may act synergistically in the degradation of host cuticular protein, enable the pathogen to operate in changing environmental conditions and counter host defensive enzyme inhibitors.

A spectrum of proteases may help a facultative pathogen exploit a wider range of substrates. They may be expressed at different stages of infection and have slightly different functions cf. proteases produced by *Leishmania* (Mottram *et al.*, 1997). The mycopathogenic fungus *Trichoderma harzianum* produces many chitinases and expression is dependent on the host infected (Haran *et al.*, 1996). Alternatively duplicate enzyme systems may help a facultative pathogen survive saprophytically in different ecological niches.

The use of inhibitors with *Metarhizium* culture filtrates showed that Pr1-like activity accounts for 92% of the insoluble cuticle-degrading ability present. The greater efficiency of Pr1 is a reflection of the higher alanine and low basic amino acid content of many insect cuticular proteins which would favour digestion by a subtilisin (St. Leger *et al.*, 1996a). However, the low activity of Pr2 enzymes against cuticle does not preclude a role in fungal penetration. Trypsins have activity against arginine and lysine residues, which are found in particular on the surface of globular proteins. Thus Pr2 may complement Pr1 by opening up proteins for further hydrolysis and providing peptides for nutrition. Pr2 could additionally be involved in cellular control mechanisms, catalysing specific proteolytic inactivation and activation processes (St. Leger *et al.*, 1987c). In this context it is interesting that inhibition of *M. anisopliae* (2575) Pr2 with tosyl-lysine-chloroketone selectivity repressed formation by germlings of infection structures, implying a role for Pr2 in control of differentiation (St. Leger, unpublished). A gene encoding one isoform of the trypsin-like enzyme Pr2 from *M. anisopliae* has been cloned; the predicted amino acid sequence is very similar to the trypsin of *Fusarium oxysporum* and clearly distinguishes Pr2 from the subtilisin subclass of serine proteases (Smithson *et al.*, 1995).

2. Exoproteases

The action of Pr1 on cuticle releases peptides with a mean residue length of 5 amino acids. Further degradation by peptidases will be required to

provide fungal nutrition. Two classes of aminopeptidase were isolated from cuticle-grown cultures of *M. anisopliae* and classified as an aminopeptidase M of broad specificity and a post-proline dipeptidyl aminopeptidase IV (St. Leger *et al.*, 1995a). The aminopeptidase M (pH optimum 7–8, 33 kDa) exists as six isozymes (pl 5–6) with optimal activity for alanine residues and side activities versus other apolar and hydrophobic amino acids. The enzyme is sensitive to typical inhibitors of metalloenzymes (e.g., EDTA, 1,10-phenanthroline). The dipeptidyl aminopeptidase (pH optimum 8, 74 kDa) exists as two isozymes (pl *ca.* 4.6) and removes X-prolyl groups from polypeptides (X = an apolar amino acid). The enzyme is inhibited by DFP (but not PMSF), indicating that it is a serine hydrolase. Without this enzyme degradation of proteinase-derived cuticle peptides by aminopeptidase would terminate at proline residues because of the low specificity of Pr1 for this residue. Neither peptidase alone hydrolysed intact insect cuticle. However, when combined with Pr1 they effected enhanced release of amino acids (St. Leger *et al.*, 1993a).

St. Leger *et al.* (1994a) purified a serine carboxypeptidase produced during growth on cockroach cuticle. The enzyme (M_r 30 kDa, pI 9.97, pH optimum 6.8) had a broad primary specificity towards amino acids with hydrophobic side groups in a series of N-terminally blocked dipeptides, with phenylalanine being most rapidly hydrolyzed. It complements Pr1, both showing a preference for cleavage of bonds C-terminal to aromatic residues. Both are produced during C and N deprivation and operate synergistically to provide nutritional amino acids.

Joshi and St. Leger (1999) isolated a cDNA clone for a zinc carboxypeptidase *MeCPA* by reverse transcriptase differential display PCR from a cockroach cuticle culture of *M. anisopliae*. It is of interest because it is a metallocarboxypeptidase, other fungal CPAs being serine enzymes including the one identified by St. Leger *et al.* (1994a) from *Metarhizium* (see above). MeCPA has a preference for branched aliphatic and aromatic COOH-terminal amino acids. This requirement is met by the peptides released by the action of Pr1 which cleaves COOH-terminal to aromatic amino acids like Phe. Thus, Pr1 and MeCPA complement each other. These enzymes are also regulated similarly viz produced under C and N derepressed conditions, but optimally in the presence of insect cuticle (see later).

C. CHITINOLYTIC ENZYMES

St. Leger *et al.* (1991a) purified endochitinase from culture filtrates of *M. anisopliae* grown on 1% ground chitin. The purified enzyme failed to

hydrolyze arylglycosides or chitobiose (N-acetylglucosamine dimer), showed only trace activity against chitotriose (trimer), but rapidly degraded chitotetraose (tetramer). Colloidal chitosan (deacetylated form of chitin) and crystalline chitin were less amenable to degradation than colloidal chitin, but activity against them was still substantial. The chitinase had many similarities to those produced by other microorganisms (e.g., Stirling *et al.*, 1979). These properties include a pH optimum of 5.3, a molecular weight of 33 kDa, and the lack of any requirement for a cofactor. Hydrolysis of crystalline chitin produced only one low-molecular-weight reaction product within 24 h, viz. N-acetylglucosamine (NAG). The absence of intermediary oligomers among chitin breakdown products probably means that NAG is released directly from insoluble chitin. Either the chitinase has an exo-acting component or alternatively the reaction proceeds by a single-chain processive mechanism as described for some other endo-acting polysaccharidases (Cooper *et al.*, 1978). This involves the random cleaving of bonds followed by release of monomers or dimers from exposed ends so that a single macromolecule is completely degraded before a new one is attacked. Such a mechanism, especially if it involved simultaneous digestion of several parallel chains, could result in the rapid degradation of chitin fibrils and in addition produce monomers for nutrition and induction for further enzyme synthesis.

An IEF study has shown large numbers of chitinase isoforms (10) produced in culture filtrates from *M. anisopliae* (St. Leger *et al.*, 1993b). The big difference in molecular weights of the enzymes suggests that they are products of different genes rather than post-translational modifications, e.g. glycosylation (St. Leger *et al.*, 1996b). The isoforms produced on cockroach cuticle by *M. anisopliae* sf. *anisopliae* 2575 and *M. anisopliae* sf. *acridum* 324 may be broadly classified by pI as basic or acidic. The latter are dominant. Two isoforms 43.5 and 45 kDa, pI 4.8 have been purified. In this case similarities between N-termini suggest that they may not be separate gene productions but rather result from post-translational modifications, particularly glycosylation.

Pinto *et al.* (1997) found a 30-kDa endochitinase in a different isolate of *M. anisopliae* with similar M_r, pH and temperature to one reported previously by St. Leger *et al.* (1991a). However, a 60-kDa endochitinase by Kang *et al.* (1998, 1999) differed from the St. Leger *et al.* (1991a, 1996b) isoforms not only in M_r but also protein N-terminus and ORF nucleotide sequence. It could be one of the eight uncharacterised chitinase isoforms identified by St. Leger *et al.* (1993b). In both the latter cases the enzymes were similar to the St. Leger *et al.* isoforms in having both endo and exoactivity. Bogo *et al.* (1998) isolated a cDNA for a chitinase gene

encoding an endochitinase of 58 kDa. The ORF encoded a 423 amino acid protein with a predicted final product of 43 kDa. This compared well with the 45-kDa chitinase purified biochemically by St. Leger *et al.* (1996b).

N-Acetylglucosaminidase activity has been partially purified from culture filtrates of *M. anisopliae* grown on 1% ground chitin. The enzyme had substantial activity against *p*-nitrophenol acetylglucosamine, as well as chitobiose, chitotriose, and chitotetraose, the major product in each case being NAG, showing that the enzyme is a true NAGase rather than a chitobiase (St. Leger *et al.*, 1991a). The enzyme had little activity against colloidal or crystalline chitins. Its size, 110-120 kDa, is within the range for similar enzymes from other sources (e.g., Reyes and Byrde, 1973).

D. LIPOLYTIC AND ESTEROLYTIC ENZYMES

There has been little work done on the enzymes that are active against lipids and esters. This is perhaps not an important omission in the dissection of the role of enzymes in cuticle penetration, because substrates for these enzymes are not important components of epi- or procuticle. Hydrocarbons that are major constituents of the wax layer are used by germinating fungi (Lecuona *et al.*, 1991). However, exocellular enzymes are not involved.

In general, esterases may be differentiated from lipases because short-chain fatty acids (C_2–C_4) are preferentially hydrolysed by the former and long-chain esters ($> C_8$) by the latter (Shnitka, 1974). Esterase activity produced by *M. anisopliae* in young cultures (3 days) was greatest against short- and intermediate-length *p*-nitrophenol esters with only trace activity occurring above C_{10} (St. Leger, Charnley, and Cooper, unpublished), suggesting that lipase is not produced extracellularly by young mycelia. Activity against C_{14} rose in older cultures (7–14 days). Late arrival of extracellular lipase *in vitro* was confirmed using the 'true' lipase substrate olive oil (St. Leger *et al.*, 1986a). The major esterase peak eluted from a Sephadex G100 column with a profile very similar to that obtained for endoprotease Pr1. As this protease also degrades *p*-nitrophenol esters, it is probably a major contributor to total esterase activity. Flat-bed IEF, however, revealed 25 distinct esterases (isozymes) from culture filtrates of *M. anisopliae* grown on locust cuticle. On the basis of their reactions with naphthyl esters, the isozymes appeared to have different substrate specificities. However, all the bands were inhibited by PMSF, indicating that they are serine carboxyesterases (esterase B) and not arylesterases (esterase A) that are inhibited by N-ethyl-maleimide. Esterases catalyse many enzymatic reactions though preferentially hydrolysing aliphatic or

aromatic esters and amides (Shnitka, 1974; Heymann, 1980). The considerable heterogeneity of esterases could account for their collective lack of specificity. Multiple enzyme strategies are believed to play an important role in the ability of an organism to adapt to different environments (Moon, 1975; Somero, 1975), presumably including that provided by an insect host.

E. REGULATION OF ENZYME PRODUCTION

Production of the right enzymes, in sufficient quantity, in an appropriate sequence, at the right place and time must be critical for successful parasitism. Prl is controlled by multiple regulatory circuits. The *Prla* promoter region has been sequenced (up to 1 kb upstream of the ATG). Binding sites similar to those of the 'nitrogen regulator' (AREA) and 'carbon regulator' (CREA) of *A. nidulans* have been identified. In addition genes coding for AREA and CREA-like regulatory proteins have been cloned and sequenced from *M. anisopliae* and have been shown to function when transformed into *A. nidulans* mutants (Screen *et al.*, 1997, 1998). Prl is the major protein product by appressoria on artificial surfaces or on host cuticle and thus there is an element of developmental regulation. Prl mRNA was not present in spores but Prl is the primary translation product in germlings forming appressoria. Radiolabelled Prl was secreted into the medium just 6.5–7.2 min after the addition of a pulse of [^{35}S] methionine. Rapid processing of the original large primary translation product was not affected by the Pr2 inhibitor TLCK, which is not consistent with a role in protein processing for this enzyme suggested earlier (St. Leger *et al.*, 1989a). In culture both Prl and Pr2 occurs with carbon and nitrogen starvation (St. Leger *et al.*, 1988c). In minimal medium (salts and no nutrients), the soluble protein BSA repressed production of Prl, while it allowed enhanced synthesis of Pr2. Generally Pr2 is less tightly regulated than Prl.

Both during production in culture or during appressorium formation Prl is repressed by readily utilised metabolites (e.g., glucose or alanine) (St. Leger *et al.*, 1988c, 1989a). The second messenger system that mediates the effects of starvation and regulates transcription of the Prl gene has not yet been established. Evidence militates against the involvement of cAMP (St. Leger *et al.*, 1988c, 1989b). A role for calcium is suggested by the fact that secretion but not synthesis of Prl in *M. anisopliae* is inhibited by agents which depress calcium-dependent protein phosphorylation (St. Leger *et al.*, 1989b). However, antagonists of

calmodulin (a sensor and effector of many Ca^{2+}-dependent messages) that are potent inhibitors of protein synthesis and phosphorylation during germination do not affect Pr1 synthesis nor secretion by mycelia (St. Leger *et al.*, 1989b).

Extracellular levels of both Pr1 and Pr2 were enhanced in cultures supplied with insect cuticle or other insoluble polymers (e.g., cellulose) that were insufficient to produce catabolite repression (CR). Under these conditions regulation of *Pr1* gene expression was again exerted at the level of transcription (St. Leger *et al.*, 1995b). By comparison with the situation on rich media up to 32 new proteins appeared on cuticle or chitin (some 19 also were less abundant); prominent among them was Pr1 though there were trypsin and chymotrypsin homologues. Pr1 is specifically induced by a peptide component of insect cuticle, but not by other soluble or insoluble proteinaceous substrates (Paterson *et al.*, 1994a,b). The feeding of elastin or collagen to derepressed established mycelia (starved for carbon and nitrogen) did not enhance Pr1 production significantly and, as found previously, soluble proteins were repressive. The carbohydrate polymers cellulose and xylan gave derepressed basal levels only. Peptides in the range 150–2000 Da, released from insect cuticle by either Pr1 or Pr2 induced Pr1 production to a similar level to that obtained with untreated cuticle. Deproteinised and lipid-extracted cuticles supported little Pr1 production. Pr2, by comparison, is induced non-specifically by protein (Paterson *et al.*, 1993).

Thus, rapid protease synthesis is only possible in host tissues where the concentration of readily metabolisable compounds is low. This is the case with insect cuticles as the components are largely insoluble until released by cuticle-degrading enzymes (St. Leger *et al.*, 1986d). However, repression could operate if ever the release from cuticle of degradation products exceeded fungal requirements. Thus, the pathogenic process involving infection-related morphogenesis and enzyme production occurs only when it is necessary for the pathogen to establish a nutritional relationship with the host.

Less is known about the regulation of exopeptidases, but as for the endoproteases production occurs under C and N derepression, optimal production on insoluble polymers (particularly protein and host cuticle) and repression by low molecular weight compounds e.g. the zinc carboxypeptidase *MeCPA* (Joshi and St. Leger, 1999).

Chitinase synthesis is regulated in *M. anisopliae* (St. Leger *et al.*, 1986e) by products of chitin degradation, through an inducer–repressor mechanism. High chitinase activity was found only in cultures supplied with chitin, but not with other polymers such as pectin, xylan, and

cellulose. Slow feeding of cultures with sugars or alanine in a carbon-deficient medium demonstrated that the most effective inducer of chitinase was N-acetylglucosamine (NAG). Glucosamine also allowed production, possibly an adaptation to the fact that chitin from natural sources (including insect cuticle) appears to be partially deacetylated (Hackman and Goldberg, 1974). It is unlikely that chitobiose could function as a major inducer of chitinase in *M. anisopliae* as e.g. cellobiose does for cellulose (Cooper and Wood, 1975) because NAGase would degrade it to NAG and the major product of chitinase activity is NAG. Interestingly, NAGase was produced constitutively and was little affected by catabolite repression. Co-ordinated expression of all endochitinase isoforms does not occur since a mutant in which all other isoforms were produced at low levels secreted large amounts of the major 48-kDa form (St. Leger *et al.*, 1996b).

St. Leger *et al.* (1998) showed that proteases and chitinases were only synthesised at the pH at which they function effectively, irrespective of whether the medium contained an inductive cuticle substrate, though for most enzymes cuticle increased activity 3-fold over that at the optimum pH. Aminopeptidases were produced at pH 7 (optimum enzyme activity = pH 7), metalloproteases produced at pH 6–8 (optimum = pH 7), trypsins and subtilisins produced at pH 8 (optimum = pH 8). Northern analysis of RNA corresponding to 7 cDNA sequences encoding proteases and chitinases confirmed that ambient pH played a major role in regulating gene expression of secreted proteins. During fungal infection the pH of the host insect cuticle rose from 6.3–7.7. Alkalinisation of the cuticle, possibly by the fungus itself, is a physiological signal that triggers the production of pathogenicity factors. Interestingly chitinase was produced at pH 5 and 8 (optimum = pH 5). pH 8 is the optimum for Pr1 which is needed for chitinase activity.

Consistent with the changes observed in cuticle pH during mycosis St. Leger *et al.* (1999) observed that *M. anisopliae* 2575 neutralises environmental pH during growth on solid media containing yeast extract by the production of ammonia or organic acids (oxalic, succinate, acetic). Acid non-producing mutants had reduced ability to grow at pH 8 suggesting that acid production is linked to the ability to grow at higher pH. Hyperproductive acid mutants had reduced protease activity because of the acidification of the medium. Ammonia is produced by deaminases that are regulated by induction and catabolite repression. While this work establishes the possibility that ammonia may be a virulence factor for pathogenic fungi it also complicates the interpretation of the role of organic acid production by these fungi.

F. PRODUCTION OF CUTICLE-DEGRADING ENZYMES BY OTHER ENTOMOPATHOGENIC FUNGI

Pr1-like enzymes have been resolved in culture filtrates from *B. bassiana*, *V. lecanii*, *N. rileyi*, and *A. aleyrodis* (St. Leger *et al.*, 1987b; El-Sayed *et al.*, 1993; Chrzanowska *et al.*, 2001). Trypsin, Pr2-like, endoproteases are also produced by these fungi, though they are specific for a Phe Val Arg group and demonstrate less sensitivity to trypsin inhibitors (St. Leger *et al.*, 1987b; Gupta *et al.*, 1993b, 1994). Endo- and exo-acting proteolytic enzymes have been identified in cultures of other entomopathogenic fungi viz. the collagenases produced by *Entomophthora coronata* (Zygomycete) (Hurion *et al.*, 1977) and *Lagenidium giganteum* (Oomycete) (Hurion *et al.*, 1979), and chymotrypsins produced by *Erynia* spp. (Entomophthorales; Zygomycete) (Samuels *et al.*, 1990).

Pr1-like genes have been cloned and sequenced from two isolates of *B. bassiana* also (Joshi *et al.*, 1995; Kim *et al.*, 1999). Both genes had strong homology with each other and with *Pr1a* from *M. anisopliae* and protease K from *T. album*. Genes that are significantly similar to *Pr1* are present in the entomopathogens *A. flavus* and *V. lecanii* (St. Leger *et al.*, 1992a; Leal *et al.*, 1997). Pr1 from *B. bassiana* is repressible by glucose and N-acetylglucosamine and enhanced by protein, though unlike the enzyme from *M. anisopliae* no specific induction by cuticle protein has been established (Bidochka and Khachatourians, 1987, 1988a,b).

Catabolite repression probably accounts for the low expression of Pr1 from *M. anisopliae* in host haemolymph during mycosis. However, this level of control may not occur in all species or all isolates. Substantial amounts of extracellular proteases were present in haemolymph of *Bombyx mori* infected with *B. bassiana*, estimated by ELISA using a polyclonal antibody. Enzyme activity in the haemolymph was low possibly due to protease inhibitors (Shimizu *et al.*, 1993a).

Bye and Charnley (unpublished) showed significant differences in regulation of Pr1-like enzymes between isoforms of the same isolate and between isolates of *V. lecanii*. Of particular note is an isoform from KV71 with a pI of 8.6 that is induced specifically by NAG. For isolate KV42 all isoforms were not repressed by low molecular weight C or N individually though they were in combination. Consistent with the reduced effect of catabolite repression *in vitro* substantial subtilisin activity was found in aphids infected with this isolate during the early stages of mycosis (Fig. 5). Pr1 from five isolates of *V. lecanii* was produced to equal extents on locust and (host) aphid cuticles, suggesting no host-specific induction.

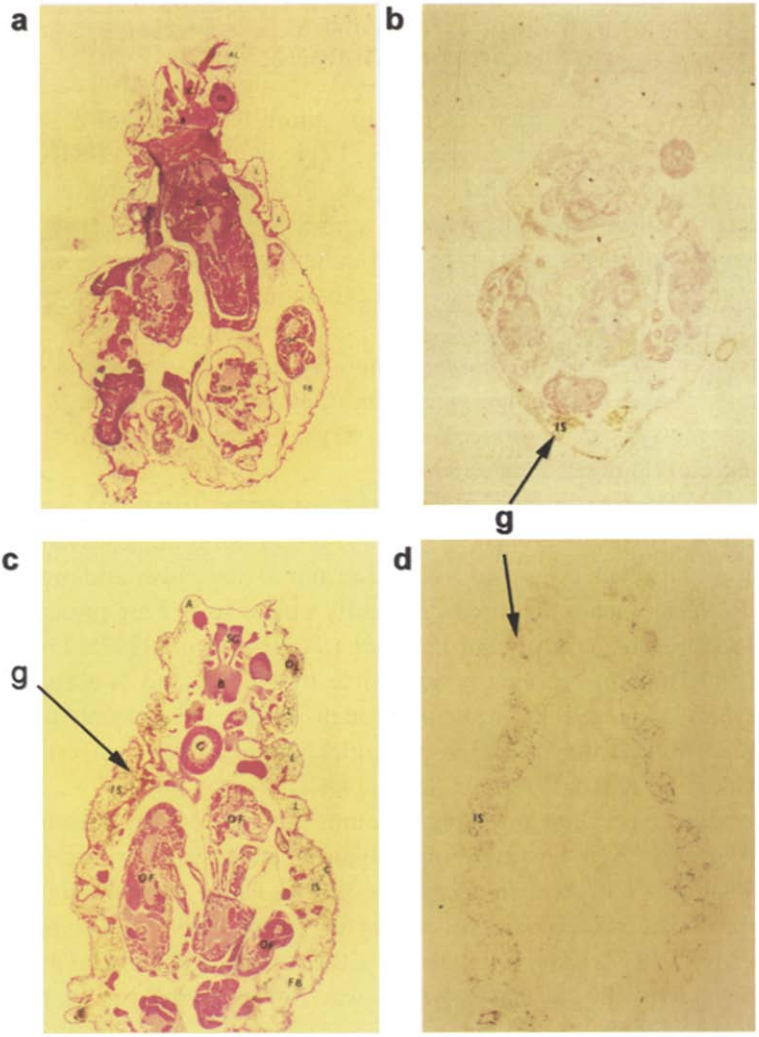


Fig. 5. Sections through whole adult peach potato aphids, *Myzus persicae*, infected with *Verticillium lecanii* (2d post inoculation). They have been immunostained with the Vectastain® ABC system using rabbit antibodies raised against Pr1 protease from either isolate KV71 or KV 42. (a) Control uninfected aphid counter stained with toluidine blue and acid fuchsin, (b) Aphid infected with isolate KV71, gold staining (g) shows location of antibody bound to protease, only a small area towards the bottom of the section, there is little protease in these insects outside of the cuticle because of catabolite repression, (c) Aphid infected with isolate KV42, gold staining (g) shows location of antibody bound to protease. It is all around the periphery of the insect in the cuticle and within subepidermal fatbody. This slide has been counterstained to show the other tissues, (d) Similar to (c) but without the counter stain so the immunostain only is visible.

Endo and exochitinases have been found consistently in culture supernatants of other entomopathogenic fungi, including *Nomuraea rileyi* and *B. bassiana* (Coudron *et al.*, 1984; Elsayed *et al.*, 1989; Bidochka *et al.*, 1993; St. Leger *et al.*, 1996b). Exochitinase from *B. bassiana* was induced by low concentrations but not repressed by high concentrations of the chitin monomer N-acetylglucosamine (Bidochka and Khachatourians, 1993b). Repression was exerted selectively by other low molecular weight compounds. Some amino acids were repressive while others were not. It appeared that the carbon skeleton of amino acids which repressed exochitinase synthesis were catabolised later in the TCA cycle i.e. α-ketoglutarate, succinyl-CoA and fumarate. Thus regulation could be where the amino acids enter the TCA cycle (Khachatourians, 1991).

In one of the few published papers on a lipase from an entomopathogenic fungus Hegedus and Khachatourians (1988) showed that lipase from *B. bassiana* was induced by olive oil but only during early stationary phase (5d post-germination) and they suggested that *in vivo* the enzyme may be important in colonisation of the haemolymph rather than the cuticle. This makes intuitive sense as most insects have high concentrations of diacylglycerol in the haemolymph.

