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Adaptation of proteases and carbohydrases of saprophytic, phytopathogenic and entomopathogenic fungi to the requirements of their ecological niches

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The abilities of isolates of saprophytes (*Neurospora crassa*, *Aspergillus nidulans*), an opportunistic human pathogen (*Aspergillus fumigatus*), an opportunistic insect pathogen (*Aspergillus flavus*), plant pathogens (*Verticillium albo-atrum*, *Verticillium dahliae*, *Nectria haematococca*), a mushroom pathogen (*Verticillium fungicola*) and entomopathogens (*Verticillium lecanii*, *Beauveria bassiana*, *Metarhizium anisopliae*) to utilize plant cell walls and insect cuticle components in different nutrient media were compared. The pathogens showed enzymic adaptation to the polymers present in the integuments of their particular hosts. Thus, the plant pathogens produced high levels of enzymes capable of degrading pectic polysaccharides, cellulose and xylan, as well as a cutinase substrate, but secreted little or no chitinase and showed no proteolytic activity against elastin and mucin. The entomopathogens and *V. fungicola* degraded a broad spectrum of proteins (including elastin and mucin) but, except for chitinase, cellulase (*V. lecanii* and *V. fungicola* only) and cutinase (*B. bassiana* only), produced very low levels of polysaccharidases. The saprophytes (*Neu. crassa* and *A. nidulans*) and the opportunistic pathogens (*A. fumigatus* and *A. flavus*) produced the broadest spectrum of protein and polysaccharide degrading enzymes, indicative of their less specialized nutritional status. *V. lecanii* and *V. albo-atrum* were compared in more detail to identify factors that distinguish plant and insect pathogens. *V. albo-atrum*, but not *V. lecanii*, grew well on different plant cell wall components. The major class of proteases produced in different media by isolates of *V. albo-atrum* and *V. dahliae* were broad spectrum basic (pI >10) trypsins which degrade Z-AA-AA-Arg-NA substrates (Z, benzoyl; AA, various amino acids; NA, nitroanilide), hide protein azure and insect (*Manduca sexta*) cuticles. Analogous peptidases were produced by isolates of *V. lecanii* and *V. fungicola* but they were specific for Z-Phe-Val-Arg-NA. *V. albo-atrum* and *V. dahliae* also produced low levels of neutral (pI ca 7) and basic (pI ca 9·5) subtilisin-like proteases active against a chymotrypsin substrate (Succinyl-Ala_2-Pro-Phe-NA) and insect cuticle. In contrast, subtilisins comprised the major protease component secreted by *V. lecanii* and *V. fungicola*. Both *V. lecanii* and *V. albo-atrum* produced the highest levels of subtilisin and trypsin-like activities during growth on collagen or insect cuticle. Results are discussed in terms of the adaptation of fungi to the requirements of their ecological niches