G. EVIDENCE FOR A ROLE FOR CUTICLE-DEGRADING ENZYMES IN FUNGAL PATHOGENESIS

St. Leger *et al.* (1987c) extracted Pr1- and Pr2-like enzymes and an aminopeptidase from wings of *C. vomitoria* and abdominal cuticle of fifth-instar larvae of *M. sexta*, about 16 h after inoculation with *M. anisopliae*. Endoprotease activity was separated into two compounds which closely resembled Pr1 and Pr2 in pI, substrate specificity, and inhibitor spectrum. Purified extracts of infected blowfly wings tested by Ouchterlony gel diffusion against specific antiserum to Pr1 gave a single precipitin line identical to that given by the pure enzyme, confirming the presence of Pr1 during infection. The translucent wings of *C. vomitoria* have also been used to locate histochemically proteolytic enzymes during penetration (St. Leger *et al.*, 1987c). Substrates and inhibitors specific for Pr1 and Pr2 established the production of these enzymes on appressoria, which developed 10–24 h after inoculation. Aminopeptidase differed from endoprotease in that it was not present on immature appressoria, and the activity extended into the mucilage surrounding mature appressoria and appressorial plates. Pulse labelling with [^{35}S] methionine in conjunction with Western blot analysis employing Pr1 antibody showed that Pr1 forms the

majority of the protein being synthesised by appressoria *in vitro* and *in vivo* (St. Leger *et al.*, 1989a).

An immunogold technique has been used to locate Pr1 in cuticle during penetration of larvae of *M. sexta* (Goettel *et al.*, 1989). Protease was present on and close to infection structures and penetrant hyphae. The label was found more diffusely in the cuticle during later stages of pathogenesis. The two main Pr2 isoforms (pI 4.4 and 4.9) from *M. anisopliae* were ultrastructurally located to cell walls of the appressoria and other pre-penetrant structures of the fungus in contact with the cuticle (St. Leger *et al.*, 1996a). Within the cuticle, label was present on penetration peg and hyphal bodies. In contrast to Pr1 the enzymes were restricted to the vicinity of the fungus, even during later stages of infection. A matrix-like or fibrous material was present around the elements of the fungus which could be mucilage. It extended beyond the deposition of the enzyme. The zinc carboxypeptidase MeCPA has a specificity that complements Pr1 and the two enzymes could work co-operatively in releasing amino acids for nutrition from cuticular proteins. Consistent with this Joshi and St. Leger (1999) used immunogold to locate MeCPA to infection structures during cuticle penetration.

Pr1 appears to be a pathogenicity determinant by virtue of its considerable ability to degrade cuticle (St. Leger *et al.*, 1986d) and its production at high levels by the pathogen *in situ* during infection (St. Leger *et al.*, 1987c). Simultaneous application of turkey egg white inhibitor (TEI) and conidia significantly delayed mortality of *Manduca* larvae compared with larvae inoculated with conidia, supporting the importance of Pr1 in penetration (St. Leger *et al.*, 1988b). The inhibitor also reduced melanisation of cuticle (a host response to infection) and invasion of the haemolymph as well as maintaining the host's growth rate. TEI or antibodies raised against Pr1 delayed penetration of the cuticle but did not affect spore viability or prevent growth and formation of appressoria on the cuticle surface. This suggests that inhibition of Pr1 reduced infection by limiting fungal penetration of the insect cuticle. *In vitro* studies using TEI showed that accumulation of protein degradation products from the cuticle, including ammonia, was dependent on active Pr1. This confirms its major part in solubilising cuticle proteins and making them available for nutrition. It is interesting to note that Pr1 is resistant to serpins (a key class of protease inhibitor present in host cuticle and haemolymph) (St. Leger and Bidochka, 1996) and melanin produced *in situ* (St. Leger *et al.*, 1988a). Attributes which are consistent with an enzyme adapted for a role in insect parasitism.

Gillespie *et al.* (1998) failed to find a correlation between subtilisin production and pathogenicity for locusts among isolates of *M. anisopliae*.

A complication in this and other studies of this kind is that the different genetic backgrounds of the isolates tested may make them polymorphic for other characteristics that play a part in pathogenesis. Bidochka and Khachatourians (1990) found that a UV-induced protease-deficient mutant of *B. bassiana* displayed reduced virulence, but pleiotropic effects cannot be ruled out. Analysis of a *Pr1A* null mutant, produced by transformation-mediated gene disruption, did not provide unambiguous demonstration of a key role for Pr1 in the disease process. In those cases where there were no additional heterologous integrations mutants still retained near-normal pathogenicity under certain bioassay conditions (St. Leger, 1995). The onset of cuticle invasion coincided with secretion of high levels of Pr1b and a metalloprotease suggesting that these other cuticle-degrading proteases, that have broadly similar specificities, might partially substitute for Pr1a (St. Leger *et al.*, 1994b). This apparent redundancy in proteases helps to explain the previous observation that simultaneous application of a Pr1 inhibitor and *Metarhizium* conidia to the insect surface did not prevent infection, but did cause a significant delay in mortality (St. Leger *et al.*, 1988b).

Targeted gene disruption has been employed extensively in investigations of the part played by cell-wall degrading enzymes in fungal pathogenesis of plants. Here too null mutants e.g. of protease, cutinase and xylanase genes have failed often to have significant effects on pathogenicity (see review by Hamer and Holden, 1997), a pattern repeated with human and animal protease genes, even when knock-outs of several genes are achieved in the same isolate. These results are disappointing given the wealth of biochemical evidence that in many cases has implicated the corresponding proteins in pathogenesis. A variety of explanations have been offered: the bioassay was not sensitive enough to reveal the effect of the mutation, there was biochemical compensation by products of related genes (isoforms), there was functional redundancy (other enzymes can carry out the same task as appears to be the case with *Metarhizium*) and new group of similar enzymes produced *in vivo*.

An alternative strategy to reverse genetics to investigate the role of a gene in pathogenicity is to determine the phenotype of mutants expressing multiple copies of the target gene. Such an experiment has been done with the subtilisin protease *Pr1a* from *M. anisopliae* (St. Leger *et al.*, 1996c). Mutants containing multiple copies of the ORF behind a heterologous constitutive promoter produced protease *in vitro* on cuticle supplemented with 1% N-acetylglucosamine. The latter adjuvant is repressive in wild-type cultures. Large amounts of Pr1a were produced in the haemolymph during mycosis whereas only trace amounts were present in wild-type infections.

The enzyme caused hydrolysis of proteins, activation of endogenous trypsins involved in the regulation of prophenoloxidase and extensive melanisation; effects mimicked by the injection of pure Pr1. Survival time for infected insects dropped from 120 to 93 h. Accelerated death was due to the toxic action of the melanin as mutants had reduced fungal growth in the haemolymph. Reduced LT_{50} was not matched by reduced LC_{50} which would be expected if the increased protease had improved efficiency of cuticle penetration. This suggests that there are other constraints on cuticle penetration and that Pr1 protease was not limiting.

Chitin constitutes 17–50% of the dry weight of insect cuticle; more pliant cuticles have a higher chitin content than stiff cuticles (Hillerton, 1984). In the main, chitin fibrils are laid down parallel to the cuticular surface and as such present a potential barrier to penetration by entomopathogenic fungi. Thus it is interesting that St. Leger *et al.* (1987c) failed to find evidence of the production of chitinase during the first critical 40 h after inoculation of *C. vomitoria* wings or abdominal cuticle of *M. sexta* larvae with *M. anisopliae*. NAGase activity was extracted from infected cuticle, but this enzyme has only trace activity against polymeric chitin. The apparent absence of chitinase from infected cuticle could be due to inadequate extraction or inhibitors in the cuticle (chitinases *in vitro* binds tightly to locust chitin in a nonionic manner (St. Leger *et al.*, 1986b). Nevertheless, failure to detect the products of chitin hydrolysis in infected cuticle indicates that the activity of chitinase, if present, is negligible compared to that of protease. The slow appearance of chitinase *in vivo* (St. Leger *et al.*, 1987c) is consistent with *in vitro* results (St. Leger *et al.*, 1986c) and is probably due to the fact that chitinase is an inducible enzyme (St. Leger *et al.*, 1986e) and cuticular chitin is masked by protein (St. Leger *et al.*, 1986d). It seems likely that chitinase functions largely to provide nutrients during the saprophytic phase of fungal growth in cuticle of moribund insect hosts.

St. Leger *et al.* (1996b) raised polyclonal antibodies against the two purified chitinases from *M. anisopliae* and used them for ultrastructural location of the enzymes. No label was found on pre-penetrant structures or around the fungus during penetration of the cuticle up to 36 h post-inoculation, at a time when high levels of Pr1 and Pr2 are present. Label appeared and increased in intensity 36–60 h post-inoculation, both on the hyphal wall and, more often, extending into the surrounding cuticle. This is consistent with biochemical evidence that prior hydrolysis of protein by proteases expose chitin fibrils which results in the production and activity of chitinases.

The importance of chitin as a mechanical barrier to penetration and as a stabiliser of the cuticular protein matrix in the absence of fungal chitinase, is

evident from studies using acylurea insecticides that specifically inhibit chitin synthesis in insects. Diflubenzuron (as Dimilin) and teflubenzuron worked synergistically with *Metarhizium* to kill *Manduca sexta* (Hassan and Charnley, 1983) and *Schistocerca gregaria* (Joshi *et al.*, 1992) respectively. Ultrastructural observations demonstrated that fungal penetration through Dimilin-treated cuticle was dramatically enhanced (Hassan and Charnley, 1983). Postecdysial Dimilin-treated cuticle (without chitin) was almost completely destroyed in contrast to pre-ecdysial cuticle (laid down prior to insecticide treatment) where hydrolysis was apparently selective (presumably protein only) and restricted to the vicinity of the fungal hyphae. Consistent with these ultrastructural observations, pharate fifth-instar *Manduca* cuticle, produced during treatment with Dimilin and thus completely disrupted by the insecticide (Hassan and Charnley, 1989), was considerably more susceptible to Prl than control cuticle (St. Leger, Charnley, and Cooper, unpublished).

However, when multiple copies of chitinase gene from a *M. anisopliae* sf. *acridum* isolate 324 under a constitutive promoter from *Aspergillus nidulans* were transformed into *M. anisopliae* sf. *anisopliae* isolate 2575, the mutant did not show altered virulence to *M. sexta* (Screen *et al.*, 2001). Since the mutant 2575 isolate produced large amounts of the isolate 324 chitinase in non-inducing and inducing (chitin containing) media, neither wild-type levels of chitinase nor its mode of regulation are limiting for cuticle penetration. It is interesting that overexpression of the heterologous gene caused early production of the endogenous chitinase, probably due to the production of soluble inducers.

Askary *et al.* (1999) used the chitin-specific lectin wheat germ agglutinin to determine the effect of *V. lecanii* on chitin within the cuticle of the aphid *Macrosiphum euphorbiae*. The lectin was tagged with gold in a TEM study. Reduced labelling around penetrant hyphae was consistent with hydrolysis of chitin by the fungus. However, these effects on chitin were only observed when the fungus was already established in the aphid tissues suggesting that as in *M. anisopliae* so in *V. lecanii* chitinase is of secondary importance to the pathogenic process.

St. Leger *et al.* (1987c) detected esterase on pregerminating and germinating conidia and appressoria of *M. anisopliae* on wings of *C. vomitoria* with naphthyl acetate and naphthyl propionate as substrates. It is very difficult to differentiate histochemically between esterase and lipase. Although many workers have identified lipase produced by entomopathogenic fungi solely on the basis of activity against Tweens (e.g., Michel, 1981), such substrates are degraded by non-specific esterases (Pearse, 1972). Most microbial lipases are serine enzymes (Brockerhoff and Jensen, 1974)

and as such would not be distinguished from the non-specific esterases produced by *M. anisopliae*. The different sites of activity against Tween 80 (localised on appressoria) and naphthyl-AS-nonanoate (localised on conidia) suggest, however, that at least for *M. anisopliae* the enzyme-degrading Tween is distinguishable from at least one medium-chain-length non-specific esterase (St. Leger *et al.*, 1986e). Doubts may be cast as to the involvement of lipases in penetration because St. Leger *et al.* (1987c) failed to extract true lipase (with activity against olive oil) from cuticles of *M. sexta* and *C. vomitoria* infected with *M. anisopliae*. Failure to detect may reflect numerous factors such as binding of lipase to fungal cell walls and host cuticles. However, notwithstanding this the possible biological role of lipase in pathogenesis is not as obvious as many have assumed. Ultrastructural studies which have demonstrated the early histolysis of the 'wax' layer on cuticles underneath infection structures might implicate lipase/esterase activity (e.g. Zacharuk, 1970a). However, in most insects 'extractable' cuticular lipids are composed mainly of a complex mixture of alkanes and alkenes with triacylglycerols and wax esters (potential substrates for lipases and esterases) being comparatively minor components (Hepburn, 1985). Lipids could only have a major role if the 'bound' surface lipids and 'non-extractable' lipid fraction contain esters. Unfortunately, however, very little is known of the chemistry of bound cuticular lipids or their esters (Blomquist, 1984). Wigglesworth (1970) suggested tentatively that the outer epicuticle (Fig. 3) is a multiple polyester cross-linked by ester bonds. If this is correct, then an esterase with characteristics somewhat similar to cutinase would be required for its hydrolysis. However, no such enzyme is produced by *M. anisopliae* or *B. bassiana* (St. Leger, Charnley, and Cooper, unpublished). Lipoprotein lipases (active against olive oil emulsion activated with human blood plasma) are secreted. This enzyme perhaps in conjunction with Pr1 would aid penetration of the inner epicuticle (polymerised lipoprotein) (Fig. 3).

H. EVOLUTIONARY CONSIDERATIONS

It has been suggested that fungi emerged onto land as endophytes of plants (Lewis, 1987). Entomopathogenic fungi could have evolved to attack herbivorous insects either from endophytes or from plant pathogens because they would associate in a common niche. Such host shifts would presumably involve adaptations of extracellular hydrolases to allow degradation of insect cuticle (St. Leger and Bidochka, 1996). Perhaps it is no coincidence that some common underlying mechanisms of fungal pathogenesis occur

against insects and plants (Charnley, 1984). However, ability to infect insects must have evolved independently several times since entomopathogens are found in several divergent fungal groups. Thus while many phyto- and entomopathogenic fungi are found in the same taxonomic grouping, the Entomophthorales have no plant-pathogenic relatives. St. Leger and Bidochka (1996) suggested alternatively that insect-pathogenic fungi evolved from alimentary canal commensals or fungi that employ insects for transmission of spores, while Evans (1988) suggested a switch to parasitisim by saprophytes living off insect cadavers.

Entomophthorales fall within the Phylum Zygomycota and as such are towards the bottom of the fungal evolutionary tree, only preceeded by the Chytridiomycota (Berbee and Taylor, 2001). Among the Entomophthorales, *Conidiobolus coronatus* is one of the least specialised and least evolved; many of the others are host-adapted and difficult to growth *in vitro* on standard media (Evans, 1989). *C. coronatus* is probably essentially an opportunistic pathogen making the most of weakened hosts of many species (Papierok, 1986). In a recent EST analysis of genes expressed by *C. coronatus* during growth on insect cuticle as with *M. anisopliae* (Freimoser *et al.*, 2003b) found chitinases, multiple subtilisins (one with homology to a gene from *Thermus aquatica* others with homology to *C. carbonum* genes), trypsin (with homology to a *C. carbonum* gene), metalloproteases (homology to a gene from *Aspergillus fumigatus*) and aspartyl protease (similar to pepsinogen from *A. niger*). In contrast to *M. anisopliae* subtilisins, those from *C. coronatus* and several other entomophthoralean fungi tested showed enhanced expression and activity rather than repression following addition of soluble protein to an inducing insoluble protein source. It would be interesting to know if this attribute allows production in host haemolymph during mycosis. A pathogen that relies primarily on biomass rather than toxins to overcome its host (see later) would benefit enormously from being able to hydrolyse host proteins early on rather than just accessing free amino acids (which are in high concentration in insect haemolymph).

Some of the more specialised of the Entomophthorales viz. *Erynia rhizospora*, *E. dipterogena* and *E. neoaphidis* have a different kind of serine protease to the subtilisins found in the Hyphomycetes, that combine chymotrypsin and trypsin-like activity (Samuels *et al.*, 1990). Unlike the subtilisins, these entomophthoralean enzymes have poor locust-cuticle degrading ability. This may be because their enzymes are highly adapted to host cuticle or that like the insect's own moulting fluid cuticle degrading proteases they inherently have low cuticle-degrading ability. Controlled degradation of the old cuticle at the moult may be best served by slow

hydrolysis. Similarly the Entomophthoralean pathogens have an extended parasitic phase which may be promoted by a less aggressive degradation of host cuticle than that adopted by the toxin-assisted, subtilisin-facilitated necrotrophic Hyphomycetes (Samuels *et al.*, 1991, 1993a,b; Samuels and Paterson, 1995).

Facultative pathogens and saprophytes, which have to contend with a greater variety of sources of nutrients, often are polymorphic for their depolymerases and overall have greater genetic variation than obligate, specialised pathogens. Saprophytes such as *Neurospora crassa*, *N. nidulans* and *Aspergillus* spp. produce the broadest spectrum of exocellular depolymerases. This versatility should help such fungi tackle a broad diet, including the remains of dead insects and plants (St. Leger and Screen, 2000). Broad-spectrum proteases (subtilisins) are produced by saprophytes as well as insect pathogens. Thus, these enzymes are not *per se* an adaptation to insect pathogenicity (Gunkel and Gassen, 1989; St. Leger and Screen, 2000). The large number of Pr1 isoforms in *M. anisopliae* compared to subtilisins in saprophytes and plant-pathogenic fungi suggests that the proliferation of Pr1 genes in *Metarhizium* took place after the split between the plant and insect-infecting Pyrenomycetes (Bagga *et al.*, 2003).

St. Leger and Screen (2000) compared proteases produced by an opportunistic human pathogen, *A. fumigatus*, an insect pathogen, *M. anisopliae* and the plant pathogen *Haematonectria haematococca*. All three fungi produced subtilisins on mucin and lung polymers but smaller amounts on plant cell walls. However, insect cuticle was only stimulatory to *M. anisopliae*. Indeed only *Metarhizium* produced a full complement of isoforms on cuticle. The production of a similar complement of enzymes by all three species grown on host-related polymers suggests that some of the underlying mechanisms of fungal pathogenesis may be similar in insects, plants and animals. But there is some development of host-specific regulation of these factors. Consistent with this Bidochka *et al.* (1999) showed that *V. lecanii* 973, produced three key subtilisin isoforms only during growth on insect cuticle. Thus adaptation from dead to live host may be accompanied by altered properties and/or regulation of key hydrolases, allowing expression under conditions in which similar enzyme genes in non-pathogens are not transcribed (St. Leger *et al.*, 1991b). It is interesting that such diverse fungi as *M. anisopliae*, *V. lecanii*, *Tolypocladium niveum*, *Paecilomyces farinosus* and *Beauveria bassiana* produce a subtilisin-like protease under nutrient limiting conditions (on chitin, cellulose, cuticle), which is repressed when low molecular weight compound such as N-acetylglucosamine is added. Regulation is at the level of transcription and is an adjustment to altered conditions and not a response to stress.

Fungal pathogens of other organisms also produce subtilisins. VCP1 produced by the nematode pathogen *Verticillium chlamydosporium* has a similar pI (*ca.* 10), M_r (*ca.* 33 kDa) to Pr1 from *M. anisopliae* and is immunologically related. The two enzymes differ in inhibitor profile, substrate specificity and N-terminal sequence (Segers *et al.*, 1999). A Pr1-like enzyme has been cloned from the mycoparasitic fungus *Trichoderma harzianum*. Intriguingly, this protease, PrB1, which has the same substrate specificity as Pr1 against synthetic peptides, is also induced in a host-related manner, in this case by fungal cell walls or chitin (Geremia *et al.*, 1993).

In contrast to subtilisins, trypsins appear to have a specific role in pathogenesis (St. Leger *et al.*, 1997). They are not produced by saprophytes *N. crassa*, *N. nidulans* and *Aspergillus* spp. They are the major proteases produced by plant-pathogenic *Verticillium* spp. (St. Leger and Screen, 2000) and at least some other plant-pathogenic fungi, e.g. *Cochliobolus carbonum* (Murphy and Walton, 1996). In contrast in *M. anisopliae* and *V. lecanii* subtilisins are produced in greater quantity than trypsins. The trypsins produced by the plant pathogens and the insect pathogens have different specificities. Whereas the insect pathogenic *B. bassiana*, *V. lecanii*, *M. anisopliae* have trypsins that are particularly active against Bz Phe Val Arg, the plant pathogens both from *Verticillium* spp and other genera have broad spectrum trypsins e.g. *Cochliobolus carbonum* (Murphy and Walton, 1996; St. Leger and Roberts, 1997; St. Leger and Screen, 2000).

Expressed sequence tag libraries (EST) from cuticle-grown cultures of *M. anisopliae* sf. *anisopliae* isolate 2575 and the grasshopper-specific pathogen *M. anisopliae* sf. *acridum* isolate ARSEF 324 confirmed the wealth of cuticle-degrading enzymes revealed by earlier biochemical studies on this species (Freimoser *et al.*, 2003a). In particular proteases comprised 36% and 20% of the ARSEF 2575 and ARSEF 324 libraries respectively, with transcripts of 11 subtilisin genes in the former and 3 in the latter. In comparison with the richness of proteases genes in these entomopathogens there is a relative paucity of such genes in the genomes of *S. cerevisiae*, *N. crassa* and *A. nidulans* and EST libraries on other Ascomycetes; no chymotrypsins, often no trypsins, and only 2 or 3 subtilisin genes. Thus while subtilisins *per se* are not an adaptation to entomopathogenicity, the production of a variety of such enzymes may be critical to success. Indeed paralogs of all 11 of the Pr1 genes expressed on cuticle by the generalist *M. anisopliae* sf. *anisopliae* isolate 2575 were found in sf. *anisopliae* isolate ARSEF820 (ex beetle) and sf. *acridum* isolate 324 (grasshopper specific) (Bagga *et al.*, 2003). Insect cuticle comprises up to several hundred different proteins and a cocktail of enzymes with slightly different specificities may accelerate host invasion. Furthermore gene duplication and divergent

evolution may facilitate subtle and individual changes in regulation that promote production of each isoform at the right time, in the right place, in the right quantity and in the right combination.

I. ROLE OF CUTICLE-DEGRADING ENZYMES IN VIRULENCE AND SPECIFICITY

The possibility that virulence among isolates may be correlated (at least in part) with cuticle-degrading enzyme activity has stimulated several studies, with conflicting results (see Charnley, 1984). El Sayed *et al.* (1989) showed a relationship between chitinase activity (exo and endoenzymes) and virulence among isolates of *B. bassiana*. The greatest difference occurred during germination, though the most rapid increase in chitinases occurred at the onset of blastospore formation. However, the numbers of isolates involved was very small. Gupta *et al.* (1994) found high virulence among five isolates of *B. bassiana* for *Galleria mellonella* was associated with high activity of chymoelastase, chymotrypsin and chitinases. Comparisons between isolates for pathogenicity and production of enzymes, however, may only reveal the great variability within a species for numerous factors, many of which may influence but be unrelated to cuticle-degrading enzyme activity. Induction of mutants within a common genetic background is an alternative approach which has also been exploited. Dasilva *et al.* (1989) found *M. anisopliae* mutants selected for high amylase and lipase that showed greater virulence for *Triatoma infestans*, whereas hyperproductive protease mutants were either similar or less virulent than wild-type. Hypoproductive protease mutants from *B. bassiana* showed reduced virulence towards *Melanoplus sanguinipes* (Bidochka and Khachatourians, 1990). Comparable studies by others do not all show similar association. Interpretation of the results is, however, complicated as pleiotropic effects cannot be ruled out; in no case was a point mutation established. Paris and Ferron (1979) found a link (which is ambiguous; see earlier) between virulence and lipase in mutants of *B. brogniartii*. However, mutants deficient in other respects were also avirulent, suggesting that, as one might expect, pathogenicity was a function of many attributes. Similarly, Pekrul and Grula (1979) found that low pathogenicity of *B. bassiana* mutants against *H. zea* larvae, was not simply a consequence of the lack of a suitable enzyme cocktail. All mutants possessed varying levels of the three major enzyme activities regardless of pathogenicity and some mutants contained very high levels of certain activities and yet were poor pathogens.

Another approach to unravelling the role of cuticle-degrading enzymes in host specificity and virulence is to look at the properties of the enzymes

concerned. As is clear from this review, at present only endoproteases for Deuteromycete entomopathogens have been studied in sufficient detail to draw any significant conclusions. Cuticle-degrading endoproteases, with similar modes of action, are produced in quantity by all species studied (St. Leger *et al.*, 1987b), and it seems unlikely that they contribute to host specificity or virulence, though the occurrence of two or three types of extracellular protease in five genera of entomopathogen implies an indispensable function for these enzymes (St. Leger *et al.*, 1987b).

Leal *et al.* (1997) using nested PCR, have demonstrated significant variation in Pr1 gene sequence among 54 isolates from seven fungal species (primarily *M. anisopliae* but including *M. flavoviride*). Furthermore Pr1-like enzymes from isolates of *M. anisopliae* and other entomopathogenic Deuteromycetes differ in amino acid sequence since they vary in their response to a polyclonal antibody raised against Pr1 from *M. anisopliae* (2575) (St. Leger *et al.*, 1987b). Even a few strategic amino acid substitutions can affect cuticle binding and hence activity of Pr1 from *M. anisopliae* (St. Leger *et al.*, 1986a, 1992a). Thus it is possible that virulence between isolates can be influenced by variations in Pr1 sequence. Furthermore protein composition of insect cuticle varies between insect species. Isolate specificity could in part depend on the impact of Pr1 enzymes on host cuticular proteins. Thus it is interesting that purified basic isoforms of Pr1 from aphid isolates of *V. lecanii* degraded aphid cuticle significantly more effectively than non-host locust cuticle (Bye and Charnley, unpublished).

Cuticles of *Hyalophora cecropia* (Cox and Willis, 1985) and *Locusta migratoria* (Hojrup *et al.*, 1986) have a non-uniform distribution of charge, with negatively charged and positively charged proteins predominating in flexible cuticle (e.g. arthrodial membranes) and rigid body-wall cuticle, respectively. Thus, it is possible that regions of the cuticle may be favourable or unfavourable to binding (and thus degradation) by individual enzymes, with consequences for the parts of the insect body which can be invaded by enzymatic action. This may influence speed of penetration and thus virulence. Gillespie *et al.* (1998) looked at the ability of partially pure cocktails of Pr1 isoforms from cuticle cultures of 19 locust-pathogenic isolates of *Metarhizium* spp. to degrade insect cuticle. The cuticle types used were *Manduca sexta* pupal cuticle, adult desert locust (*Schistocerca gregaria*) wing cuticle, abdominal cuticle and pharate adult locust abdominal cuticle. These cuticle types differ in their protein composition and degree of sclerotisation. Pr1 hydrolysed the locust cuticle to different degrees. The data suggest a hierarchy for the susceptibility to hydrolysis in the order pharate adult abdominal > adult abdominal > wing. There was no significant

correlation between the ability of the enzymes to degrade any of the cuticle types and median lethal time of the isolates against the desert locust. Bidochka and Khachatourians (1994) also found that proteases from *M. anisopliae* and *B. bassiana* had differential action against acid and basic proteins from different parts of grasshopper cuticle.

The possibility that particular protease isoforms may play a part in host specificity is suggested by a recent paper by Wang *et al.* (2002). They found three spontaneous mutants of *M. anisopliae* that did not produce Pr1A or Pr1B; mutants were identified by nested PCR and confirmed by Southern analysis. These mutants had wild-type virulence to *Galleria mellonella* but 20% reduction in pathogenicity for *Tenebrio molitor*.

The possible importance of regulation not only in determining entompathogenicity *per se* but also species specificity is suggested by the observation that an EST library of the grasshopper-pathogen *M. anisopliae* sf *acridum* isolate ARSEF 324 grown on non-host cuticle (cockroach) contained transcripts of only a few of the trypsin and subtilisin genes identified in the genome whereas the EST library of the generalist isolate ARSEF 2575 was fully representative of the genome (Freimoser *et al.*, 2003a).

The virulence of facultative pathogens like *M. anisopliae* is the result of many factors that operate in concert to overcome the host. Identifying putative virulence determinants is potentially even more problematic for an opportunistic fungus like *A. fumigatus* in which there has probably been no selection for specific virulence genes (St. Leger and Screen, 2000).

VI. TOXINS

A. OVERVIEW

The definition of what constitutes a 'toxin' depends on the scientific discipline. A bacteriologist working on mammalian pathogens would use the term to describe high molecular weight proteins that have detrimental effects on their hosts (Roberts, 1980). Plant pathologists have restricted the term 'toxin' largely to low molecular weight compounds that are bioactive in low concentrations and are often products of so-called 'secondary metabolism' (Graniti, 1972). In this review no restrictions will be made on what can or cannot be called a toxin. Though, reflecting the state of knowledge, the focus will be primarily on small molecules, the potential effects of fungal proteases in the haemolymph during mycosis will also be considered thus bringing together both aspects of this review.

Compounds that have no apparent role in the primary metabolic processes of living things have been termed secondary metabolites; a name that reflects early views of their significance as a means of disposing of waste metabolic intermediates. 'Secondary metabolism' is particularly well developed in higher plants and fungi (Vining, 1990). Amongst the latter the most prolific producers occur in groups with adaptability to changing environments e.g. Basidiomycetes and Ascomycetes. Thus, it has been suggested that secondary metabolism is an area where biochemical evolution is taking place. In this view occasionally substances are manufactured that are of benefit to the producer. The problem with this approach is that it implies that some of the complex molecules synthesised by fungi may have no functional significance. This is hard to equate with the metabolic costs involved in their production. The range of biological activities of diverse and often complex molecules has ensured a large interest and commensurate literature.

B. INCIDENCE OF INSECTICIDAL TOXINS AMONGST ENTOMOPATHOGENIC FUNGI

A key problem for a pathologist is how to make sense of the bewildering diversity of toxic metabolites produced *in vitro* by entomopathogenic fungi in the context of the disease process. In part this is a technical problem viz. how to detect small quantities of chemicals in small insects. Additionally as 60 years of research on the mode of action of synthetic chemical insecticides have shown, detailed analysis of the *in vitro* effects of biocides may be hard to reconcile with symptoms expressed *in vivo*.

A *priori* it seems reasonable that insect pathogenic fungi might produce metabolites that would serve one or more of the following functions: toxic to the host and help to cause death; immunosuppressive to aid the fungus overcome host defence; antibiotic to suppress competition from other pathogens or the saprophytic flora on the cadaver *post mortem*; toxic to mycophagous organisms and thus provide a defence outside of the host.

Early commentators concluded from a consideration of histopathology that entomopathogens among the lower fungi (e.g. of the genera *Coelomomyces*, *Lagenidium*, *Entomophthora*) overcome susceptible hosts by utilising available nutrients in the haemolymph, followed by colonisation of parts or all of the living host (Evans, 1989). They may need to be fast growing when they are pathogens of rapidly developing insects or those which have short-lived adult stages e.g. aphids. Sporulation occurs very soon after host death – the saprophytic phase is minimal or completely absent. Locusts and grasshoppers infected with *Entomophthora grylli* climb

to the tops of plants prior to death. This so-called 'summit disease' may promote spore dispersal. It is certainly not in the best interests of the host which expose themselves to predation by this behaviour. While it has been suggested that fungal metabolites may control this behaviour, summit disease occurs in a number of insect species infected with viruses or fungi suggesting possibly a preprogrammed insect behaviour that is triggered non-specifically by microbial pathogens.

Consistent with these observations on mycosis, no toxic secondary metabolites have been described in cultures from *Entomophaga*, *Erynia*, *Lagenidium* or *Harposporium* species (Anke and Sterner, 2002). Initial reports of a high molecular weight toxin produced by *Entomophaga aulicae* (=*Entomophthora egressa*) (Dunphy and Nolan, 1982), have not been confirmed (Tyrrell, 1990). A 32-kDa cell lytic factor has been semi-purifed from larvae of the spruce budworm *Choristoneura fumiferana* mycosed with *E. aulicae* (Milne *et al.*, 1994). However claims that this protein is primarily responsible for symptoms observed in moribund larvae and host death are premature since it has not been established that the protein comes from the fungus.

Some of the most primitive of the Entomophthorales like *Conidiobolus* spp. may produce toxic secondary metabolites *in vitro* (Roberts, 1980) e.g. 4, 4′ hydroxymethyl azoxybenzene carboxylic acid (Fig. 6b) from *C. thromboides* (Claydon, 1978) and a 30-kDa insecticidal protein (Bogus and Scheller, 2002) from *C. coronatus*. Furthermore symptoms of wax moth larvae infected with *C. coronatus* were consistent with the effects of a toxin (Bogus and Szczepanik, 2000). Thus it is interesting that (Freimoser *et al.*, 2003b) in their EST study of gene expression in *C. coronatus* found few genes for toxic enzymes (e.g. no phospholipases), insecticidal secondary metabolite or antibiotic synthesising enzymes. Instead the high proportion of ESTs encoding ribosomal proteins and enzymes of intermediary metabolism suggest a facility for rapid growth. Of course, as these authors state this does not preclude the existence of a novel protein toxin.

In contrast to most of the Entomophthorales, the Deuteromycete and Ascomycete entomopathogens tend to have wider host ranges and kill their hosts rapidly. For many of these fungi circumstantial evidence is consistent with the involvement of fungus-derived biocides in pathogenesis: sparse growth of the fungus in the haemolymph of the host prior to death; changes in host behaviour such as reduced activity, paralysis, reduced feeding; pathogenic changes in the ultrastructure of host tissues in advance of penetrating hyphae; compromised immune system; reduced microbiota on the cadaver. Rapid killing consequent upon the activity of toxins, resulting in a low fungal biomass at the time of host death may reduce

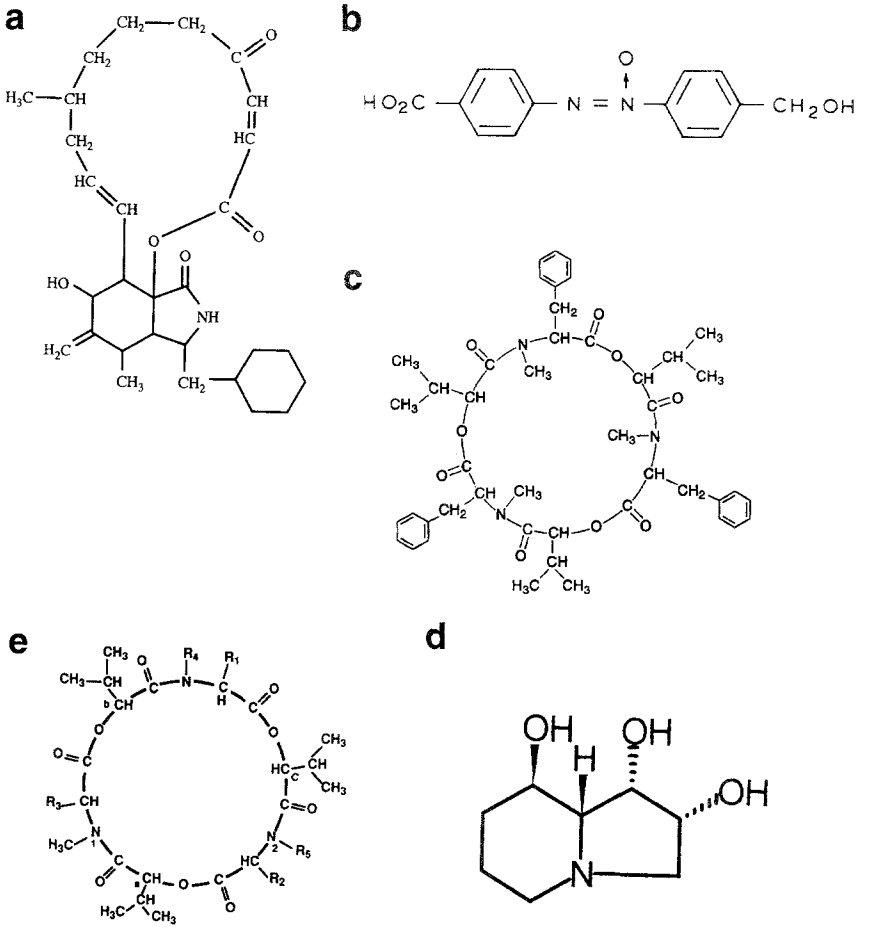


Fig. 6. (a) Cytochalasin (from *Metarhizium anisopliae*) a perhydroindole with a macrocyclic ring, (b) 4, hydroxymethyl azoxybenzene 4 carboxylic acid (from *Conidiobolus thromboides* (= *Entomophora virulenta*), (c) Beauvericin (from *Beauveria bassiana*, *B. brogniartii*, *V. lecanii*). A cyclic hexadepsipeptide comprising three residues of D-α-hydroxyvaleric acid alternating with three molecules of N-methyl-L-phenylalanine, (d) Swainsonine (from *Metarhizium anisopliae*), an indolizidine alkaloid, (e) Enniatin (from *Fusarium* spp.). Cyclohexadepsipeptides comprising three molecules of d-2-hydroxyisovaleric acid alternating with L-amino acids or N-methyl-L-amino acids. Enniatin A has 3 N-methylvaline residues (R1, R2, R3). R4 and R5 = CH_2.

the ability of the fungus to compete with the saprophytic microbiota unless antibiotic secondary metabolites are also produced. In common with other members of the Entomophthorales, *C. coronatus* uses rapid growth to overcome its host and exploit the cadaver before competitors overrun it.

What follows is a review of the most important of the toxic secondary compounds that have been identified in cultures of entomopathogenic fungi and an assessment of their actual or potential role in the development of disease. Evidence for the involvement of secondary metabolites in pathogenesis is limited to just a handful of compounds. Since some of these fungi are facultatively pathogenic with an alternative saprophytic existence in soil, the adaptive significance of these compounds in some cases may be to deter fungivorous insects or other Arthropods. Furthermore horizontal transfer of genes may result in the acquisition by entomopathogens of a variety of useful defensive compounds originally evolved by saprophytes to prevent mammalian and insect mycophagy. A further interesting ecological aspect of tritrophic relationships is seen in the periodic population decline of spruce budworm caused by the insecticidal cyclic peptide enniatin A/A1 (Fig. 6e), produced by the phylloplane fungus *Fusarium avanaceum* (Strongman *et al.*, 1987). There are other examples of plants gaining protection from insect and mammalian grazers through toxins produced by endophytic fungi (Clay, 1988). If, has been suggested, that fungi emerged onto land as endophytes of plants (Lewis, 1987) and entomopathogenic fungi evolved to attack herbivorous insects from endophytes because they associate in a common niche, then they would have a ready-made arsenal of insecticidal toxins. Interestingly *B. bassiana* exists endophytically in certain genotypes of maize (Vakili, 1990). Furthermore translocation within plants of conidia initially applied externally can provide season-long control of the European cornborer, *Ostrinia nubilalis* (Bing and Lewis, 1991).

Though dealing with microbial competitors on or off the host or its cadaver must be a key issue for an insect pathogenic fungus, there are comparatively few reports of antibiotic activity. This of course probably reflects a failure to look.

C. A SURVEY OF TOXIC METABOLITES PRODUCED BY ENTOMOPATHOGENIC FUNGI

A huge variety of toxic metabolites are produced by entomopathogenic fungi (see Table II which is not exhaustive, and Figs. 6 and 7). Of particular interest among non-protein metabolites are the viridoxins from ethanolic extract of mycelia of *M. anisopliae* var. *flavoviride*, which are closely related to Colletochin and Colletotrichin produced by the plant pathogen *Colletotrichum nicotianae* (Gupta *et al.*, 1993a). The viridoxins are insecticidal against the Colorado beetle *Leptinotarsa decemlineata*. Oosporein, a red-pigmented dibenzoquinone antimicrobial pigment,

is produced by isolates of *B. bassiana* and *B. brogniartii* and some other non-entomopathogenic soil fungi (Eyal *et al.*, 1994; Strasser *et al.*, 2000). Oosporein is antibiotic against gram-positive bacteria and to a lesser extent gram-negative also (Brewer *et al.*, 1984). Oosporein appears to be produced in cuticle but not haemolymph during mycosis of *Manduca sexta* by *B. bassiana* so it could help to suppress the saprophytic surface microbiota of the host, though there was no significant correlation between isolate virulence and incidence of oosporein in cadavers, which militates against a significant role in pathogenesis (Foley, Reynolds and Charnley, unpublished). Oosporein disrupts membranes (Jeffs and Khachatourians, 1997) which may account for inhibitory effects on Na^+/K^+ and Ca^{2+} ATPases. Interestingly oosporein was the only secondary metabolite produced in quantity by isolates of *B. brogniartii* (Strasser *et al.*, 2000).

Oxalic acid is a pathogenicity determinant of some plant pathogens (Godoy *et al.*, 1990) and this organic acid is also produced by *Beauveria bassiana* (Kodaira, 1961). Twelve isolates of *B. bassiana* all produced significant quantities of oxalic acid in Czapek-Dox liquid medium (mean of $400 \pm 46\,mg\,l^{-1}$) (Foley, Charnley and Reynolds, unpublished). Although oxalic acid ($108 \pm 9.5\,\mu g\,ml^{-1}$) was present in the haemolymph of *Manduca* caterpillars infected with a virulent isolate of *B. bassiana* 48 h after inoculation, significant amounts were also present in uninfected controls ($36 \pm 2.2\,\mu g\,ml^{-1}$). Oxalic acid could help facilitate invasion of the cuticle. Bidochka and Khachatourians (1991) showed that oxalic acid can solubilise cuticular protein and suggested that it may synergise with cuticle-degrading enzymes. However, subsequently they established that hyperproductive oxalic acid mutants were no different in pathogenicity for grasshoppers than wild-type (Bidochka and Khachatourians, 1993a).

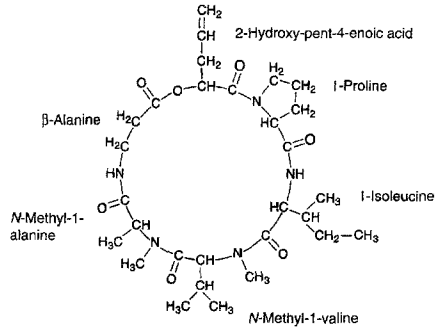
Isolates of *Aspergillus flavus* and *Aspergillus parasiticus* may be saprophytic, plant-pathogenic or insect-pathogenic. Isolates of all three nutritional strategies produce aflatoxins. The toxic effects of these compounds on insects include delayed development, diminutive pupal and adult size, reduced fecundity and sterility (Wright *et al.*, 1982). Fifteen pathogenic strains of *A. flavus* of silkworm (*Bombyx mori*) produced aflatoxins *in vitro* and aflatoxins have been extracted from infected larvae, though it is not clear whether in sufficient quantities to be the cause of death (Murakoshi *et al.*, 1977). Aflatoxin production *in vivo* has been demonstrated also by Drummond and Pinnock (1990). Although $14.2\,\mu g\,g^{-1}$ of aflatoxin wet weight of insect was extracted from infected mealybugs 7 d after inoculation, not all pathogenic isolates produced aflatoxins and a highly virulent isolate lost the ability to produce aflatoxin without any reduction in pathogenicity. Entomopathogenic strains of *Aspergillus* spp.

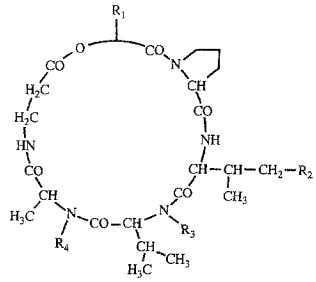
TABLE II
Toxins produced by entomopathogenic fungi

Chemistry	Name of compound	Fungus	Effect	Reference
Indolizidine alkaloid	swainsonine	*M. anisopliae*	α mannosidase inhibitor	Tamerler *et al.* (1998)
Perhydroindole with a macrocyclic ring	cytochalasins	ditto	Inhibit cell movement	Aldridge and Turner (1969)
A dibenzoquinone	oosporein	*B. bassiana*	Red pigment, antibacterial (gram positive > gram negative)	Vining *et al.* (1962)
Diterpene derivatives of polysubstituted γ-pyrones	viridoxins	*M. flavoviride*	Insecticidal	Gupta *et al.* (1993a)
Organic acid	oxalic acid	*B. bassiana*	Hydrolyses protein	Kodaira (1961)
Hydoxamic acid	tolypocin	*T. geodes*	Siderophore	Jegorov *et al.* (1993)
3-(6,8-dimethyl-E, E, E-deca-2, 4,6,-trienoyl)-1,4-dihydroxy-5-(p-hydroxyphenyl)-2(1H)-pyridone	bassianin	*B. bassiana*	Yellow pigment	Wat *et al.* (1977)
3-(4,6-dimethyl-E, E-octa-2, 4-dienoyl)-1, 4-dihydroxy-5-(p-hydroxyphenyl)-2(1H)-pyridone	tenellin	*B. bassiana*	Yellow pigment	ditto
5-n-butylpyridine-2-carboxylic acid	fusaric acid	*Fusarium solani*	Insecticidal	Claydon *et al.* (1977)
5-hydroxy-2-(hydroxymethyl)-4H-pyran-4-one	kojic acid	*Aspergillus flavus*	Insecticidal, inhibits insect phenoloxidase	Beard and Walton (1969); Dowd (1999)
Polyketide	aflatoxin B1	ditto	Insecticidal, interferes with growth and development	Murakoshi *et al.* (1977)
6-(1-prophenyl)-5,-6-dihydro-5-hydroxypyran-2-one)	phomalactone	*Hirsutella thompsonii*	Insecticidal, antifungal (inhibits germination of *B. bassiana*)	Krasnoff and Gupta (1994)
4′-hydroxymethylazoxybenene-4-carboxylic acid		*Entomophthora virulenta* (syn *Conidiobulus thromboides* Drechseler)	Insecticidal by injection	Claydon (1978)

triterpenoid	helvolic acid	*M. anisopliae*	Antibiotic	Turner and Aldridge (1983)
(3S,6R)-4-methyl-6-(1-methylethyl)-3-phenylmethyl-1, 4-perhydrooxazine-2,5-dione	bassiatin	*B. bassiana*	Platelet aggregation inhibitor	Kagamizono *et al.* (1995)
Pyridine-2,6-dicarboxylic acid	dipicolinic acid	*V. lecanii*	Insecticidal	Claydon and Grove (1982)
Two C_{25} keto acids		ditto	ditto	ditto
Diterpene derivatives of polysubstituted - γ - pyrones	viridoxins	*M. anisopliae* var *flavoviride*	Insecticidal	Gupta *et al.* (1993a)
Linear peptide	efrapeptins	*Tolypocladium niveum*	Insecticidal, antimicrobial, inhibitor of ATPase	Krasnoff and Gupta (1991)
Protein	M_r 14 kDa, hirsutellin	*H. thompsonii*	Ribosomal inhibitor	Mazet and Vey (1995); Liu *et al.* (1996)
Protein	M_r 10 kDa	*B. bassiana*	Immunosuppressive	Mazet *et al.* (1994)
Protein	TF2	*Beauveria sulfurescens*	Cytotoxic to insect but not mammalian cell lines	Mollier *et al.* (1994a)
Protein	M_r 100–129 kDa	*Beauveria sulfurescens*	Insecticidal, causes cuticle melanisation	Mollier *et al.* (1994b)
Protein	hydrophobin	*M. anisopliae*	Insecticidal	St. Leger *et al.* (1992c)
Cyclic peptide	cyclosporin	*Tolypocladium inflatum*, *Tolypocladium spp*, *B. bassiana*, *B. brogniartii*, *V. lecanii*	Immunosuppressive	Weiser and Matha (1988a); Jegorov *et al.* (1990)
Cyclic peptide	beauvericin	*B. bassiana*, *P. fumoso-roseus*, *Fusarium spp.*	Insecticidal, antibacterial (gram positive), antifungal, ionophore	Hamil *et al.* (1969)
Cyclic peptide	destruxin	*M. anisopliae*	Immunosuppressive	Kodaira (1961); Vilcinskas *et al.* (1997c)
Cyclic peptide	beauverolides	*B. bassiana*, *B. brogniartii*, *P. fumoso-roseus*, *Isaria* sp.,	Immunosuppressive	Grove (1980)
Cyclic peptide	bassianolides	*B. bassiana*, *V. lecanii*,	Insecticidal	Suzuki *et al.* (1977)
Cyclic peptide	enniatins	*Fusarium spp.*, *Alternaria kikuchiana*	Insecticidal	Visconti *et al.* (1992)

a Destruxin A **b Destruxin backbone**





Stuctures of key members of the destruxin series (see b). above

destruxins	R_1	R_2	R_3	R_4	MW
E_d	$-CH_2-CH(OH)-CH_2OH$	CH_3	CH_3	CH_3	611
D	$-CH_2-CH(CH_3)-CO_2H$	CH_3	CH_3	CH_3	623
E	$-CH_2-CH\overset{\diagup O \diagdown}{-}CH_2$	CH_3	CH_3	CH_3	593
C	$-CH_2-CH(CH_3)-CH_2OH$	CH_3	CH_3	CH_3	609
A	$CH_2-CH=CH_2$	CH_3	CH_3	CH_3	577
B	$-CH_2-CH(CH_3)_2$	CH_3	CH_3	CH_3	593
E_2	$-CH_2-CH\overset{\diagup O \diagdown}{-}CH_2$	H	CH_3	CH_3	579
C_2	$-CH_2-CH(CH_3))-CH_2OH$	H	CH_3	CH_3	595
A_2	$-CH_2-CH=CH_2$	H	CH_3	CH_3	563
B_2	$-CH_2-CH(CH_3)_2$	H	CH_3	CH_3	579
Ch1	$-CH_2-CH(OH)-CH_2CL$	CH_3	CH_3	CH_3	630
desMeB	$-CH_2-CH(CH_3)_2$	CH_3	H	CH_3	579
ProtoB	$-CH_2-CH(CH_3)_2$	H	H	H	565

Fig. 7. Structures of key members of the destruxin series

produce a variety of secondary metabolites including cyclopiazonic acid, aflatrem (Richard and Gallagher, 1979) and Kojic acid (Beard and Walton, 1969). The last named is insecticidal and can act synergistically with aflatoxin B-1 (Dowd, 1988).

Cytochalasins are a family of perhydro-indoles with a macrocyclic ring (Table II, Fig. 6a). They were first isolated from *M. anisopliae* (Aldridge and Turner, 1969) though other fungi are now known to produce them. An injected dose of up to $150\,\mu g\,g^{-1}$ has no detrimental effects on *Galleria mellonella* larvae (Roberts, 1980). However, cytochalasins are inhibitors of filamentous actin formation and *in vitro* reduce phagocytosis and spreading of insect haemocytes (Vilcinskas *et al.*, 1997b,c). Thus by interfering with the host cellular immune response these compounds could still have a role in mycosis.

A variety of proteinaceous toxins have been found. Cyclic peptides will be considered separately in the next section. The linear molecules will be surveyed here. Efrapeptins are a group of linear peptides, comprising 15 amino acids. They are produced only by *Tolypocladium* spp. (Krasnoff and Gupta, 1991) and its possible teleomorph *Cordyceps subsessilis* (Hodge *et al.*, 1996). There is intra and interspecific variation in efrapeptin production (Krasnoff and Gupta, 1992). Efrapeptins have limited antifungal and antibacterial activity as well as being insecticidal by injection and/or or by contact (Matha *et al.*, 1988; Krasnoff *et al.*, 1991). Antifeedant and growth inhibitory properties have been noted in treated insects also (Bandani *et al.*, 2000). They have been extracted from the haemolymph and whole bodies of *G. mellonella* infected with *T. niveum* but not in concentrations sufficient to cause host death. Furthermore, though limited growth of *T. niveum* in non-natural hosts such as *G. mellonella* prior to death is restricted, consistent with a toxin (Bandani *et al.*, 2000), hyphae ramify through mycosed mosquitoes and Chaoboridae (natural hosts of *Tolypocladium* spp.) and death has been attributed to starvation (Turlington *et al.*, 1990). Weiser and Matha (1988b) suggested that efrapeptins probably evolved as broad scale antibiotics rather than as components of the pathogenic process. Efrapeptins are potent inhibitors of ATPases from bacteria, and chloroplasts and mitochondria from a variety of organisms (Krasnoff *et al.*, 1991; Fricaud *et al.*, 1992). They also blocked cell surface expression of viral glycoproteins in a cell line infected with Newcastle disease virus and vesicular stomatitis virus but not the uptake of ricin and diptheria toxin from which Muroi *et al.* (1996) concluded that efrapeptins block exocytic but not endocytic trafficking of proteins. Interestingly though, efrapeptins are inhibitors of F-ATPase (Cross and Kohlbrenner, 1978), a key enzyme in ATP regeneration from ADP.

The mite pathogen *Hirsutella thompsonii* produces Hirsutellin A, a 15-kDa, pI 10.5, non-glycosylated, thermostable protein (Liu *et al.*, 1995) that is toxic by contact to its host, the citrus rust mite *Phyllocoptruta oleivora* (Omoto and McCoy, 1998). Hirsutellin is also insecticidal by

injection or by mouth to a range of insects, including aphids, fruit flies, mosquitoes (Mazet and Vey, 1995) and Lepidoptera which it kills slowly (within 8d) (Liu *et al.*, 1995). It is cytolytic to insect cells, but no other eukaryotic or prokaryotic cells tested (Liu *et al.*, 1996). It inhibits protein synthesis by specific cleavage of rRNA and as such is similar to the well-characterised ribosomal-inhibiting proteins (RIPs), α sarcin, mitogelin and restrictocin (Liu *et al.*, 1996; Boucias *et al.*, 1998). However, the hirsutellin gene is unique. The cloned cDNA does not contain the RNAase motif of fungal RIPs but does have other features in common with these proteins e.g. a series of consensus phosphorylation and myristoylation sites (Boucias *et al.*, 1998). Furthermore it encodes a single polypeptide rather than the two observed in some RIPs (Liu *et al.*, 1996). Hirsutellin A was isolated from 162 mite-associated isolates of *H. thompsonii*. Most isolates (100/162) contained the HtA gene. However, there was a lack of correlation between either the presence of the HtA gene or the amount of hirsutellin protein (semi-quantified using a monoclonal antibody) and mortality induced by culture filtrates or pathogenicity of isolates for *Galleria mellonella*. Thus HtA is not required for survival or pathogenicity and strains are likely to produce other toxins not yet characterised (Maimala *et al.*, 2002).

Cell-free haemolymph of larvae of the lepidopteran *S. exigua* in the later stages of infection with *B. bassiana* contains a >10 kDa protein that is highly toxic by injection to healthy larvae. The protein caused a permanent reduction in the numbers of spreading plasmatocytes though haemocyte viability and phagocytcic competence (determined *in vitro*) was not affected; symptoms also seen in haemocytes from mycosed insects. Insects appeared normal but died later in development (Mazet *et al.*, 1994).

D. CYCLICPEPTIDE TOXINS

1. Overview

Depsipeptides are a very varied group of compounds that comprise hydroxy and amino acids joined by amide and ester linkages to form a cyclic structure (Visconti *et al.*, 1992). The amino acids include methylated and other non-protein forms. The same compounds can be produced by a number of species (see Table III). Each compound often comprises a family of similar molecules which differ in one or a few residues. In addition there are a number of related depsipeptide families e.g. beauverolides (produced by *B. bassiana*, *B. brogniartii*, *P. fumoso-roseus*, *Isaria* sp.), bassianolides (*B. bassiana*, *V. lecanii*), beauvericins (*B. bassiana*, *P. fumoso-roseus*, *Fusarium* spp.) beauvaricins (*B. bassiana*), isarolids (= beauverolides), isariins (*Isaria*

TABLE III
Insecticidal secondary metabolites common to both entomopathogenic and non-entomopathogenic fungi*

Compound	Chemistry	Species	Reference
Beauvericin	Cyclic peptide	*Beauveria bassiana**	Hamil *et al.* (1969)
		*Paecilomyces fumoso-roseus**	Peeters *et al.* (1983)
		*Paecilomyces tenuipes**	Nilanonta *et al.* (2002)
		Fusarium proliferatum	Logrieco *et al.* (1997)
		F. semitectum	Gupta *et al.* (1991)
		F. subglutians	Logrieco *et al.* (1998)
Destruxins	Cyclic peptides	*Metarhizium anisopliae**	Kodaira (1961)
		*Aschersonia sp**	Krasnoff *et al.* (1996)
		Ophiosphaerella herpotricha	Venkatsubbaiah *et al.* (1994)
		Alternaria brassicae	Buchwaldt and Jensen (1991)
Enniatins	Cyclic peptides	*Fusarium oxysporum**	Madry *et al.* (1983)
		F. acuminatum	Visconti *et al.* (1992)
		F. avenaceum	Strongman *et al.* (1987)
		*F. lacteritium**	Tsantrizos *et al.* (1993)

fwelina, *Isaria cretacea*) and enniatins (*Fusarium* spp., *Alternaria kikuchiana*) (Anke and Sterner, 2002).

Beauvericin (see Fig. 6c) is a hexadepsipeptide, comprising a cyclic repeating sequence of three molecules of phenylalanine alternating with three molecules of hydroisovaleric acid. It is closely related to the enniatins (Fig. 6e) (Visconti *et al.*, 1992). It was first identified from *B. bassiana* (Grove and Pople, 1980; Gupta *et al.*, 1995) though it has recently been identified also in some plant pathogens e.g. *Fusarium* spp. (Gupta *et al.*, 1991; Logrieco *et al.*, 1998). Not all isolates of *B. bassiana* produce beauvericin (Frappier *et al.*, 1975). Several analogues of beauvericin have been described (Gupta *et al.*, 1995). Beauvericin is cationophoric and will increase permeability of membranes to Na^+ and K^+ ions (Steinrauf, 1985) and uncouple oxidative phosphorylation in isolated mitochondria. Apoptosis-like cell death was initiated in a murine cell line by beauvericin as a consequence of an increase in the cytoplasmic calcium concentration (Ojcius *et al.*, 1991). Beauvericin is bacteriocidal (Ovchinnikov *et al.*, 1971) and toxic to some insects (Gupta *et al.*, 1995) but not all (Champlin and Grula, 1979).

Fusarium lateritium, a pathogen of the scale insect, *Hemiberlesia rapax*, produces enniatins (Tsantrizos *et al.*, 1993). These are less active against the mosquito *Aedes aegypti*, than beauvericin but are more insecticidal against the blowfly *Calliphora vicina* (Grove and Pople, 1980). Plant-pathogenic *Fusarium* spp. also produce beauvericin and enniatin and both toxins have been implicated in disease (Hohn, 1997).

Beauverolide L was isolated by Jegorov *et al.* (1990) from mycelia of *B. brogniartii*. It is a tetradepsipeptide with a M_r of 516. Fifteen members of the family have been characterised.

Bassianolide, a cyclo-octadepsipeptide that comprises four molecules each of L-N-methyl leucine and D-α-hydroxyisovaleric acid, was extracted from mycelia of *B. bassiana* (Suzuki *et al.*, 1977). It is lethal to *B. mori* by ingestion. An injected dose caused atonic symptoms in *B. mori*, similar symptoms are seen in larvae infected with the fungus (Murakoshi *et al.*, 1978).

Cyclosporins are cyclic undecapeptides with immunosuppressive and anti-inflammatory activity in humans as well as having antiparasitic and antifungal properties (Wartburg and Traber, 1988). They were originally isolated from *Trichoderma polysporum* (Ruegger *et al.*, 1976) and many kinds of soil fungi, but more recently from *Tolypocladium cylindrosporium*, a pathogen of mosquito larvae. *B. nivea*, *B. bassiana*, *B. brogniartii* and *Verticillium* sp are among many entomopathogenic fungi that have subsequently been found to produce cyclosporins, though they were absent from isolates of *Hirsutella* sp, *Metarhizium* sp, *Paecilomyces farinosus* and *P. fumoso-roseus* (Jegorov *et al.*, 1990). Cyclosporins A, B and C have insecticidal properties (Weiser and Matha, 1988a). Swollen mitochondria and vacuolated rough endoplasmic reticulum occur in toxin-treated cells of mosquito larvae and in Malpighian tubules incubated *in vitro* (Dumas *et al.*, 1996b). However, injection or feeding of cyclosporin to *G. mellonella* and *Spodoptera exigua* had no effect, probably because of rapid removal from the haemolymph. Haemolymph proteins, particularly lipophorin, bind cyclosporin and remove it to the fatbody (Vilcinskas *et al.*, 1997a).

2. Destruxins: A Case History

The entomopathogenic fungus *Metarhizium anisopliae* produces a family of cyclic peptide toxins known as the destruxins (DTX) (see Fig. 7). To date some 30 variants have been identified in cultures of this fungus; all comprise five amino acids and an hydroxyacid (Kodaira, 1961; Suzuki *et al.*, 1970; Suzuki and Tamura, 1972; Pais *et al.*, 1981; Gupta *et al.*, 1989; Wahlman and Davidson, 1993; Chen *et al.*, 1995; Yeh *et al.*, 1996; Jegorov *et al.*, 1998). DTXs are produced during active fungal growth (Roberts, 1966a; Samuels

et al., 1988a; Amiri-Besheli *et al.*, 2000) and DTX A, E and B tend to predominate in culture. Destruxins have been described also from the entomopathogenic fungus *Aschersonia* sp. (Krasnoff *et al.*, 1996). It is interesting that DTXs (including two not found in *M. anisopliae* cultures) are produced by three unrelated plant pathogenic fungi, *Alternaria brassicae* (Bains and Tewari, 1987), *Trichothecium roseum* (Springer *et al.*, 1984) and *Ophiosphaerella herpotricha* (Venkatsubbaiah *et al.*, 1994). Natural analogues of destruxins have also been found e.g. roseotoxin (Engstrom *et al.*, 1975) and bursephalocids (Kawazu *et al.*, 1993).

Injection of DTX into lepidopteran larvae and adult Diptera causes immediate, tetanic muscular paralysis, followed by flaccidity; insects recover from low doses while high doses are lethal (Kodaira, 1961; Roberts, 1966b; Samuels *et al.*, 1988a,b,c). However, DTX has a wide range of other effects. DTXs inhibit Malpighian tubule fluid secretion in *Schistocerca gregaria* (James *et al.*, 1993) and ecdysteroid secretion by the prothoracic glands of *Manduca sexta* (Sloman and Reynolds, 1993) while stimulating the heart beat of *M. sexta* (Samuels, 1998). Cytopathological effects have been observed in cultured cells (Quiot *et al.*, 1985; Odier *et al.*, 1992) and in cells of the midgut and Malpighian tubules treated *in vitro* or *in vivo* with low doses of DTX (Vey and Quiot, 1989a; Dumas *et al.*, 1996b). Symptoms include vacuolisation of the cytoplasm caused by dilation of the endoplasmic reticulum, appearance of vesicles on microvilli and aggregation of chromatin in nuclei (Quiot *et al.*, 1985; Dumas *et al.*, 1996a). DTX also inhibits viral replication in culture cells (Quiot *et al.*, 1980; Yeh *et al.*, 1996) via an effect on RNA and DNA synthesis. Several studies have shown immunomodulatory effects of DTXs including inhibition of nodule formation around injected spores of *Aspergillus niger* (Vey, 1985; Huxham *et al.*, 1989; Vilcinskas *et al.*, 1997c).

DTXs can be toxic by injection, ingestion (Amiri *et al.*, 1999) and/or topical application (Poprawski *et al.*, 1994), depending on variant and host, though it is not clear how penetration of the cuticle is achieved. Lepidoptera and adult Diptera are particularly susceptible; on the whole other insects less so. The injected 24 h-median lethal dose for DTX A and B on *Bombyx mori* caterpillars was 0.015–0.030 mg g^{-1} (Kodaira, 1961) but *Galleria mellonella* was 10–30 times less susceptible (Roberts, 1966b). DTX A, B, C are repellant and antifeedant for a number of insects including *Plutella xylostella* and *Phaedon cochleariae* (Robert and Riba, 1989; Amiri *et al.*, 1999). DTX may, however, have a direct effect on growth also (Brousseau *et al.*, 1996; Amiri *et al.*, 1999). DTXs exhibit toxicity towards vertebrates. The injected intraperitoneal toxicity against mice is 1–1.35 mg kg^{-1} for DTX A and 13.2–16.9 mg kg^{-1} for DTX B (Kodaira, 1961) though effects

on fish and amphibians maybe less marked (Debeaupuis and Lafont, 1985; Genthner and Middaugh, 1992; Genthner *et al.*, 1998).

The first critical experiments on the effects of DTXs on lepidopteran muscle indicated that paralysis was due to calcium-dependent depolarisation of the muscle membrane (Samuels *et al.*, 1988c; Bradfisch and Harmer, 1990). Both studies established that DTX specifically and reversibly gated calcium channels in the plasma membrane. Destruxin-induced degranulation of crayfish (*Pacifastacus leniuseulus*) haemocytes was also calcium dependent, being abolished in calcium-free conditions and by cadmium ions (Cerenius *et al.*, 1990). Dumas *et al.* (1996a) found that DTXs induce calcium influx and phosphorylation of intracellular proteins within lepidopteran cell lines. The effects on intracellular calcium appeared secondary and may be brought about by the binding of DTX to an unidentified receptor (Dumas *et al.*, 1994).

Ultrastructural changes in Malpighian tubules and midgut epithelial cells *in vitro* caused by DTE E were Ca^{2+} dependent viz. the effects were absent in calcium-free media and in the presence of $CdCl_2$, a calcium channel blocker (Dumas *et al.*, 1996a). Although experiments by Samuels *et al.* (1988c) suggested that DTX did not exhibit ionophoric activity, structurally related molecules, the enniatins and beauvericin, move ions across membranes (Steinrauf, 1985) and Hinaje *et al.* (2002) demonstrated that DTX A can move calcium across liposomal membrane barriers.

A direct involvement for secondary messengers appears to be ruled out for the inhibition of ecdysone secretion by lepidopteran prothoracic glands (Sloman and Reynolds, 1993) and fluid secretion by locust Malpighian tubules (James *et al.*, 1993) because in both cases DTX inhibits responses to treatments elevating both cAMP and calcium. It is interesting that DTX B has been shown to be a specific, dose-dependent and reversible inhibitor of vacuolar-type ATPase from the yeast *Saccharomyces cerevisiae*, (Muroi *et al.*, 1994). This enzyme maintains acidic homeostasis in membrane-bound organelles in eukaryotic cells. Acidification of intracellular compartments, a pivotal event in many aspects of cell physiology, was also found to be blocked by DTX B. However, while DTX inhibited V-ATPase from mung bean and yeast (Muroi *et al.*, 1994) it had little effect on V-ATPase from barley and critically V-ATPase of brush border membrane vesicles from midgut columnar cells of *G. mellonella* (Bandani *et al.*, 2001).

In comparative studies DTX E often proves to be the most toxic of the DTX family probably because of its reactive epoxide group (Dumas *et al.*, 1994). A number of studies have synthesised analogues of DTX to investigate structure–activity relationship. D-Lac-destruxin E, with lactic acid in place of the hydroxy acid in DTX E, has lower activity than the

parent molecule. Synthetic analogues in which the ester bond is replaced by an amide bond are not active, showing the importance of the depsipeptide bond. Substituting the hydroxy acid with an alcohol or carboxy acid on side chain R resulted in a relatively inactive compund. Opening up the epoxide sidechain in DTX E to form a diol (which occurs *in vivo* during detoxification) reduces activity. None of the analogues were better than the parent (Cavelier *et al.*, 1996, 1997, 1998). Also in a sequence of hemisynthetic analogues, none was as good as the parent (Dumas *et al.*, 1994). The naturally occurring polar DTX D and DTX Ed are less active than DTX A or DTX E. Thus the epoxy group or double bond confer a high toxicity; a radical with free $COOH^-$ is weaker.

The ability of insects to detoxify DTX may have a bearing on host specificity of isolates. Removal of DTX from the haemolymph occurs much more quickly *in vivo* than *in vitro* suggesting that detoxification occurs primarily outside the haemolymph (Cherton *et al.*, 1991). The half-life of DTX in *G. mellonella* is 1 h. This corresponds to the timescale of recovery from paralysis from a comparable dose (Jegorov *et al.*, 1992). Injected ^3H destruxin was found in cuticle, haemolymph, midgut and hindgut; with high concentration in the middle two (Jegorov *et al.*, 1992). This is consistent with ultrastructural observations that cytotoxicity is greatest in midgut and haemocytes (Vey and Quiot, 1989b). DTX E is detoxified by hydrolysis and conjugation with glutathione in the migratory locust, *Locusta migratoria* (Loutelier *et al.*, 1994); the fat body in particular is responsible. Opening up the epoxide sidechain in DTX E forms a diol which is then excreted through the Malpighian tubules (Cherton *et al.*, 1991). Detoxification of DTX E occurs by similar mechanisms in *G. mellonella* (Hubert *et al.*, 1999) and E-diol has little activity upon injection in this insect (Dumas *et al.*, 1994). Detoxification of DTX A occurs by linearising the molecule (Lange *et al.*, 1991, 1992).

Interference with the cellular immune system seems the most likely impact of low doses of DTX during mycosis. DTXs reduced zymosan-induced nodule formation and activation of phenoloxidase in *Periplaneta americana* and *Schistocerca gregaria* (Huxham *et al.*, 1989). Injected blastospores of *M. anisopliae* cause morphological alterations to plasmatocytes (a class of insect blood cell involved with phagocytic uptake of microorganisms) of *G. mellonella*. The cells from injected insects, when presented with particles that normally evoke phagocytosis, remained rounded, did not attach to glass surfaces, had surface blebs and failed to form actin filaments and filipodia. *In vitro* DTX A and DTX E had similar effects on the morphology and behaviour of plasmatocytes (Vilcinskas *et al.*, 1997b). Additional features of DTX-treated cells viz swollen nuclei with clumped chromatin and blebbing are typical of cells undergoing programmed cell death (apoptosis). Thus the

toxin may be inducing apoptosis in haemocytes via the stimulation of key intracellular proteins. Consistent with this DTX E and concanamycin, another V-ATPase inhibitor, induced apoptosis in human tumor cells overexpressing epidermal growth factor receptor and stimulated by epidermal growth factor (Yoshimoto and Imoto, 2002).

Cytochalasin D, also produced by *M. anisopliae in vitro* (see earlier), like DTX, inhibited attachment. However, in contrast to DTX, spreading was not affected and haemocytes had a somewhat different appearance. Thus whereas the effects of DTX on haemocytes mirrored that seen in larvae infected with *M. anisopliae*, the same was not true of cytochalasin. Suggesting that the latter has at best a minor role in pathogenesis, consistent with this view, cytochalasins have not been found yet in mycosed insects (Vilcinskas *et al.*, 1997b).

Hydrophobic DTXs are enriched on cell walls of growing fungi (Matha *et al.*, 1992). If such DTXs are co-located with the equally hydrophobic cytochalasins on the surface of fungi they could work synergistically against the haemocytes (Vilcinskas *et al.*, 1997b). This strategy could also work for other cyclic peptides such as beauverolides or cyclosporins that are probably too hydrophobic to be found free in quantity in the haemolymph. The retention of secondary metabolites to the fungal cell-wall would not only place the toxins in a prime spot to target immunocompetent haemocytes, but also minimise the amount needed for activity. Injecting silica beads coated with either beauverolide or cyclosporin into *Galleria mellonella* provoked nodule formation, synthesis of antimicrobial peptides, as well as attachment and spreading of plasmatocytes; but no phagocytosis. Direct injection caused a smaller effect, probably because the toxins were taken up by haemolymph proteins (lipophorins) and removed from circulation (in the fat body). Conversely *in vitro* the toxins inhibit phagocytosis of silica beads, yeast cell and blastospores of *B. bassiana* in a dose-dependent manner but did not affect haemocyte attachment or spreading though cytoskeleton of haemocytes was impeded (Vilcinskas *et al.*, 1999). However, the effects of beauverolide and cyclosporin on the immune response are not seen *in vivo* during mycosis by *B. bassiana*, arguing against a role for these compounds in pathogenesis.

In summary, of all the cyclic peptide toxins produced by entomopathogenic fungi the best evidence exists for an involvement of DTXs in pathogenesis of *M. anisopliae*. However, there is considerable variation in the quantity of DTX produced *in vitro* by isolates of *M. anisopliae* (Pais *et al.*, 1981; Samuels *et al.*, 1988a; Loutelier *et al.*, 1994). Some isolates produce little or no DTX particularly those from *M. anisopliae* var. *majus* and *M. anisopliae* var. *acridum* (Kaijiang and Roberts, 1986;

Kershaw *et al.*, 1999). Production of destruxins is not dramatically affected by the composition of the culture medium (Roberts, 1966a; Jegorov *et al.*, 1989). This suggests that DTX is produced constitutively and thus it is likely that *in vitro* studies are a true reflection of isolate capability and thus DTX production is not a prerequisite for pathogenicity.

Identifying fungal metabolites in mycosed insects is the first step to establishing a role for the chemicals in pathogenesis. DTX B and desmethyl destruxin B were identified in *B. mori* infected with *M. anisopliae* by Suzuki *et al.* (1971) and quantified by the amount of β-alanine present (assuming all the β-alanine comes from DTX, though cuticle may also contain β-alanine). The amount present in dead insects was sufficient to have caused mortality. Vey *et al.* (1986) also extracted biologically significant quantities of DTX from the haemolymph of *Oryctes rhinoceros* and *B. mori* using HPLC. Trace amounts of DTX A and B were found in *G. mellonella* infected with *M. anisopliae* var. *anisopliae* and var. *majus* but not var. *flavoviride* isolates (Amiri-Besheli *et al.*, 2000). Samuels *et al.* (1988b) extracted DTX A from *M. sexta* infected with the virulent isolate 2575 but not from insects infected with two pathogenic but less virulent isolates. Onset of paralysis in diseased insects, similar to that seen in DTX-injected caterpillars, has suggested a direct link between DTXs and death from mycosis in some cases (Roberts, 1966b; Suzuki *et al.*, 1971; Vey *et al.*, 1986; Samuels *et al.*, 1988b). Paralysis could interfere with haemolymph circulation, gaseous exchange and other vital functions. Given the variety of tissues susceptible to DTX the lethal lesion may not involve the muscles. Failure of an isolate to produce *in vivo* a sufficient quantity of DTX to induce muscular paralysis, therefore, does not preclude an involvement for DTX in pathogenesis. Samuels *et al.* (1988b) showed that among insects from 5 orders only Lepidoptera and adult Diptera were knocked down by low doses of DTX. This may reflect the sensitivity of the muscles from these different types of insects though other factors such as detoxification may also be important. If DTX has a role in pathogenesis of *M. anisopliae* then in most cases tissues other than muscles must be targeted.

A positive correlation between virulence and destruxin production among hyper- or hypoproductive mutants would constitute good evidence for the involvement of DTX in mycosis. Indeed Al-Aidroos and Roberts (1978) found a spontaneous mutant of *M. anisopliae*, significantly more virulent for mosquito larvae than wild-type, that had enhanced *in vitro* destruxin production. However, the mutation probably involved several loci since the mutant also had more dense sporulation and more rapid *in vitro* germination than the wild-type.

Amiri-Besheli *et al.* (2000) found that most virulent isolates produced large quantities of DTX but some low toxin producers were also pathogen.

Kershaw *et al.* (1999) also studied the relationship between DTX production and isolate virulence using three species of insect and isolates of *Metarhizium*. A significant negative correlation was found between the titre of DTX produced *in vitro* by isolates of *M. anisopliae* var. *anisopliae* pathogenic for the vine weevil *Otiorhynchus sulcatus* and median lethal time, suggesting a role for the toxin in isolate virulence. The same was true for isolates active against *M. sexta*. Growth form of the fungus in the haemolymph was clearly associated with virulence towards *M. sexta*. The more virulent isolates tended to grow more profusely as blastospores than hyphal fragments; the reverse was true for the weaker pathogens. The larger size of the hyphal fragments compared to blastospores suggested that there was greater growth of less virulent isolates in the haemolymph of insects prior to death. The appearance of infected insects was consistent with this interpretation. Five of the seven most virulent isolates all displayed flaccid paralysis prior to death. This symptom is also caused by injection of DTX (Samuels *et al.*, 1988b,c). A key exception was isolate 703. This is highly virulent for *M. sexta* yet does not produce DTX *in vitro*, grows largely as hyphal fragments in the haemolymph of infected insects and does not cause host paralysis. A recent study, using the fungal-specific ergosterol as a measure of fungal biomass, revealed a much greater growth of 703 than 2575 (a virulent, high DTX producer) in mycosed *Manduca* prior to death (Graystone and Charnley, unpublished). Thus there are are two possible virulence strategies among isolates of *M. anisopliae* var. *anisopliae* pathogenic for *Manduca* viz the 'toxin strategy' and the 'growth strategy'. For desert locusts, *Schistocerca gregaria*, a strong positive correlation was found only between *in vitro* toxin production and % mortality of individuals where sporulation did not occur on the cadaver. To account for this Kershaw *et al.* (1999) suggested that if DTX kills locusts before the fungus has established itself then the pathogen may not compete effectively with the saprophytic flora and as a result fails to sporulate. They concluded that, in the pathogenesis of *M. anisopliae* var. *anisopliae* for all three insects, there was a relationship between the titre of DTX production by isolates *in vitro* and killing power. The fact that little or no DTX is produced by certain specialist isolates of *M. anisopliae* e.g. *M. anisopliae* var. *majus*, pathogenic for scarabaeid beetles (Kaijiang and Roberts, 1986), *M. anisopliae* var. *album*, hemipteran specific (Amiri-Besheli *et al.*, 2000), *M. anisopliae* var. *acridum*, pathogens of locusts and grasshoppers (Kershaw *et al.*, 1999) is also consistent for a role for DTX in isolate virulence.

Definitive evidence for the involvement of destruxin in virulence would come from the use of transformation-mediated gene disruption to produce a single lesion mutant of the 'destruxin gene'. In common with other

cyclic peptide antibiotics produced by fungi e.g. beauvericin, enniatins and cyclosporins, DTXs are probably synthesised non-ribosomally by a multifunctional peptide synthetase (for review, see Kleinkauf and von Dohren, 1990). These enzymes possess a multidomain structure and employ the thiotemplate mechanism to activate, modify and link together by amide or ester bonds the constituent amino acids (Stachelhaus and Marahiel, 1995). The domains (one for each amino acid residue) act as independent enzymes. The order of the domains defines the sequence of the eventual peptide. Type I domains handle hydroxy and amino acids, type II domains are bigger and have the additional function of methylating the residue they handle. The advantage of this in comparison to ribosomal synthesis is a 6-fold lower consumption of ATP per peptide bond, though substrate fidelity is sacrificed, as evidenced by the variety of molecules produced in the series. The destruxin synthetase gene has not yet been cloned, though recent work in our lab has resulted in the cloning and sequencing of a cyclic peptide synthetase (of unknown function) from *M. anisopliae* (isolate 2575) and has provided evidence for the existence of others (Bailey *et al.*, 1996; Bailey *et al.*, unpublished).

In an express sequence tag study of *M. anisopliae* isolates grown on insect cuticle, (Freimoser *et al.*, 2003a) found several transcripts encoding enzymes (cyclic peptide synthetases, reductases) involved in the synthesis of toxic metabolites in the library of *M. anisopliae* sf. *anisopliae* 2575 and the absence of counterparts in the library of *M. anisopliae* sf. *acridum*. The former as has been noted above kills its hosts quickly apparently through the action of toxins, while the specific grasshopper/locust pathogen *M. anisopliae* sf. *acridum* invades all tissues of the host and the insect dies when it is filled with fungal biomass (Inglis *et al.*, 2001). This provides further support of the 'toxin' and 'growth' strategies employed by isolates of *Metarhizium* spp. suggested by Kershaw *et al.* (1999) (see above).

A number of fungal genes involved in the production of cyclic peptide toxins have been cloned and sequenced. They tend to be clustered, often being positioned with regulatory elements and autoresistance genes less than 2 kb from each other. Most fungal genes for other biosynthetic pathways follow the normal eukaryotic model of dispersion throughout the genome. Walton (2000) has argued that the grouping of genes in this way is an aid to retention during horizontal transmission; they are more likely to persist in an active form if they are moved together. Horizontal movement of fungal genes between isolates and species through hyphal anastamosis is important for the retention of the characteristic in the light of the relative inefficiency of vertical transmission due to the instability of fungal genomes. The alternative is the clustering of genes to aid co-regulation. However,

secondary metabolite genes are controlled by trans-acting transcription factors which can control the expression of dispersed or clustered genes. The horizontal movement hypothesis accounts for the presence of the toxin in some isolates of a species and not others.

Some species have an arsenal of toxins e.g. *B. bassiana* has beauverolides, bassianolides, beauvericins and cyclosporins. The gene clusters coding for these attributes may have been acquired by horizontal transfer. This would account for the occurrence of the same molecule in a number of different fungal species e.g. destruxin in the insect pathogens *M. anisopliae* and *Aschersonia* sp. and in three unrelated plant pathogenic fungi, *Alternaria brassicae* (Bains and Tewari, 1987) , *Trichothecium roseum* (Springer *et al.*, 1984) and *Ophiosphaerella herpotricha* (Venkatsubbaiah *et al.*, 1994). What we don't know yet is whether the cocktail of toxins acquired by a particular species and/or isolate influences host range.

E. PROTEASES AND OTHER ENZYMES AS TOXINS

Although subtilisins and metalloproteases from *M. anisopliae* and other entomopathogenic Deuteromycotina are particularly active against insect cuticle their broad specificity could make them effective weapons during later stages of mycosis. Kucera (1980, 1981) was the first to demonstrate the insecticidal effects of *M. anisopliae* proteases (partially purified serine and cystiene enzymes) upon injection into wax moth larvae. More recently several authors (Vilcinskas *et al.*, 1997b,c; Vilcinskas and Wedde, 1997; Griesch and Vilcinskas, 1998; Griesch *et al.*, 2000) have shown that *in vitro* proteases from *B. bassiana* and *M. anisopliae* impaired attachment, spreading, cytoskeleton formation and inhibited phagocytosis of plasmatocytes from *G. mellonella*. Metalloproteases were more effective than serine enzymes (trypsin and chymotrypsin). Similar symptoms were seen also *in vivo* during mycosis (Vilcinskas *et al.*, 1997b)

The role of proteases in the toxic activities of pathogens and predators is well established outside of the fungi e.g. proteases produced by *Pseudomonas aeruginosa* play an important part in pathogenesis (Lysenko and Kucera, 1971) and snake venoms comprise proteases and other enzymes (Zeller, 1977). However, the problem with seeking a role for fungal proteases in pathogenesis outside of cuticle invasion is that there have been few reports of significant activity in haemolymph and other tissues during the pathogenic phase. A number of studies have established the importance of catabolite repression in the regulation of fungal proteases (St. Leger *et al.*, 1988c; Paterson *et al.*, 1994b). Thus the low

Pr1 activity observed in haemolymph during later stages of infection of *M. sexta* with *M. anisopliae* (St. Leger *et al.*, 1996c) could be due to the repressive effects of amino acids and other low molecular weight metabolites. That this may not be true for all isolates of all species of entomopathogenic fungi is shown by the fact that Shimizu *et al.* (1993a,b) detected elastase-type ('subtilisin') *B. bassiana* protease serologically in the haemolymph of mycosed *B. mori* larvae. However, activity was low, they suggested, because of host protease inhibitors. We have found recently that regulation of Pr1 enzymes can vary between isolates and isoforms within isolates of *V. lecanii*. Thus while Pr1s from KV42 are not subject to C or N repression *in vitro*, KV71 are. As a consequence *in vivo* during pathogenesis of the peach potato aphid *Myzus persicae*, significant Pr1 activity appeared early in aphids infected with KV42, but not KV71 (Bye and Charnley, unpublished). Furthermore immunochemical staining of whole body sections of infected aphids using specific antibodies showed the widespread presence of Pr1 in cuticle and peripheral fatbody of insects in the early stages (2 days) of mycosis with KV42, but little in aphids infected with KV71 (see Fig. 5) (Bye and Charnley, unpublished).

The subtilisins of *C. coronatus* are less subject to catabolite repression (Freimoser *et al.*, 2003b). Thus they could be produced in the haemolymph during mycosis. Consistent with this the blackening of the haemolymph in mycosed insects infected with this fungus is reminiscent of the activation of host prophenoloxidase by *Metarhizium* Pr1 subtilisin experimentally and *in vivo* by hyperproductive, constituent mutants (St. Leger *et al.*, 1996c).

The presence of an array of protease inhibitors in insect haemolymph with activity against pathogenic enzymes implies an essential function and thus the potential importance of fungal enzymes in disease development. *M. sexta* has 15 different plasma serpins (Kanost, 1999). One of these has 12 alternatively spliced versions of exon 9, each encoding a different reactive site with a different proteinase targeting. Interestingly Vilcinskas and Wedde (1997) showed a protease inhibitor in the haemolymph of *G. mellonella* induced by the injection of *B. bassiana* spores. This inhibitor inhibited pathogen proteases and germination *in vitro*. Fungal invasion through the cuticle following topical application (a 'natural infection') did not elicit production of the inhibitor.

Thus fungal proteases may have a part to play in pathogenicity other than during host invasion through the cuticle and destruction of the cadaver *post mortem*. The effects of a hyperproductive Pr1 mutant under the direction of a constitutive promoter suggest reasons why fungi have not generally evolved to release highly active proteases during the haemolymph growth phase. St. Leger *et al.* (1996c) found the extracellular enzyme produce

during mycosis caused in *M. sexta* by these mutants caused widespread activation of the phenoloxidase cascade and the production of large amounts of melanin. This contributed to early death of the insect and a large significant reduction in spore production by the fungus on the cadaver.

Phospholipases are important cytotoxins produced by insect and mammalian bacterial pathogens (Lysenko, 1985). While *M. anisopliae* and other entomopathogenic Deuteromycotina are known to secrete phospholipases *in vitro* and transcripts of the corresponding genes have been found in EST libraries of isolates grown on cuticle (Freimoser *et al.*, 2003a), the importance of these enzymes in mycosis is yet to be established.

Undoubtedly the increasing application of molecular techniques to entomopathogenic fungi will reveal other novel specific toxins, some of which may even be host-specific as has been found with phytopathogenic fungi. Recent research has shown that *Nomuraea rileyi* secretes a specific enzyme that oxidises the hydroxyl group at position C22 of haemolymph ecdysteroids and prevents moulting in larvae of the Japanese silkmoth *Bombyx mori* (Kiuchi *et al.*, 2003). If this mirrors what happens in mycosis then posssibly *N. rileyi* in common with insect nuclearpolyhedrosis viruses has evolved a way to interfere with moulting by which it keeps its host feeding and thus in an optimum nutritional state. Interestingly DTX from *M. anisopliae* inhibits production of ecdysone by isolated prothoracic glands from *M. sexta in vitro* (Sloman and Reynolds, 1993). This could account for instances where development is disrupted in caterpillars infected with *M. anisopliae* (Sloman and Reynolds, 1993).

F. MULTIPLE TOXINS – SYNERGY OR SPECIFICITY?

There appear to have been few experimental demonstrations of synergy between toxic insecticidal secondary metabolites produced by entomopathogenic fungi. In one of the few Dowd (1988) showed enhanced toxicity of combined application of two co-occuring fungal metabolites, aflatoxin B- and kojic acid produced by *A. flavus*, to two species of lepidopteran caterpillar. Hydrophobic DTXs are enriched on cell walls of growing fungi (Matha *et al.*, 1992). Also, as stated earlier, if such DTXs are co-located with the equally hydrophobic cytochalasins on the surface of fungi they could work synergistically against the haemocytes (Vilcinskas *et al.*, 1997b).

Yoder and Turgeon (2001) have suggested that pathogens carry more genes involved in the production of secondary metabolites, particularly non-ribosomal multifunctional peptide synthetases (NRPSs) and polyketide

synthetases, than saprophytes. Their survey of the literature is enriched for phytopathogens. But it is clear from the present review that Deuteromycete entomopathogens in common with their plant pathogenic cousins produce a range of cyclic peptide toxins that derive from NRPSs.

As has been noted several times in this review useful analogies can be made between fungal pathogenesis in insects and plants. Good evidence exists for the role of NRPSs as virulence factors in phytopathogens (Herrmann *et al.*, 1996; Walton, 1996; Johnson *et al.*, 2000). The abundance of NRPS genes in these plant pathogens (Yoder and Turgeon, 2001), appears to be matched by the situation in *M. anisopliae*.

We have cloned and sequenced an NRPS from *M. anisopliae* (PES1) comprising a 23733 bp ORF encoding a protein of 5158 amino acids (Bailey, Reynolds, Charnley and Clarkson, unpublished). Analysis shows that this polypeptide contains only four domains with no evidence for amino acid methylation motifs. The inferred amino acid sequence shows similarity to those of other peptide synthetases. The conserved domain region contains sequences similar to those of the six core motifs and this domain shows most similarity (36% identity) to the second domain of the gene encoding the HC tetrapeptide toxin from the plant pathogenic fungus *Cochliobolus carbonum*. DTX contains six residues and we predict that the gene encoding DTX synthetase will contain six domains and include methylation motifs associated with two of the domains. The sequenced gene is therefore unlikely to encode DTX synthetase. Southern analysis of a wide range of isolates of *M. anisopliae* demonstrated that all contained DNA that hybridised strongly to a probe based on PES1. No clear difference in pattern or intensity of hybridisation was seen between DTX producers and non-producers. This observation adds weight to the contention that the sequenced gene encodes a peptide synthetase other than DTX synthetase. No Northern analysis with this clone was carried out. However, an RT-PCR demonstrated that this gene is expressed in *M. anisopliae* (2575). Through a PCR strategy using primers based on those described by Nikolskaya *et al.* (1995) we found sequences consistent with at least 17 peptide synthetase domains including the four in PES1, some of which include methylation motifs. Thus, *M. anisopliae* may have multiple NRPS genes that synthesise several families of cyclic peptide.

VII. THE FUTURE

The 21st century is an exciting time to be involved in research on microbial pathogenesis of eukaryotes. The availability of the sequences of whole

genomes from an increasing number of organisms is giving realistic expectation of the resolution of long-standing far-reaching questions on the nature of pathogenesis. Where do pathogens acquire pathogenicity genes from? There is evidence from human pathogenic bacteria for horizontal transfer of chromosomal genes ('pathogenicity islands') and plasmids. A similar phenomenon may occur in phytopathogens (Rosewich and Kistler, 2000). Do pathogens have unique pathogenicity genes or are 'house-keeping' genes co-opted (Yoder and Turgeon, 2001)? Evidence for phytopathogenic fungi suggests both situations prevail. It is still too early to expect answers to such searching questions for entomopathogenic fungi, though there are some tantalising pieces of evidence.

CHY1 a chymotrypsin produced by *M. anisopliae* belongs to the S2 group of chymotrypsins. It is most closely related to protease C from the actinomycete *Streptomyces griseus*. Both have only 15% identity to mammalian S1 chymotrypsin. Plant- and insect-pathogenic Ascomycotina and actinomycete bacteria are the only known microbial sources of S1 trypsins. Furthermore, S1 and S2 homologues appear to be absent from the ascomycete yeasts *S. cerevisiae* and the saprophytic filamentous ascomycetes *N. crassa* and *A. nidulans*. So *M. anisopliae* may have acquired CHY1 by lateral gene transfer from an actinomycete. Such a process would be facilitated by the sharing, in soil, of a common habitat (Screen and St. Leger, 2000). This study was an outcome of the production of expressed sequence tag libraries (EST) from cuticle-grown cultures of two isolates of *M. anisopliae* (Freimoser *et al.*, 2003a). Several other ESTs were found with closer homology to bacterial than fungal genes, again consistent with gene transfer.

ACKNOWLEDGEMENTS

I would like to thank R. J. St. Leger and A. Vey for helpful comments on a draft of the manuscript, R. J. St. Leger for providing preprints of unpublished papers, S. Fairhurst for drawing the super cartoons and D. Steinkraus, H. Evans and C. Prior for generously allowing me use of some of their photographs. I am grateful also to the BBSRC and the EC for funding.

REFERENCES

Al-Aidroos, K. and Roberts, D. W. (1978). Mutants of *Metarhizium anisopliae* with increased virulence toward mosquito larvae. *Canadian Journal of Genetics and Cytology* **20**, 211–220.

Aldridge, D. C. and Turner, W. B. (1969). Structures of Cytochalasin C and Cytochalasin D from *Metarhizium anisopliae*. *Journal of the Chemical Society Section C Organic Chemistry*, 923–928.

Amiri, B., Ibrahim, L. and Butt, T. M. (1999). Antifeedant properties of destruxins and their potential use with the entomogenous fungus *Metarhizium anisopliae* for improved control of crucifer pests. *Biocontrol Science and Technology* **9**, 487–498.

Amiri-Besheli, B., Khambay, B., Cameron, S., Deadman, M. L. and Butt, T. M. (2000). Inter- and intra-specific variation in destruxin production by insect pathogenic *Metarhizium* spp., and its significance to pathogenesis. *Mycological Research* **104**, 447–452.

Anke, H. and Sterner, O. (2002). Insecticidal and nematicidal metabolites from fungi. In 'The Mycota volume X – Industrial applications' (H.D. Osiewacz, ed.), pp. 109–127, Springer, Heidelberg.

Askary, H., Benhamou, N. and Brodeur, J. (1999). Ultrastructural and cytochemical characterization of aphid invasion by the hyphomycete *Verticillium lecanii*. *Journal of Invertebrate Pathology* **74**, 1–13.

Bagga, S., Screen, S. E. and St. Leger, R. J. (2003). Reconstructing the diversification of subtilisins in the pathogenic fungus *Metarhizium anisopliae*. Gene (In press).

Bailey, A. M., Kershaw, M. J., Hunt, B. A., Paterson, I. C., Charnley, A. K., Reynolds, S. E. and Clarkson, J. M. (1996). Cloning and sequence analysis of an intron-containing domain from a peptide synthetase-encoding gene of the entomopathogenic fungus *Metarhizium anisopliae*. *Gene* **173**, 195–197.

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

Bandani, A. R., Amiri, B., Butt, T. M. and Gordon-Weeks, R. (2001). Effects of efrapeptin and destruxin, metabolites of entomogenous fungi, on the hydrolytic activity of a vacuolar type ATPase identified on the brush border membrane vesicles of *Galleria mellonella* midgut and on plant membrane bound hydrolytic enzymes. *Biochimica Et Biophysica Acta–Biomembranes* **1510**, 367–377.

Bandani, A. R., Khambay, B. P. S., Faull, J. L., Newton, R., Deadman, M. and Butt, T. M. (2000). Production of efrapeptins by *Tolypocladium* species and evaluation of their insecticidal and antimicrobial properties. *Mycological Research* **104**, 537–544.

Bassi, A. (1835). Del mal del segno. Reproduced in 'Phytopathological Classics' (G. C. Ainsworth and P. J. Yarrow, eds.), American Phytopathological Society.

Beard, R. L. and Walton, G. S. (1969). Kojic acid as an insecticidal mycotoxin. *Journal of Invertebrate Pathology* **14**, 53–59.

Berbee, M. L. and Taylor, J. W. (2001). Fungal molecular evolution: gene trees and geological time. In 'The Mycota Vol 7A – Systematics and Evolution' (D. J. McLaughlin, E. G. McLaughlin and P. A. Lemke, eds.), pp. 229–245, Springer, Berlin.

Bidochka, M. J. and Khachatourians, G. G. (1987). Purification and properties of an extracellular protease produced by the entomopathogenic fungus *Beauveria bassiana*. *Applied and Environmental Microbiology* **53**, 1679–1684.

Bidochka, M. J. and Khachatourians, G. G. (1988a). N-acetyl-D-glucosamine-mediated regulation of extracellular protease in the entomopathogenic fungus *Beauveria bassiana*. *Applied and Environmental Microbiology* **54**, 2699–2704.

Bidochka, M. J. and Khachatourians, G. G. (1988b). Regulation of extracellular protease in the entomopathogenic fungus *Beauveria bassiana*. *Experimental Mycology* **12**, 161–168.

Bidochka, M. J. and Khachatourians, G. G. (1990). Identification of *Beauveria bassiana* extracellular protease as a virulence factor in pathogenicity toward the migratory grasshopper, *Melanoplus sanguinipes*. *Journal of Invertebrate Pathology* **56**, 362–370.

Bidochka, M. J. and Khachatourians, G. G. (1991). The implication of metabolic acids produced by *Beauveria bassiana* in pathogenesis of the migratory grasshopper, *Melanoplus sanguinipes*. *Journal of Invertebrate Pathology* **58**, 106–117.

Bidochka, M. J. and Khachatourians, G. G. (1993a). Oxalic-acid hyperproduction in *Beauveria bassiana* mutants is related to a utilizable carbon source but not to virulence. *Journal of Invertebrate Pathology* **62**, 53–57.

Bidochka, M. J. and Khachatourians, G. G. (1993b). Regulation of extracellular N-acetyl-D-glucosaminidase production in the entomopathogenic fungus *Beauveria bassiana*. *Canadian Journal of Microbiology* **39**, 6–12.

Bidochka, M. J. and Khachatourians, G. G. (1994). Protein hydrolysis in grasshopper cuticles by entomopathogenic fungal extracellular proteases. *Journal of Invertebrate Pathology* **63**, 7–13.

Bidochka, M. J. and Melzer, M. J. (2000). Genetic polymorphisms in three subtilisin-like protease isoforms (Pr1A, Pr1B, and Pr1C) from *Metarhizium* strains. *Canadian Journal of Microbiology* **46**, 1138–1144.

Bidochka, M. J., Tong, K. I. and Khachatourians, G. G. (1993). Partial purification and characterization of two extracellular N-acetyl-D-glucosaminidases produced by the entomopathogenic fungus, *Beauveria bassiana*. *Canadian Journal of Microbiology* **39**, 40–45.

Bidochka, M. J., Burke, S. and Ng, L. (1999). Extracellular hydrolytic enzymes in the fungal genus *Verticillium*: adaptations for pathogenesis. *Canadian Journal of Microbiology* **45**, 856–864.

Bing, L. A. and Lewis, L. C. (1991). Suppression of *Ostrinia nubilalis* (Hubner) (Lepidoptera, Pyralidae) by Endophytic *Beauveria bassiana* (Balsamo) Vuillemin. *Environmental Entomology* **20**, 1207–1211.

Blomquist, G. J. (1984). Cuticular lipids of insects. In Conference Report (D. W. Roberts and J. R. Aist, eds.), pp. 54-60: Rockerfeller Foundation.

Bogo, M. R., Rota, C. A., Pinto, H., Ocampos, M., Correa, C. T., Vainstein, M. H. and Schrank, A. (1998). A chitinase encoding gene (chit1 gene) from the entomopathogen *Metarhizium anisopliae*: Isolation and characterization of genomic and full-length cDNA. *Current Microbiology* **37**, 221–225.

Bogus, M. I. and Scheller, K. (2002). Extraction of an insecticidal protein fraction from the parasitic fungus *Conidiobolus coronatus* (Entomophthorales). *Acta Parasitologica* **47**, 66–72.

Bogus, M. I. and Szczepanik, M. (2000). Histopathology of *Conidiobolus coronatus* (Entomophthorales) infection in *Galleria mellonella* (Lepidoptera) larvae. *Acta Parasitologica* **45**, 48–54.

Boucias, D. G. and Pendland, J. C. (1991). Attachment of mycopathogens to cuticle: the initial event of mycosis in arthropod hosts. In 'The Fungal Spore and Disease Initiation in Plants and Animals.' (G. T. Cole and H. C. Hoch, eds.), pp. 101–128. Plenum Press, New York.

Boucias, D. G., Farmerie, W. G. and Pendland, J. C. (1998). Cloning and sequencing of cDNA of the insecticidal toxin Hirsutellin A. *Journal of Invertebrate Pathology* **72**, 258–261.

Bradfisch, G. A. and Harmer, S. L. (1990). Omega-Conotoxin Gvia and Nifedipine inhibit the depolarizing action of the fungal metabolite, destruxin-B on muscle from the tobacco budworm (*Heliothis virescens*). *Toxicon* **28**, 1249–1254.

Brewer, D., Jen, W. C., Jones, G. A. and Taylor, A. (1984). The antibacterial activity of some naturally occuring 2,5-dihydroxy-1,4-benzoquinones. *Canadian Journal of Microbiology* **30**, 1068–1072.

Brey, P. T., Latge, J. P. and Prevost, M. C. (1986). Integumental penetration of the Pea Aphid, *Acyrthosiphon pisum*, by *Conidiobolus obscurus* (Entomophthoraceae). *Journal of Invertebrate Pathology* **48**, 34–41.

Brobyn, P. J. and Wilding, N. (1983). Invasive and developmental processes of *Entomophthora muscae* infecting houseflies. *Transactions of the British Mycological Society* **81**, 72–74.

Brockerhoff, H. and Jensen, R. G. (1974). 'Lipolytic Enzymes'. Academic Press, New York.

Brousseau, C., Charpentier, G. and Belloncik, S. (1996). Susceptibility of spruce budworm, *Choristoneura fumiferana* Clemens, to destruxins, cyclodepsi-peptidic mycotoxins of *Metarhizium anisopliae*. *Journal of Invertebrate Pathology* **68**, 180–182.

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

Butt, T. M. (1987). A fluorescence microscopy method for the rapid localization of fungal spores and penetration sites on insect cuticle. *Journal of Invertebrate Pathology* **50**, 72–74.

Carruthers, R. I. and Soper, R. S. (1987). Fungal diseases. In 'Epizootology of Insects Diseases.' (J. R. Fuxa and Y. Tanada, eds.), pp. 357–416. Wiley and Sons, New York.

Cavelier, F., Jacquier, R., Mercadier, J. L. and Verducci, J. (1996). Destruxin analogues: Depsi peptidic bond replacement by amide bond. *Tetrahedron* **52**, 6173–6186.

Cavelier, F., Jacquier, R., Mercadier, J. L., Verducci, J., Traris, M. and Vey, A. (1997). Destruxin analogs: variations of the alpha-hydroxy acid side chain. *Journal of Peptide Research* **50**, 94–101.

Cavelier, F., Verducci, J., Andre, F., Haraux, F., Sigalat, C., Traris, M. and Vey, A. (1998). Natural cyclopeptides as leads for novel pesticides: Tentoxin and destruxin. *Pesticide Science* **52**, 81–89.

Cerenius, L., Thornquist, P.-O., Vey, A., Johansson, M.W. and Soderhall, K. (1990). The effect of the fungal toxin destruxin E on isolated crayfish haemocytes. *J. Insect. Physiol.* **36**, 785–789.

Champlin, F. R. and Grula, E. A. (1979). Noninvolvement of Beauvericin in the entomopathogenicity of *Beauveria bassiana*. *Journal of Invertebrate Pathology* **37**, 1122–1125.

Charnley, A. K. (1984). Physiological aspects of destructive pathogenesis in insects by fungi: a speculative review. In 'Invertebrate–Microbial Interactions' (J. M. Anderson, A. D. M. Rayner and D. W. H. Walton, eds.), pp. 229–276. Cambridge University Press, Cambridge.

Charnley, A. K. (1989). Mechanisms of fungal pathogenesis in insects. In 'The Biotechnology of Fungi for Improving Plant Growth' (J. M. Whipps and R. D. Lumsden, eds.), pp. 85–127. Cambridge University Press, Cambridge.

Charnley, A. K. (1997). Entomopathogenic fungi and their role in pest control. In 'The Mycota Vol IV, Environmental and Microbial Relationships'

(D. Wicklow and M. Soderstrom, eds.), pp. 185–201. Springer-Verlag, Heidelberg.

Charnley, A. K. and St. Leger, R. J. (1991). The role of cuticle-degrading enzymes in fungal pathogenesis of insects. In 'The Fungal Spore and Disease Initiation in Plants and Animals' (G. T. Cole and H. C. Hoch, eds.), pp. 129–156, Plenum press, London.

Chen, H. C., Yeh, S. F., Ong, G. T., Wu, S. H., Sun, C. M. and Chou, C. K. (1995). The Novel Desmethyldestruxin B–2 from *Metarhizium anisopliae*, that suppresses hepatitis-B virus surface-antigen production in human hepatoma-cells. *Journal of Natural Products-Lloydia* **58**, 527–531.

Cherton, J. C., Lange, C., Mulheim, C., Pais, M., Cassier, P. and Vey, A. (1991). Direct in vitro and in vivo monitoring of destruxins metabolism in insects using internal surface reversed-phase high- performance liquid-chromatography. 1. Behavior of E-destruxin in locusts. *Journal of Chromatography-Biomedical Applications* **566**, 511–524.

Chrzanowska, J., Banas, J. and Kolaczkowska, M. (2001). Purification and characterization of *Beauveria bassiana* proteinases. *Acta Biotechnologica* **21**, 73–81.

Clarkson, J. M. and Charnley, A. K. (1996). New insights into the mechanisms of fungal pathogenesis in insects. *Trends in Microbiology* **4**, 197–203.

Clay, K. (1988). The effect of fungi on the interaction between host plants and their herbivores. *Canadian Journal of Plant Pathology* **9**, 380–388.

Claydon, N. (1978). Insecticidal secondary metabolites from entomogenous fungi: *Entomophthora virulenta*. *Journal of Invertebrate Pathology* **32**, 319–324.

Claydon, N. and Grove, J. F. (1982). Insecticidal secondary metabolic products from the entomogenous fungus *Verticillium lecanii*. *Journal of Invertebrate Pathology* **40**, 413–418.

Claydon, N., Grove, J. F. and Pople, M. (1977). Insecticidal secondary metabolic products from the entomogenous fungus *Fusarium solani*. *Journal of Invertebrate Pathology* **30**, 216–223.

Cole, S. C. J., Charnley, A. K. and Cooper, R. M. (1993). Purification and partial characterization of a novel trypsin-like cysteine protease from *Metarhizium anisopliae*. *FEMS Microbiology Letters* **113**, 189–196.

Cooper, R. M. and Wood, R. K. S. (1975). Regulation of synthesis of cell-wall degrading enzymes by *Verticillium lecanii* and *Fusarium oxysporum* f. sp. *lycopersici*. *Physiological Plant Pathology* **5**, 135–156.

Cooper, R. M., Rankin, B. and Wood, R. K. S. (1978). Cell-wall degrading enzymes of vascular wilt fungi II. Properties and modes of action of polysaccharidases of *Verticillium albo-atrum* and *Fusarium oxysporum* f. sp. *lycopersici*. *Physiological Plant Pathology* **13**, 101–134.

Coudron, T. A., Kroha, M. J. and Ignoffo, C. M. (1984). Levels of chitinolytic activity during development of 3 entomopathogenic fungi. *Comparative Biochemistry and Physiology B* **79**, 339–348.

Cox, D. L. and Willis, J. H. (1985). The cuticular proteins of *Hyalophora cecropia* from different anatomical regions and metamorphic stages. *Insect Biochemistry* **15**, 349–362.

Cross, R. L. and Kohlbrenner, W. E. (1978). The mode of inhibition of oxidative phosphorylation by efrapeptin A-23871 evidence for an alternating site mechanism for ATP synthesis. *Journal of Biological Chemistry* **253**, 4865–4873.

Dasilva, J. C., Desiqueira, J. P. and Marcondes, C. B. (1989). Selection of *Metarhizium anisopliae* for extracellular enzyme-production and virulence toward *Triatoma infestans*. *Revista Brasileira De Genetica* **12**, 1–7.

Debeaupuis, J. P. and Lafont, P. (1985). Effet de quelques mycotoxines et substances genotoxiques de synthese sur le developpement de l'embryon et de la *Brachydanio rerio*. *Comptes Rendus De L Academie Des Sciences Serie 3 - Sciences De La Vie-Life Sciences* **300**, 167–170.

Dowd, P. F. (1988). Synergism of aflatoxin B-1 toxicity with the co-occuring fungal metabolite kojic acid to two caterpillars. *Entomologia Experimentalis et Applicata* **47**, 69–72.

Dowd, P. F. (1999). Relative inhibition of insect phenoloxidase by cyclic fungal metabolites from insect and plant pathogens. *Natural Toxins* **7**, 337–341.

Drummond, J. and Pinnock, D. E. (1990). Aflatoxin production by entomopathogenic isolates of *Aspergillus parasiticus* and *Aspergillus flavus*. *Journal of Invertebrate Pathology* **55**, 332–336.

Dumas, C., Robert, P., Pais, M., Vey, A. and Quiot, J. M. (1994). Insecticidal and cytotoxic effects of natural and hemisynthetic destruxins. *Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology* **108**, 195–203.

Dumas, C., Matha, V., Quiot, J. M. and Vey, A. (1996a). Effects of destruxins, cyclic depsipeptide mycotoxins, on calcium balance and phosphorylation of intracellular proteins in lepidopteran cell lines. *Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology* **114**, 213–219.

Dumas, C., Ravallec, M., Matha, V. and Vey, A. (1996b). Comparative study of the cytological aspects of the mode of action of destruxins and other peptidic fungal metabolites on target epithelial cells. *Journal of Invertebrate Pathology* **67**, 137–146.

Dunphy, G. B. and Nolan, R. A. (1982). Mycotoxin production by the protoplast stage of *Entomophthora egressa*. *Journal of Invertebrate Pathology* **39**, 261–263.

Elsayed, G. N., Coudron, T. A., Ignoffo, C. M. and Riba, G. (1989). Chitinolytic activity and virulence associated with native and mutant isolates of an entomopathogenic fungus, *Nomuraea rileyi*. *Journal of Invertebrate Pathology* **54**, 394–403.

El-Sayed, G. N., Ignoffo, C. M., Leathers, T. D. and Gupta, S. C. (1993). Cuticular and non-cuticular substrate influence on expression of cuticle-degrading enzymes from conidia of an entomopathogenic fungus *Nomuraea rileyi*. *Mycopathologia* **122**, 79–87.

Engstrom, G., Delance, J., Richard, A. L. and Baetz, J. (1975). Purification and characterisation of roseotoxin B, a toxic cyclodepsipeptide from *Trichothecium roseum*. *Journal of Agriculture and Food Chemistry* **23**, 244–253.

Evans, H. C. (1988). Coevolution of entomogenous fungi and their insect hosts. In 'Coevolution of fungi with plants and animals' (K. A. Pirozynski and D. L. Hawksworth, eds.). pp. 149–171. Academic Press, London.

Evans, H. C. (1989). Mycopathogens of insects of epigeal and aerial habitats. In 'Insect Fungus Interactions', (N. Wilding, N. M. Collins, P. M. Hammond and J. F. Weber, eds.), pp. 205–238. Academic Press, London.

Eyal, J., Mabud, A., Fischbein, K. L., Walter, J. F., Osborne, L. S. and Landa, Z. (1994). Assessment of *Beauveria bassiana* Nov. EO-1 strain, which produces a red pigment for microbial control. *Applied Biochemistry and Biotechnology* **44**, 65–80.

Frappier, F., Ferron, P. and Pais, M. (1975). Chemistry of entomopathogenic fungi: beauvellide, a new cyclodepsipeptide isolated from *Beauveria tenella* Phytochemistry **14**, 2703–2705.

Freimoser, F. M., Screen, S., Bagga, S., Hu, G. and St. Leger, R. J. (2003a). Expressed sequence tag (EST) analysis of two subspecies of *Metarhizium anisopliae* reveals a plethora of secreted proteins with potential activity in insect hosts. *Microbiology* **149**, 1–9.

Freimoser, F. M., Screen, S. E., Hu, G. and St. Leger, R. J. (2003b). EST analysis of genes expressed by the zygomycete pathogen *Conidiobolus coronatus* during growth on insect cuticle. *Microbiology* **149**, 1893–1900.

Fricaud, A. C., Walters, A. J., Whitehouse, D. G. and Moore, A. L. (1992). The roles of adenylate kinase and the adenylate carrier in the regulation of plant mitochondrial respiratory activity. *Biochimica et Biophysica Acta* **1099**, 253–261.

Genthner, F. J. and Middaugh, D. P. (1992). Effects of *Beauveria bassiana* on Embryos of the Inland Silverside Fish (*Menidia beryllina*). *Applied and Environmental Microbiology* **58**, 2840–2845.

Genthner, F. J., Chancy, C. A., Couch, J. A., Foss, S. S., Middaugh, D. P., George, S. E., Warren, M. A. and Bantle, J. A. (1998). Toxicity and pathogenicity testing of the insect pest control fungus *Metarhizium anisopliae*. *Archives of Environmental Contamination and Toxicology* **35**, 317–324.

Geremia, R. A., Goldman, G. H., Jacobs, D., Ardiles, W., Vila, S. B., Vanmontagu, M. and Herreraestrella, A. (1993). Molecular characterization of the proteinase-encoding gene, Prb1, related to mycoparasitism by *Trichoderma harzianum*. *Molecular Microbiology* **8**, 603–613.

Gillespie, A. T. (1988). Use of fungi to control pests of agricultural importance. In 'Fungi in Biological Control Systems.' (M. N. Burge, ed.), pp. 37–60. Manchester University Press, Manchester.

Gillespie, A. T. and Claydon, N. (1989). The use of entomogenous fungi for pest control and the role of toxins in pathogenesis. *Pesticide Science* **27**, 203–215.

Gillespie, J. P., Bateman, R. and Charnley, A. K. (1998). Role of cuticle-degrading proteases in the virulence of *Metarhizium* spp. for the desert locust, *Schistocerca gregaria*. *Journal of Invertebrate Pathology* **71**, 128–137.

Godoy, G., Steadman, J. R., Dickman, M. B. and Dam, R. (1990). Use of mutants to demonstrate the role of oxalic acid in pathogenicity of *Sclerotinia sclerotiorum*. *Physiological and Molecular Plant Pathology* **37**, 179–191.

Goettel, M. S., St. Leger, R. J., Rizzo, N. W., Staples, R. C. and Roberts, D. W. (1989). Ultrastructural-localization of a cuticle-degrading protease produced by the entomopathogenic fungus *Metarhizium anisopliae* During Penetration of Host (*Manduca sexta*) cuticle. *Journal of General Microbiology* **135**, 2233–2239.

Graniti, A. (1972). The evolution of the toxin concept in plant pathology. In 'Phytotoxins in Plant Diseases' (R. K. S. Woods, A. Ballio and A. Graniti, eds.), pp. 1–18. Academic Press, New York.

Griesch, J. and Vilcinskas, A. (1998). Proteases released by entomopathogenic fungi impair phagocytic activity, attachment and spreading of plasmatocytes isolated from haemolymph of the greater wax moth *Galleria mellonella*. *Biocontrol Science and Technology* **8**, 517–531.

Griesch, J., Wedde, M. and Vilcinskas, A. (2000). Recognition and regulation of metalloproteinase activity in the haemolymph of *Galleria mellonella*: a new

pathway mediating induction of humoral immune responses. *Insect Biochemistry and Molecular Biology* **30**, 461–472.

Grove, J. F. (1980). Cyclodepsipeptides from *Beauveria bassiana*. 3. The isolation of Beauverolides Ba, Ca, Ja. and Ka. *Journal of the Chemical Society-Perkin Transactions 1*, 2878–2880.

Grove, J. F. and Pople, M. (1980). The insecticidal activity of beauvericin and the enniatin complex. *Mycopathologia* **70**, 103–105.

Gunkel, F. A. and Gassen, H. G. (1989). Proteinase K from *Tritirachium album* Limber. Characterisation of the chromosomal gene and expression of the cDNA in *Escherichia coli*. *European Journal of Biochemistry* **179**, 185–194.

Gupta, S., Roberts, D. W. and Renwick, J. A. A. (1989). Preparative isolation of destruxins from *Metarhizium anisopliae* by high-performance liquid-chromatography. *Journal of Liquid Chromatography* **12**, 383–395.

Gupta, S., Krasnoff, S. B., Underwood, N. L., Renwick, J. A. A. and Roberts, D. W. (1991). Isolation of Beauvericin as an insect toxin from *Fusarium semitectum* and *Fusarium moniliforme* var *subglutinans*. *Mycopathologia* **115**, 185–189.

Gupta, S., Krasnoff, S. B., Renwick, J. A. A., Roberts, D. W., Steiner, J. R. and Clardy, J. (1993a). Viridoxin-a and Viridoxin-B – Novel toxins from the fungus *Metarhizium flavoviride*. *Journal of Organic Chemistry* **58**, 1062–1067.

Gupta, S. C., Leathers, T. D., Elsayed, G. N. and Ignoffo, C. M. (1993b). Purification and characterization of trypsin from an Entomopathogen, *Nomuraea rileyi* Nrrl-13755. *Current Microbiology* **27**, 103–107.

Gupta, S. C., Leathers, T. D., El-Sayed, G. N. and Ignoffo, C. M. (1994). Relationships among enzyme activities and virulence parameters in *Beauveria bassiana* infections of *Galleria mellonella* and *Trichoplusia ni*. *Journal of Invertebrate Pathology* **64**, 13–17.

Gupta, S., Montllor, C. and Hwang, Y. S. (1995). Isolation of Novel Beauvericin Analogs from the Fungus *Beauveria bassiana*. *Journal of Natural Products-Lloydia* **58**, 733–738.

Hackman, R. H. and Goldberg, M. (1974). Light scattering and infra-red spectrophotometric studies of chitin and chitin derivatives. *Carbohydrate Research* **38**, 35–45.

Hajek, A. E. and St. Leger, R. J. (1994). Interactions between fungal pathogens and insect hosts. *Annual Review of Entomology* **39**, 293–322.

Hamer, J. E. and Holden, D. W. (1997). Linking approaches in the study of fungal pathogenesis: a commentary. *Fungal Genetics and Biology* **21**, 11–16.

Hamil, R. L., Higgins, C. E., Boaz, H. E. and Gorman, M. (1969). The structure of beauvericin, a new depsipeptide antibiotic toxic to *Artemia salina*. *Tetrahedron Letters* **49**, 4255–4258.

Haran, S., Schickler, H., Oppenheim, A. and Chet, I. (1996). Differential expression of *Trichoderma harzianum* chitinases during mycoparasitism. *Phytopathology* **86**, 980–985.

Hassan, A. E. M. and Charnley, A. K. (1983). Combined effects of diflubenzuron and the entomopathogenic fungus *Metarhizium anisopliae* on the tobacco hornworm *Manduca sexta*. In *10th International Congress on Plant Protection*, pp. 790. Brighton: BCPC.

Hassan, A. E. M. and Charnley, A. K. (1987). The effect of dimilin on the ultrastructure of the integument of *Manduca sexta*. *Journal of Insect Physiology* **33**, 669 et seq.

Hassan, A. E. M. and Charnley, A. K. (1989). Ultrastructural-study of the penetration by *Metarhizium anisopliae* through dimilin-affected cuticle of *Manduca sexta*. *Journal of Invertebrate Pathology* **54**, 117–124.

Hegedus, D. D. and Khachatourians, G. G. (1988). Production of an extracellular lipase by *Beauveria bassiana*. *Biotechnology Letters* **10**, 637–642.

Hepburn, H. R. (1985). Structure of the integument. In 'Comprehensive Insect Physiology, Biochemistry and Pharmacology' (G. A. Kerkut and L. I. Gilbert, eds.). Pergammon Press, Oxford.

Herrmann, M., Zocher, R. and Haeses, A. (1996). The effect of disruption of the enniatin synthetase gene on the virulence of *Fusarium avenaceum*. *Molecular Plant Microbe Interactions* **9**, 226–232.

Heymann, E. (1980). Carboxyesterases and amidases. In 'Enzymatic Basis of Detoxification' (W. B. Jakoby, ed.), pp. 291–323. Academic Press, New York.

Hillerton, J. E. (1984). Cuticle: mechanical properties. In 'Biology of the Integument .I. Invertebrates' (J. Bereiter-Hahn, A. G. Matolsty and K. S. Richards, ed.), pp. 583–625. Springer-Verlag, Berlin.

Hinaje, M., Ford, M., Banting, L., Arkle, S. and Khambay, B. (2002). An investigation of the ionophoric characteristics of destruxin A. *Archives of Biochemistry and Biophysics* **405**, 73–77.

Hodge, H. T., Krasnoff, S. B. and Humber, R. A. (1996). *Tolypocladium inflatum* is the anamorph of *Cordyceps subsessilis*. *Mycologia* **88**, 715–719.

Hohn, T. M. (1997). Fungal phytotoxins: biosynthesis and activity. In 'Plant Relationships' (G. C. Caroll and P. Tudzynski, eds.), pp. 129–144. Springer, New York.

Hojrup, P., Andersen, S. O. and Roepstorff, P. (1986). Isolation, Characterization, and N-terminal sequence studies of cuticular proteins from the migratory locust, *Locusta migratoria*. *European Journal of Biochemistry* **154**, 153–159.

Hubert, M., Cherton, J. C., Vey, A., Lange, C. (1999). Metabolites of A and E destruxins in *Galleria mellonella* larvae using negative-ion fast-atom bombardment mass spectrometry. Rapid communications in mass spectrometry **13**, 179–184.

Hurion, N., Fromentin, H. and Keil, B. (1977). Proteolytic enzymes of *Entomophthora coronata* characterization of a collagenase. *Comparative Biochemistry and Physiology b- biochemistry* **56B**, 259–264.

Hurion, N., Fromentin, H. and Keil, B. (1979). Specificity of a collagenolytic enzyme from the fungus *Entomophthora coronata*: comparison with the bacterial collagenase from *Achromobacter iophagus*. *Archives of Biochemistry and Biophysics* **192**, 438–445.

Huxham, I. M., Lackie, A. M. and McCorkindale, N. J. (1989). Inhibitory effects of cyclodepsipeptides, destruxins, from the fungus *Metarhizium anisopliae*, on cellular-immunity in insects. *Journal of Insect Physiology* **35**, 97–105.

Inglis, G. D., Goettel, M. S., Butt, T. M. and Strasser, H. (2001). Use of hyphomycetous fungi for managing insect pests. In 'Fungi as Biocontrol Agents' (T. M. Butt, C. W. Jackson and N. Magan, eds.), pp. 23–69. CAB International, Wallingford.

James, P. J., Kershaw, M. J., Reynolds, S. E. and Charnley, A. K. (1993). Inhibition of desert locust (*Schistocerca gregaria*) malpighian tubule fluid secretion by destruxins, cyclic peptide toxins from the insect pathogenic fungus *Metarhizium anisopliae*. *Journal of Insect Physiology* **39**, 797–804.

Jeffs, L. B. and Khachatourians, G. G. (1997). Toxic properties of *Beauveria* pigments on erythrocyte membranes. *Toxicon* **35**, 1351–1356.

Jegorov, A., Matha, V. and Hradec, H. (1992). Detoxification of Destruxins in *Galleria mellonella* L Larvae. *Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology* **103**, 227–229.

Jegorov, A., Sedmera, P., Havlicek, V. and Mat'ha, V. (1998). Destruxin Ed(1) a cyclopeptide from the fungus *Metarhizium anisopliae*. *Phytochemistry* **49**, 1815–1817.

Jegorov, A., Matha, V., Sedmera, P. and Roberts, D. W. (1989). Destruxins from *Metarhizium anisopliae*. In *Proceedings of the International Conference on Biopesticides, Theory and Practice* (Ed. by A. Jegorov and V. Matha), pp. 64–70.

Jegorov, A., Matha, V. and Weiser, J. (1990). Production of Cyclosporins by Entomopathogenic Fungi. *Microbios Letters* **45**, 65–70.

Jegorov, A., Matha, V., Husak, M., Kratochvil, B., Stuchlik, J., Sedmera, P. and Havlicek, V. (1993). Iron uptake system of some members of the genus *Tolypocladium*: crystal structure of the ligand and its iron (III) complex. *Journal of Chemical Society Dalton Transactions* **1**, 1287–1293.

Johnson, R. D., Johnson, L., Itoh, Y., Kodama, M., Otani, H. and Kohmoto, K. (2000). Cloning and characterization of a cyclic peptide synthetase gene from *Alternaria alternaria* apple pathotype whose product is involved in AM-toxin synthesis and pathogenicity. *Molecular Plant Microbe Interactions* **13**, 742–753.

Joshi, L., Charnley, A. K., Arnold, G., Brain, P. and Bateman, R. (1992). Synergism between entomopathogenic fungi, *Metarhizium* spp., and the benzoylphenyl urea insecticide, teflubenzuron, against the desert locust, *Schistocerca gregaria*. *Brighton Crop Protection Conference: Pests and Diseases, Vols. 1, 2 and 3*, 369–374.

Joshi, L. and St. Leger, R. J. (1999). Cloning, expression, and substrate specificity of MeCPA, a zinc carboxypeptidase that is secreted into infected tissues by the fungal entomopathogen *Metarhizium anisopliae*. *Journal of Biological Chemistry* **274**, 9803–9811.

Joshi, L., St. Leger, R. J. and Bidochka, M. J. (1995). Cloning of a cuticle-degrading protease from the entomopathogenic fungus, *Beauveria bassiana*. *FEMS Microbiology Letters* **125**, 211–217.

Joshi, L., St. Leger, R. J. and Roberts, D. W. (1997). Isolation of a cDNA encoding a novel subtilisin-like protease (Pr1B) from the entomopathogenic fungus, *Metarhizium anisopliae* using differential display–RT-PCR. *Gene* **197**, 1–8.

Kagamizono, T., Nishino, E., Matsumoto, K., Kawashima, A., Kishimoto, M., Sakai, N., He, B. M., Chen, Z. X., Adachi, T., Morimoto, S. and Hanada, K. (1995). Bassiatin, a new platelet aggregation inhibitor produced by *Beauveria bassiana* K-717. *Journal of Antibiotics* **48**, 1407–1412.

Kaijiang, L. and Roberts, D. W. (1986). The Production of destruxins by the entomogenous fungus *Metarhizium anisopliae* var *major*. *Journal of Invertebrate Pathology* **47**, 120–122.

Kang, S. C., Park, S. and Lee, D. G. (1998). Isolation and characterization of a chitinase cDNA from the entomopathogenic fungus, *Metarhizium anisopliae*. *FEMS Microbiology Letters* **165**, 267–271.

Kang, S. C., Park, S. and Lee, D. G. (1999). Purification and characterization of a novel chitinase from the entomopathogenic fungus, *Metarhizium anisopliae*. *Journal of Invertebrate Pathology* **73**, 276–281.

Kanost, M. R. (1999). Serine proteinase inhibitors in arthropod immunity. *Developmental and Comparative Immunology* **23**, 291–301.

Kawazu, K., Murakami, T., Ono, Y., Kanzaki, H., Kobayashi, A., Mikawa, T. and Yoshikawa, N. (1993). Isolation and characterization of 2 novel nematocidal depsipeptides from an imperfect fungus, Strain D1084. *Bioscience Biotechnology and Biochemistry* **57**, 98–101.

Kershaw, M. J., Moorhouse, E. R., Bateman, R., Reynolds, S. E. and Charnley, A. K. (1999). The role of destruxins in the pathogenicity of *Metarhizium anisopliae* for three species of insect. *Journal of Invertebrate Pathology* **74**, 213–223.

Khachatourians, G. G. (1991). Physiology and genetics of entomopathogenic fungi. In 'Humans, Animals and Insects' (D. K. Arora, L. Ajello and K. G. Mukerji, eds.), pp. 613–661. Marcel Dekker, New York.

Khachatourians, G. G. (1996). Biochemistry and molecular biology of entomopathogenic fungi. In 'Human and Animal Relationships' (R. J. Howard and J. S. Miller, eds.), pp. 331–363. Springer-Verlag, Heidelberg.

Kim, H. K., Hoe, H. S., Suh, D. S., Kang, S. C., Hwang, C. and Kwon, S. T. (1999). Gene structure and expression of the gene from *Beauveria bassiana* encoding bassiasin I, an insect cuticle-degrading serine protease. *Biotechnology Letters* **21**, 777–783.

Kiuchi, M., Yasui, H., Hayasaka, S. and Kamimura, M. (2003). Entomogenous fungus *Nomuraea rileyi* inhibits host insect moulting by C22 oxidising inactivation of haemolymph ecdysteroids. *Archives of Insect Biochemistry and Physiology* **52**, 35–44.

Kleinkauf, H. and von Dohren, H. (1990). Non-ribosomal biosynthesis of peptide antibiotics. *European Journal of Biochemistry* **192**, 1–15.

Kodaira, Y. (1961). Biochemical studies on the muscardine fungi in the silkworms, *Bombyx mori* L. *Journal of Faculty Textile Technology Sinshu University*, **5**, 1–68.

Krasnoff, S. B. and Gupta, S. B. (1991). Identification and biosynthesis of efrapeptins in the fungus *Tolypocladium geodes* Gams (Deuteromycotina: Hyphomycetes). *Journal of Chemical Ecology* **17**, 1953–1960.

Krasnoff, S. B. and Gupta, S. (1992). Efrapeptin production by *Tolypocladium* fungi (Deuteromycotina, Hyphomycetes) – intraspecific and interspecific variation. *Journal of Chemical Ecology* **18**, 1727–1741.

Krasnoff, S. B. and Gupta, S. (1994). Identification of the antibiotic phomalactone from the entomopathogenic fungus *Hirsutella thompsonii* var *synnematosa*. *Journal of Chemical Ecology* **20**, 293–302.

Krasnoff, S. B., Gupta, S., St. Leger, R. J., Renwick, J. A. A. and Roberts, D. W. (1991). Antifungal and insecticidal properties of the efrapeptins – metabolites of the fungus *Tolypocladium niveum*. *Journal of Invertebrate Pathology* **58**, 180–188.

Krasnoff, S. B., Gibson, D. M., Belofsky, G. N., Gloer, K. B. and Gloer, J. B. (1996). New destruxins from the entomopathogenic fungus *Aschersonia* sp. *Journal of Natural Products* **59**, 485–489.

Kucera, M. (1980). Proteases from the fungus *Metarhizium anisopliae* toxic for *Galleria mellonella* larvae. *Journal of Invertebrate Pathology* **35**, 304–310.

Kucera, M. (1981). The production of toxic protease by the entomopathogenic fungus *Metarhizium anisopliae* in submerged culture. *Journal of Invertebrate Pathology* **38**, 33–38.

Lange, C., Loutelier, C., Cherton, J. C., Cassier, P., Vey, A. and Pais, M. (1992). Desorption of ions from locust tissues. 2. Metabolites of E-destruxin using negative-ion fast–atom-bombardment mass-spectrometry. *Rapid Communications in Mass Spectrometry* **6**, 28–31.

Lange, C., Mulheim, C., Cherton, J. C., Cassier, P., Vey, A. and Pais, M. (1991). Desorption of ions from locust tissues. 1. Behavior of E- destruxin using positive-ion fast-atom-bombardment mass-spectrometry. *Rapid Communications in Mass Spectrometry* **5**, 169–174.

Leal, S. C. M., Bertioli, D. J., Butt, T. M., Carder, J. H., Burrows, P. R. and Peberdy, J. F. (1997). Amplification and restriction endonuclease digestion of the Pr1 gene for the detection and characterization of *Metarhizium strains. Mycological Research* **101**, 257–265.

Lecuona, R., Riba, G., Cassier, P. and Clement, J. L. (1991). Alterations of insect epicuticular hydrocarbons during infection with *Beauveria bassiana* or *Beauveria brongniartii. Journal of Invertebrate Pathology* **58**, 10–18.

Lewis, D. H. (1987). Evolutionary aspects of mutualistic associations between fungi and photosynthetic organisms. In 'Evolutionary Biology of the Fungi' (A. D. M. Rayner, C. M. Brasier and D. Moore, eds.), pp. 161–178. Cambridge University Press, Cambridge.

Liu, W. Z., Boucias, D. G. and McCoy, C. W. (1995). Extraction and characterization of the insecticidal toxin Hirsutellin A produced by *Hirsutella thompsonii* var *thompsonii. Experimental Mycology* **19**, 254–262.

Liu, J. C., Boucias, D. G., Pendland, J. C., Liu, W. Z. and Maruniak, J. (1996). The mode of action of hirsutellin A on eukaryotic cells. *Journal of Invertebrate Pathology* **67**, 224–228.

Logrieco, K., Ritieni, A., Moretti, A., Randazzo, G. and Bottalico, A. (1997). Beauvericin and fusaproliferin: New emerging *Fusarium* toxins. *Cereal Research Communications* **25**, 407–413.

Logrieco, A., Moretti, A., Castella, G., Kostecki, M., Golinski, P., Ritieni, A. and Chelkowski, J. (1998). Beauvericin production by *Fusarium* species. *Applied and Environmental Microbiology* **64**, 3084–3088.

Loutelier, C., Lange, C., Cassier, P., Vey, A. and Cherton, J. C. (1994). Non-extractive metabolism study of E-destruxin and a-destruxin in the locust, *Locusta migratoria* L. 3. Direct high-performance liquid-chromatographic analysis and parallel fast-atom- bombardment mass-spectrometric monitoring. *Journal of Chromatography B-Biomedical Applications* **656**, 281–292.

Lysenko, O. (1985). Non-sporeforming bacteria pathogenic to insects: incidence and mechanisms. *Annual Review of Microbiology* **39**, 673–695.

Lysenko, O. and Kucera, M. (1971). Microorganisms as sources of new insecticidal chemicals – toxins. In 'Microbial Control of Insects and Mites' (H. D. Burges and N. W. Hussey, eds.), pp. 205–227. Academic Press, London.

Madry, N., Zocker, R. and Kleinkauf, H. (1983). Enniatin production by *Fusarium oxysporum* in chemically defined media. *European Journal of Applied Microbiology and Biotechnology* **17**, 103–105.

Maimala, S., Tartar, A., Boucias, D. and Chandrapatya, A. (2002). Detection of the toxin Hirsutellin A from *Hirsutella thompsonii. Journal of Invertebrate Pathology*.

Matha, V., Weiser, J. and Olejnicek, J. (1988). The effect of tolypin in *Tolypocladium niveum* crude extract against mosquito and blackfly larvae in the laboratory. *Folia Parasitologia* **35**, 381–383.

Mazet, I., Hung, S. Y. and Boucias, D. G. (1994). Detection of toxic metabolites in the haemolymph of *Beauveria bassiana* infected *Spodoptera exigua* larvae. *Experientia* **50**, 142–147.

Mazet, I. and Vey, A. (1995). Hirsutellin-a, a Toxic Protein Produced *in vitro* by *Hirsutella thompsonii*. *Microbiology* **141**, 1343–1348.

McCoy, C. W., Samson, R. A. and Boucias, D. G. (1988). Entomogenous fungi. In 'Vol V Microbial Insecticides, Part A Entomogenous Protozoa and Fungi' (C. M. Ignoffo, ed.), pp. 151–236. CRC Press, Boca Ratan.

Michel, B. 1981. Recherches experimentales sur la penetration des chapignons pathogens chez les insectes., pp. 170: University of Montpellier.

Milne, R., Wright, T., Welton, M., Budau, C., Gringorten, L. and Tyrrell, D. (1994). Identification and partial purification of a cell-lytic factor from *Entomophaga aulicae*. *Journal of Invertebrate Pathology* **64**, 253–259.

Mollier, P., Lagnel, J., Fournet, B., Aioun, A. and Riba, G. (1994a). A glycoprotein highly toxic for *Galleria mellonella* larvae secreted by the entomopathogenic fungus *Beauveria sulfurescens*. *Journal of Invertebrate Pathology* **64**, 200–207.

Mollier, P., Lagnel, J., Quiot, J. M., Aioun, A. and Riba, G. (1994b). Cytotoxic activity in culture filtrates from the entomopathogenic fungus *Beauveria sulfurescens*. *Journal of Invertebrate Pathology* **64**, 208–213.

Moon, T. W. (1975). Temperature adaptation, isozymic function and the maintenance of heterogeneity. In 'Isozymes II. Physiological Function' (A. P. Marbet, ed.). Academic Press, New York.

Mottram, J. C., Frame, M. J., Brooks, D. R., Tetley, L., Hutchison, J. E., Souza, A. E. and Coombs, G. H. (1997). The multiple cpb cysteine proteinase genes of *Leishmania mexicana* encode isoenzymes that differ in their stage regulation and substrate preferences. *Journal of Biological Chemistry* **272**, 14285–14293.

Murakoshi, S., Ichinoe, M., Kumata, H. and Kurata, H. (1977). Productivity of aflatoxins and some biological effects in *Aspergillus flavus* link isolated from cadavers of the silkworm *Bombyx mori* L. *Applied Entomology and Zoology* **12**, 255–259.

Murakoshi, S., Ichinoe, M., Suzuki, A., Kanaoka, M., Isogai, A. and Tamura, S. (1978). Presence of toxic substance in fungus fruiting bodies of the entomopathogenic fungi, *Beauveria bassiana* and *Verticillium lecanii*. *Applied Entomology and Zoology* **13**, 97–102.

Muroi, M., Shiragami, N. and Takatsuki, A. (1994). Destruxin B, a specific and readily reversible inhibitor of vacuolar type H^+ translocating ATPase. *Biochemical and Biophysical Research Communications* **205**, 1358–1365.

Muroi, M., Kaneko, N., Suzuki, K., Nishio, T., Oku, T., Sato, T. and Takatsuki, A. (1996). Efrapeptins block exocytic but not endocytic trafficking of proteins. *Biochemical and Biophysical Research Communications* **227**, 800–809.

Murphy, J. M. and Walton, J. D. (1996). Three extracellular proteases from *Cochliobolus carbonum*: Cloning and targeted disruption of ALP1. *Molecular Plant-Microbe Interactions* **9**, 290–297.

Neville, A. C. (1984). Cuticle: organisation. In 'Biology of the Integument, I. Invertebrates' (J. Bereiter-Hahn, A. G. Matolsty and K. S. Richards, eds.). Springer-Verlag, Berlin.

Nikolskaya, A. N., Panaccione, D. G. and Walton, J. D. (1995). Identification of peptide synthetase-encoding genes from filamentous fungi producing host-selective phytotoxins or analogs. *Gene* **165**, 207–211.

Nilanonta, C., Isaka, M., Kittakoop, P., Trakulnaleamsai, S., Tanticharoen, M. and Thebtaranonth, Y. (2002). Precursor-directed biosynthesis of beauvericin analogs by the insect pathogenic fungus *Paecilomyces tenuipes* BCC 1614. *Tetrahedron* **58**, 3355–3360.

Nnakumusana, E. S. (1985). Laboratory infection of mosquito larvae by entomopathogenic fungi with particular reference to *Aspergillus parasiticus* and its effects on fecundity and longevity of mosquitoes exposed to conidial infections in larval stages. *Current Science* **54**, 1221–1228.

Odier, F., Vey, A. and Bureau, J. P. (1992). *In vitro* effect of fungal cyclodepsipeptides on leukemic-cells – study of destruxin-A, destruxin-B and destruxin-E. *Biology of the Cell* **74**, 267–271.

Ojcius, D. M., Zychlinsky, A., Zheng, L. M. and Young, J. D.-E. (1991). Ionophore-induced apoptosis: role of DNA fragmentation and calcium fluxes. *Experimental Cell Research* **197**, 43–49.

Omoto, C. and McCoy, C. W. (1998). Toxicity of purified fungal toxin hirsutellin A to the citrus rust mite *Phyllocoptruta oleivora*. *Journal of Invertebrate Pathology* **72**, 319–322.

Ovchinnikov, Y. A., Ivanov, V. T. and Mikhaleva, I. I. (1971). The synthesis and some properties of beauvericin. *Tetrahedron Letters* **2**, 159–162.

Pais, M., Das, B. C. and Ferron, P. (1981). Depsipeptides from *Metarhizium anisopliae*. *Phytochemistry* **20**, 715–723.

Papierok, B. (1986). Some noteworthy biological problems within the genus *Conidiobolus*. In 'Fundamental and Applied Aspects of Invertebrate Pathology' (R. A. Samson, J. M. Vlak and D. Peter, eds.), pp. 197–200. Foundation of the Fourth International Colloquium of Invertebrate Pathology, Wageningen.

Paris, S. and Ferron, P. (1979). Study of the virulence of some mutants of *Beauveria brogniartii*. *Journal of Invertebrate Pathology* **34**, 71–77.

Paterson, I. C., Charnley, A. K., Cooper, R. M. and Clarkson, J. M. (1993). Regulation of production of a trypsin-like protease by the insect pathogenic fungus *Metarhizium anisopliae*. *FEMS Microbiology Letters* **109**, 323–327.

Paterson, I. C., Charnley, A. K., Cooper, R. M. and Clarkson, J. M. (1994a). Partial characterization of specific inducers of a cuticle-degrading protease from the insect pathogenic fungus *Metarhizium anisopliae*. *Microbiology* **140**, 3153–3159.

Paterson, I. C., Charnley, A. K., Cooper, R. M. and Clarkson, J. M. (1994b). Specific induction of a cuticle-degrading protease of the insect pathogenic fungus *Metarhizium anisopliae*. *Microbiology* **140**, 185–189.

Pearse, A. G. E. (1972). 'Histochemistry, Theoretical and Applied'. Churchill Livingstone, Edinburgh.

Peeters, H., Zocher, R., Madry, N. and Kleinkauf, H. (1983). Incorporation of radioactive precursors into beauvericin produced by *Paecilomyces fumosoroseus*. *Phytochemistry* **22**, 1719–1720.

Pekrul, S. and Grula, E. A. (1979). Mode of infection of the earworm (*Heliothis zea*) by *Beauveria bassiana* as revealled by scanning electron microscopy. *Journal of Invertebrate Pathology* **34**, 238–247.

Pinto, A. D. S., Barreto, C. C., Schrank, A., Ulhoa, C. J. and Vainstein, M. H. (1997). Purification and characterization of an extracellular chitinase from the entomopathogen *Metarhizium anisopliae*. *Canadian Journal of Microbiology* **43**, 322–327.

Poprawski, T. J., Robert, P. H. and Maniania, N. K. (1994). Contact activity of the mycotoxin destruxin E to *Empoasca vitis*. *Journal of Applied Entomology* **117**, 135–143.

Quiot, J. M., Vey, A. and Vago, C. (1985). Effects of mycotoxins on invertebrate cells *In vitro*. *Advances in Cell Research* **4**, 203–212.

Quiot, J. M., Vey, A., Vago, C. and Pais, M. (1980). Anti-viral activity of a mycotoxin – study of the toxin of the hyphomycete *Metarhizium anisopliae*

(Metsch) Sorok in lepidoptera cell-culture. *Comptes Rendus Hebdomadaires Des Seances De L Academie Des Sciences Serie D* **291**, 763–766.

Reyes, F. and Byrde, R. J. W. (1973). Partial purification and properties of N-acetylglucosaminidase from the fungus *Sclerotina fructigena*. *Biochemical Journal* **131**, 381–388.

Richard, J. L. and Gallagher, R. T. (1979). Multiple toxin production by an isolate of *Aspergillus flavus*. *Mycopathologia* **67**, 161–163.

Robert, P. and Riba, G. (1989). Toxic and repulsive effects of spray, *per os* and systemic applications of destruxin-E to aphids. *Mycopathologia* **108**, 179–183.

Roberts, D. W. (1966a). Toxins from the entomogenous fungus *Metarhizium anisopliae* 1. Production in submerged and surface cultures, and in inorganic and organic nitrogen media. *Journal of Invertebrate Pathology* **8**, 212–221.

Roberts, D. W. (1966b). Toxins from the entomogenous fungus *Metarhizium anisopliae* II. symptoms and detection in moribund hosts. *Journal of Invertebrate Pathology* **8**, 222–227.

Roberts, D. W. (1980). Toxins of entomopathogenic fungi. In 'Microbial Control of Insects, Mites and Plant Diseases' (H. D. Burges, ed.), pp. 441–463. Academic Press, New York.

Robinson, R. K. (1966). Studies on penetration of insect integument by fungi. *Pest Articles and News Summaries* **12**, 131–142.

Rosewich, U. L. and Kistler, H. C. (2000). Role of horizontal gene transfer in the evolution of fungi. *Annual Review of Phytopathology* **38**, 325–363.

Ruegger, A., Kuhn, M., Lichti, H., Loosli, H. R., Huguenin, R., Quiqierez, C. and Wartburg, V. A. (1976). Cyclosporin A, ein immunosuppressiv wirksamer Peptidmetabolit aus *Trichoderma polysporum*. *Helvetica Chimica Acta* **59**, 1075–1092.

Samsinakova, A., Misikova, S. and Leopold, J. (1971). Action of enzymatic system of *Beauveria bassiana* on the cuticle of the greater wax moth larvae *Galleria mellonella*. *Journal of Invertebrate Pathology* **18**, 322–330.

Samson, R. A., Evans, H. C. and Latge, J. P. (1988). 'Atlas of Entomopathogenic Fungi.' Springer-Verlag, New York.

Samuels, R. I. (1998). A sensitive bioassay for destruxins, cyclodepsipeptides from the culture filtrates of the entomopathogenic fungus *Metarhizium anisopliae* (Metsch.) Sorok. *Anais da Sociedade Entomologica do Brasil* **27**, 229–235.

Samuels, R. I., Charnley, A. K. and Reynolds, S. E. (1988a). Application of reversed-phase hplc in separation and detection of the cyclodepsipeptide toxins produced by the entomopathogenic fungus *Metarhizium anisopliae*. *Journal of Chromatographic Science* **26**, 15–19.

Samuels, R. I., Charnley, A. K. and Reynolds, S. E. (1988b). The role of destruxins in the pathogenicity of 3 strains of *Metarhizium anisopliae* for the tobacco hornworm *Manduca sexta*. *Mycopathologia* **104**, 51–58.

Samuels, R. I., Reynolds, S. E. and Charnley, A. K. (1988c). Calcium-channel activation of insect muscle by destruxins, insecticidal compounds produced by the entomopathogenic fungus *Metarhizium anisopliae*. *Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology* **90**, 403–412.

Samuels, R. I., Charnley, A. K. and St. Leger, R. J. (1990). The partial characterization of endoproteases and exoproteases from 3 species of entomopathogenic entomophthorales and 2 species of deuteromycetes. *Mycopathologia* **110**, 145–152.

Samuels, R. I., Charnley, A. K. and Reynolds, S. E. (1991). A comparison of insect cuticle degrading proteases from insect moulting fluid and from cultures of entomopathogenic fungi. In *Proceedings of the Vth International Colloquium on Invertebrate Pathology and Microbial Control*, pp. 313. Adelaide, Australia.

Samuels, R. I., Charnley, A. K. and Reynolds, S. E. (1993a). An aminopeptidase from the molting fluid of the tobacco hornworm, *Manduca sexta*. *Insect Biochemistry and Molecular Biology* **23**, 615–620.

Samuels, R. I., Charnley, A. K. and Reynolds, S. E. (1993b). A cuticle-degrading proteinase from the molting fluid of the tobacco hornworm, *Manduca sexta*. *Insect Biochemistry and Molecular Biology* **23**, 607–614.

Samuels, R. I. and Paterson, I. C. (1995). Cuticle degrading proteases from insect molting fluid and culture filtrates of entomopathogenic fungi. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* **110**, 661–669.

Screen, S. E. and St. Leger, R. J. (2000). Cloning, expression, and substrate specificity of a fungal chymotrypsin – Evidence for lateral gene transfer from an actinomycete bacterium. *Journal of Biological Chemistry* **275**, 6689–6694.

Screen, S., Bailey, A., Charnley, K., Cooper, R. and Clarkson, J. (1997). Carbon regulation of the cuticle-degrading enzyme PR1 from *Metarhizium anisopliae* may involve a trans-acting DNA binding protein CRR1, a functional equivalent of the *Aspergillus nidulans* CREA protein. *Current Genetics* **31**, 511–518.

Screen, S., Bailey, A., Charnley, K., Cooper, R. and Clarkson, J. (1998). Isolation of a nitrogen response regulator gene (nrr1) from *Metarhizium anisopliae*. *Gene* **221**, 17–24.

Screen, S. E., Hu, G. and St. Leger, R. J. (2001). Transformants of *Metarhizium anisopliae* sf *anisopliae* overexpressing chitinase from *Metarhizium anisopliae* sf *acridum* show early induction of native chitinase but are not altered in pathogenicity to *Manduca sexta*. *Journal of Invertebrate Pathology* **78**.

Segers, R., Butt, T. M., Carder, J. H., Keen, J. N., Kerry, B. R. and Peberdy, J. F. (1999). The subtilisins of fungal pathogens of insects, nematodes and plants: distribution and variation. *Mycological Research* **103**, 395–402.

Seyoum, E., Moore, D. and Charnley, A. K. (1995). Reduction in flight activity and food consumption by the desert locust, *Schistocerca gregaria*, after infection with *Metarhizium flavoviride*. *Zeitschrift fur Angewandte Entomologie* **118**, 310–315.

Shimizu, S., Tsuchitani, Y. and Matsumoto, T. (1993a). Production of an extracellular protease by *Beauveria bassiana* in the hemolymph of the silkworm, *Bombyx mori*. *Letters in Applied Microbiology* **16**, 291–294.

Shimizu, S., Tsuchitani, Y. and Matsumoto, T. (1993b). Serology and substrate-specificity of extracellular proteases from 4 Species of entomopathogenic hyphomycetes. *Journal of Invertebrate Pathology* **61**, 192–195.

Shnitka, T. K. (1974). Esterases-nonspecific esterases. In 'Electron Microscopy of Enzymes' (M. A. Hayat, ed.), pp. 1–53. Van Nostrand-Reinhold. Princeton, N.J.

Sloman, I. S. and Reynolds, S. E. (1993). Inhibition of ecdysteroid secretion from *Manduca* prothoracic glands in vitro by destruxins – cyclic depsipeptide toxins from the insect pathogenic fungus *Metarhizium anisopliae*. *Insect Biochemistry and Molecular Biology* **23**, 43–46.

Smith, R. J. and Grula, E. A. (1983). Chitinase is an inducible enzyme in *Beauveria bassiana*. *Journal of Invertebrate Pathology* **42**, 319–326.

Smith, R. J., Pekrul, S. and Grula, E. A. (1981). Requirements for sequential enzymatic activities for penetration of the integument of the cornworm *Helithis zea*. *Journal of Invertebrate Pathology* **38**, 335–344.

Smithson, S. L., Paterson, I. C., Bailey, A. M., Screen, S. E., Hunt, B. A., Cobb, B. D., Cooper, R. M., Charnley, A. K. and Clarkson, J. M. (1995). Cloning and characterization of a gene encoding a cuticle- degrading protease from the insect pathogenic fungus *Metarhizium anisopliae*. *Gene* **166**, 161–165.

Soledade, M., Pedras, C., Zahira, L. I. and Ward, D. E. (2002). The destruxins: synthesis, biosynthesis, biotransformation, and biological activity. *Phytochemistry* **59**, 579–596.

Somero, I. (1975). The role of isoenzymes in adaptation to varying temperatures. In 'Isozymes II. Physiological Function' (A. P. Marbet, ed.). Academic Press, New York.

Springer, J. P., Cole, R. J., Dorner, J. W., Cox, R. H., Richard, J. L., Barnes, C. L. and van der Helm, D. (1984). Structure and conformation of roseiotoxin. *Journal of American Chemical Society* **106**, 2388–2392.

St. Leger, R. J. (1991). Integument as a barrier to microbial infections. In 'Physiology of the Insect Epidermis' (K. Binnington and A. Retnakaran, eds.), pp. 284–306.

St. Leger, R. J. (1993). Biology and mechanisms of insect-cuticle invasion by Deuteromycete fungal pathogens. In 'Parasites and Pathogens of Insects' (N. E. Beckage, S. N. Thompson and B. A. Federici, eds.), pp. 211–229. Academic Press.

St. Leger, R. J. (1995). The role of cuticle-degrading proteases in fungal pathogenesis of insects. *Canadian Journal of Botany* **73**, S1119–S1125.

St. Leger, R. J. and Bidochka, M. J. (1996). Insect-fungus interactions. In 'Invertebrate Immunology' (K. Soderhall, Vasta, G. and Iwanaga, S, eds.), pp. 441–478. SOS Publications, Fairhaven, NJ.

St. Leger, R. J. and Roberts, D. W. (1997). Engineering improved mycoinsecticides. *Trends in Biotechnology* **15**, 83–85.

St. Leger, R. J. and Screen, S. E. (2000). In vitro utilization of mucin, lung polymers, plant cell walls and insect cuticle by *Aspergillus fumigatus*, *Metarhizium anisopliae* and *Haematonectria haematococca*. *Mycological Research* **104**, 463–471.

St. Leger, R. J., Charnley, A. K. and Cooper, R. M. (1986a). Cuticle-degrading enzymes of entomopathogenic fungi – mechanisms of interaction between pathogen enzymes and insect cuticle. *Journal of Invertebrate Pathology* **47**, 295–302.

St. Leger, R. J., Charnley, A. K. and Cooper, R. M. (1986b). Cuticle-degrading enzymes of entomopathogenic fungi mechanisms of interaction between pathogen enzymes and insect cuticle. *Journal of Invertebrate Pathology* **47**, 295–302.

St. Leger, R. J., Charnley, A. K. and Cooper, R. M. (1986c). Cuticle-degrading enzymes of entomopathogenic fungi: synthesis in culture on cuticle. *Journal of Invertebrate Pathology* **48**, 85–95.

St. Leger, R. J., Cooper, R. M. and Charnley, A. K. (1986d). Cuticle-degrading enzymes of entomopathogenic fungi cuticle degradation *in vitro* by enzymes from entomopathogens. *Journal of Invertebrate Pathology* **47**, 167–177.

St. Leger, R. J., Cooper, R. M. and Charnley, A. K. (1986e). Cuticle-degrading enzymes of entomopathogenic fungi regulation of production of Chitinolytic Enzymes. *Journal of General Microbiology* **132**, 1509–1518.

St. Leger, R. J., Charnley, A. K. and Cooper, R. M. (1987a). Characterization of cuticle-degrading proteases produced by the entomopathogen *Metarhizium anisopliae*. *Archives of Biochemistry and Biophysics* **253**, 221–232.

St. Leger, R. J., Cooper, R. M. and Charnley, A. K. (1987b). Distribution of chymoelastases and trypsin-like enzymes in five species of entomopathogenic deuteromycetes. *Archives of Biochemistry and Biophysics* **258**, 123–131.

St. Leger, R. J., Cooper, R. M. and Charnley, A. K. (1987c). Production of cuticle-degrading enzymes by the entomopathogen *Metarhizium anisopliae* during infection of cuticles from *Calliphora vomitoria* and *Manduca sexta*. *Journal of General Microbiology* **133**, 1371–1382.

St. Leger, R. J., Charnley, A. K. and Cooper, R. M. (1988a). Production of polyphenol pigments and phenoloxidase by the entomopathogen *Metarhizium anisopliae*. *Journal of Invertebrate Pathology* **52**, 215–220.

St. Leger, R. J., Durrands, P. K., Charnley, A. K. and Cooper, R. M. (1988b). Role of extracellular chymoelastase in the virulence of *Metarhizium anisopliae* for *Manduca sexta*. *Journal of Invertebrate Pathology* **52**, 285–293.

St. Leger, R. J., Durrands, P. K., Cooper, R. M. and Charnley, A. K. (1988c). Regulation of production of proteolytic enzymes by the entomopathogenic fungus *Metarhizium anisopliae*. *Archives of Microbiology* **150**, 413–416.

St. Leger, R. J., Butt, T. M., Staples, R. C. and Roberts, D. W. (1989a). Synthesis of proteins including a cuticle-degrading protease during differentiation of the entomopathogenic fungus *Metarhizium anisopliae*. *Experimental Mycology* **13**, 253–262.

St. Leger, R. J., Roberts, D. W. and Staples, R. C. (1989b). Calcium-mediated calmodulin-mediated protein synthesis and protein phosphorylation during germination growth and protease production by *Metarhizium anisopliae*. *Journal of General Microbiology* **135**, 2141–2154.

St. Leger, R. J., Cooper, R. M. and Charnley, A. K. (1991a). Characterization of chitinase and chitobiase produced by the entomopathogenic fungus *Metarhizium anisopliae*. *Journal of Invertebrate Pathology* **58**, 415–426.

St. Leger, R. J., Staples, R. C. and Roberts, D. W. (1991b). Changes in translatable messenger RNA species associated with nutrient deprivation and protease synthesis in *Metarhizium anisopliae*. *Journal of General Microbiology* **137**, 807–816.

St. Leger, R. J., Frank, D. C., Roberts, D. W. and Staples, R. C. (1992a). Molecular cloning and regulatory analysis of the cuticle-degrading protease structural gene from the entomopathogenic fungus *Metarhizium anisopliae*. *European Journal of Biochemistry* **204**, 991–1001.

St. Leger, R. J., May, B., Allee, L. L., Frank, D. C., Staples, R. C. and Roberts, D. W. (1992b). Genetic differences in allozymes and in formation of infection structures among isolates of the entomopathogenic fungus *Metarhizium anisopliae*. *Journal of Invertebrate Pathology* **60**, 89–101.

St. Leger, R. J., Staples, R. C. and Roberts, D. W. (1992c). Cloning and regulatory analysis of starvation-stress gene, Ssga, encoding a hydrophobin-like protein from the entomopathogenic fungus, *Metarhizium anisopliae*. *Gene* **120**, 119–124.

St. Leger, R. J., Cooper, R. M. and Charnley, A. K. (1993a). Analysis of aminopeptidase and dipeptidylpeptidase Iv from the entomopathogenic fungus *Metarhizium anisopliae*. *Journal of General Microbiology* **139**, 237–243.

St. Leger, R. J., Staples, R. C. and Roberts, D. W. (1993b). Entomopathogenic isolates of *Metarhizium anisopliae*, *Beauveria bassiana*, and *Aspergillus*

flavus produce multiple extracellular chitinase isozymes. *Journal of Invertebrate Pathology* **61**, 81–84.

St. Leger, R. J., Bidochka, M. J. and Roberts, D. W. (1994a). Characterization of a novel carboxypeptidase produced by the entomopathogenic fungus *Metarhizium anisopliae*. *Archives of Biochemistry and Biophysics* **314**, 392–398.

St. Leger, R. J., Bidochka, M. J. and Roberts, D. W. (1994b). Isoforms of the cuticle-degrading PR1 protease and production of a metalloprotease by *Metarhizium anisopliae*. *Archives of Biochemistry and Biophysics* **313**, 1–7.

St. Leger, R. J., Joshi, L., Bidochka, M. J. and Roberts, D. W. (1995a). Multiple aminopeptidases produced by *Metarhizium anisopliae*. *Journal of Invertebrate Pathology* **65**, 313–314.

St. Leger, R. J., Joshi, L., Bidochka, M. J. and Roberts, D. W. (1995b). Protein-synthesis in *Metarhizium anisopliae* growing on host cuticle. *Mycological Research* **99**, 1034–1040.

St. Leger, R. J., Joshi, L., Bidochka, M. J., Rizzo, N. W. and Roberts, D. W. (1996a). Biochemical characterization and ultrastructural localization of two extracellular trypsins produced by *Metarhizium anisopliae* in infected insect cuticles. *Applied and Environmental Microbiology* **62**, 1257–1264.

St. Leger, R. J., Joshi, L., Bidochka, M. J., Rizzo, N. W. and Roberts, D. W. (1996b). Characterization and ultrastructural localization of chitinases from *Metarhizium anisopliae*, *Metarhizium flavoviride*, and *Beauveria bassiana* during fungal invasion of host (*Manduca sexta*) cuticle. *Applied and Environmental Microbiology* **62**, 907–912.

St. Leger, R. J., Joshi, L., Bidochka, M. J. and Roberts, D. W. (1996c). Construction of an improved mycoinsecticide overexpressing a toxic protease. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 6349–6354.

St. Leger, R. J., Joshi, L. and Roberts, D. W. (1997). Adaptation of proteases and carbohydrases of saprophytic, phytopathogenic and entomopathogenic fungi to the requirements of their ecological niches. *Microbiology (UK)* **143**, 1983–1992.

St. Leger, R. J., Joshi, L. and Roberts, D. (1998). Ambient pH is a major determinant in the expression of cuticle-degrading enzymes and hydrophobin by *Metarhizium anisopliae*. *Applied and Environmental Microbiology* **64**, 709–713.

St. Leger, R. J., Nelson, J. O. and Screen, S. E. (1999). The entomopathogenic fungus *Metarhizium anisopliae* alters ambient pH, allowing extracellular protease production and activity. *Microbiology-UK* **145**, 2691–2699.

Stachelhaus, T. and Marahiel, M. A. (1995). Modular structure of genes encoding multifunctional peptide synthetases required for non-ribosomal peptide synthesis. *FEMS Microbiology Letters* **125**, 3–14.

Steinrauf, L. K. (1985). Beauvericin and the other enniatins. *Metal Ions in Biological Systems* **19**, 139–171.

Stirling, J. L., Cook, E. A. and Pope, A. M. S. (1979). Chitin and its degradation. In 'Fungal walls and hyphal growth' (J. A. Burnett and C. Trinci, eds.), pp. 169–188, Cambridge University Press, Cambridge.

Strasser, H., Abendstein, D., Stuppner, H. and Butt, T. M. (2000). Monitoring the distribution of secondary metabolites produced by the entomogenous fungus *Beauveria brongniartii* with particular reference to oosporein. *Mycological Research* **104**, 1227–1233.

Strongman, D. B., Strunz, G. M., Giguere, P., Yu, C.-M. and Calhoun, L. (1987). Enniatins from *Fusarium avenaceum* isolated from balsam fir foliage and their toxicity to spruce budworm larvae, *Choristoneura fumiferana*. *Journal of Chemical Ecology* **14**, 753–764.

Suzuki, A. and Tamura, S. (1972). Isolation and structure of protodestruxin from *Metarrhizium anisopliae*. *Agricultural and Biological Chemistry* **36**, 896–898.

Suzuki, A., Taguchi, H. and Tamura, S. (1970). Isolation and structure elucidation of three new insecticidal cyclodepsipeptides, destruxins C and D and desmethyldestruxin B produced by *Metarhizium anisopliae*. *Agricultural and Biological Chemistry* **34**, 813–816.

Suzuki, A., Kawakami, K. and Tamura, S. (1971). Detection of destruxins in silkworm larvae infected with *Metarhizium anisopliae*. *Agricultural and Biological Chemistry* **35**, 1641–1643.

Suzuki, A., Kanaoka, M., Isogai, A., Murakoshi, S., Ichinoe, M. and Tamura, S. (1977). Bassianolide, a new insecticidal cyclodepsipeptide from *Beauveria bassiana* and *Verticillium lecanii*. *Tetrahedron Letters* **25**, 2167–2170.

Tanada, Y. and Kaya, H. K. (1993). 'Insect Pathology'. Academic Press, San Diego.

Tsantrizos, Y.S., Xu, X.J., Sauriol, F. and Hynes, R.C. (1993) Novel quinazolinones and enniatins from *Fusarium lateritium*. *Canadian Journal of Chemistry* **71**, 1362–1367.

Tunlid, A. and Talbot, N. J. (2002). Genomics of parasitic and symbiotic fungi. *Current Opinion in Microbiology* **5**, 513–519.

Turlington, L. W., Woodward, D. L. and Colwell, A. E. (1990). Pathogenesis of *Tolypocladium cylindrosporium* (Deuteromycotina: Hyphomycetes) in *Chaoborus astictopus* (Diptera: Chaoboridae). *Journal of Invertebrate Pathology* **55**, 126–129.

Turner, W. B. and Aldridge, D. C. (1983). Triterpenoids and steroids. In 'Fungal Metabolites 2nd Edition (W. B. Turner and D. C. Aldridge, eds.), pp. 225–336. Academic Press, London.

Tyrrell, D. (1990). Pathogenesis of *Entomophaga aulicae*. 1. Disease symptoms and effect of infection on weight-gain of infected *Choristoneura fumiferana* and *Malacosoma disstria* Larvae. *Journal of Invertebrate Pathology* **56**, 150–156.

Vakili, N. G. (1990). Biocontrol of stalk rot in corn. In *Proceedings of the 44th annual corn and sorgum research conference*, pp. 87–105.

Venkatsubbaiah, P., Tisserat, N. A. and Chilton, W. S. (1994). Metabolites of *Ophiosphaerella herpotricha*. *Mycopathologia* **128**, 155–159.

Vey, A. (1985). Efficiency of the encapsulation reaction in insects – effect on the structure and the viability of the encapsulated fungus. *Developmental and Comparative Immunology* **9**, 174–174.

Vey, A. and Quiot, J. M. (1989a). Cytotoxic effect of destruxins cyclodepsipeptide toxins produced by the entomopathogenic fungus *Metarhizium anisopliae* investigated *in-vitro* and in the insect host. *Canadian Journal of Microbiology* **35**, 1000–1008.

Vey, A. and Quiot, J. M. (1989b). *In vitro* and insect-host cyto-toxicity of destruxins, cyclodepsipeptide mycotoxins produced by the entomopathogen, *Metarhizum anisopliae*. *Canadian Journal of Microbiology* **35**, 1000–1008.

Vey, A., Quiot, J. M. and Pais, M. (1986). Toxemia of a fungal origin in invertebrates and its cytotoxic consequences – studies on the infection caused by *Metarhizium anisopliae* (Hyphomycete, Moniliales) in lepidoptera and coleoptera. *Comptes Rendus Des Seances De La Societe De Biologie Et De Ses Filiales* **180**, 105–112.

Vilcinskas, A. and Wedde, M. (1997). Inhibition of *Beauveria bassiana* proteases and fungal development by inducible protease inhibitors in the

haemolymph of *Galleria mellonella* larvae. *Biocontrol Science and Technology* **7**, 591–601.

Vilcinskas, A., Kopacek, P., Jegorov, A., Vey, A. and Matha, V. (1997a). Detection of lipophorin as the major cyclosporin-binding protein in the hemolymph of the greater wax moth *Galleria mellonella*. *Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology* **117**, 41–45.

Vilcinskas, A., Matha, V. and Gotz, P. (1997b). Effects of the entomopathogenic fungus *Metarhizium anisopliae* and its secondary metabolites on morphology and cytoskeleton of plasmatocytes isolated from the greater wax moth, *Galleria mellonella*. *Journal of Insect Physiology* **43**, 1149–1159.

Vilcinskas, A., Matha, V. and Gotz, P. (1997c). Inhibition of phagocytic activity of plasmatocytes isolated from *Galleria mellonella* by entomogenous fungi and their secondary metabolites. *Journal of Insect Physiology* **43**, 475–483.

Vilcinskas, A., Jegorov, A., Landa, Z., Gotz, P. and Matha, V. (1999). Effects of beauverolide L and cyclosporin A on humoral and cellular immune response of the greater wax moth, *Galleria mellonella*. *Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology* **122**, 83–92.

Vining, L. C. (1990). Functions of secondary metabolites. *Annual Review of Microbiology* **44**, 395–427.

Vining, L. C., Kelleher, W. J. and Schwarting, A. E. (1962). Osporein production by a strain of *Beauveria bassiana* originally identified as *Amanita muscaria*. *Canadian Journal of Microbiology* **8**, 931–933.

Visconti, A., Blais, L. A., ApSimon, J. W., Greenhalgh, R. and Miller, J. D. (1992). Production of enniatins by *Fusarium acuminatum* and *Fusarium compactum* in liquid culture: isolation and characterization of three new enniatins, B_2, B_3, B_4. *Journal of Agriculture and Food Chemistry* **40**, 1076–1082.

Wahlman, M. and Davidson, B. S. (1993). New destruxins from the entomopathogenic fungus *Metarhizium anisopliae*. *Journal of Natural Products* **56**, 643–647.

Walton, J. D. (1996). Host-selective toxins: agents of compatibility. *Plant Cell* **8**, 1723–1733.

Walton, J. D. (2000). Horizontal gene transfer and the evolution of secondary metabolite gene clusters in fungi: an hypothesis. *Fungal Genetics and Biology* **30**, 167–171.

Wang, C. S., Typas, M. A. and Butt, T. M. (2002). Detection and characterisation of pr1 virulent gene deficiencies in the insect pathogenic fungus *Metarhizium anisopliae*. *FEMS Microbiology Letters* **213**, 251–255.

Wartburg, V. A. and Traber, R. (1988). Cyclosporins, fungal metabolites with immunosuppressive activities. In 'Progress in Medicinal Chemistry' (G. P. Ellis and G. B. West, eds.), pp. p133. Elsevier Science Publishers.

Wat, C.-K., McInnis, A. G., Smith, D. G., Wright, J. L. C. and Vining, L. C. (1977). The yellow pigments of *Beauveria bassiana* species. Structures of tenellin and bassianin. *Canadian Journal of Chemistry* **55**, 4090–4098.

Waterfield, N. R., Daborn, P. J. and ffrench-Constant, R. H. (2002). Genomic islands in *Photorhabdus*. *Trends in Microbiology* **10**, 541–545.

Weiser, J. and Matha, V. (1988a). The insecticidal activity of cyclosporines on mosquito larvae. *Journal of Invertebrate Pathology* **51**, 143–150.

Weiser, J. and Matha, V. (1988b). Tolypin, a new insecticidal metabolite of fungi of the genus *Tolypocladium*. *Journal of Invertebrate Pathology* **51**, 94–96.

Wigglesworth, V. B. (1970). Structural lipids in the insect cuticle and the function of of oenocytes. *Tissue & Cell* **2**, 155–179.

Wright, V. F., Vesonder, R. F. and Ciegler, A. (1982). In 'Microbial and Viral Pesticides' (E. Kurstak, ed.). Marcel Dekker.

Yeh, S. F., Pan, W., Ong, G. T., Chiou, A. J., Chuang, C. C., Chiou, S. H. and Wu, S. H. (1996). Study of structure-activity correlation in destruxins, a class of cyclodepsipeptides possessing suppressive effect on the generation of hepatitis B virus surface antigen in human hepatoma cells. *Biochemical and Biophysical Research Communications* **229**, 65–72.

Yoder, O. C. and Turgeon, B. G. (2001). Fungal genomics and pathogenicity. *Current Opinion in Plant Biology* **4**, 315–321.

Yoshimoto, Y. and Imoto, M. (2002). Induction of EGF-dependent apoptosis by vacuolar-type H^+-ATPase inhibitors in A431 cells overexpressing the EGF receptor. *Experimental Cell Research* **279**, 118–127.

Zacharuk, R. Y. (1970a). Fine structure of the fungus *Metarrhizium anisopliae* infecting 3 species of larval elateridae coleoptera Part 2 conidial germ tubes and appressoria. *Journal of Invertebrate Pathology* **15**, 81–91.

Zacharuk, R. Y. (1970b). Fine structure of the fungus *Metarrhizium anisopliae* infecting 3 species of larval elateridae coleoptera Part 3 penetration of the host integument. *Journal of Invertebrate Pathology* **15**, 372–396.

Zeller, E. A. (1977). Snake venom action: are enzymes involved in? *Experientia* **33**, 143–150.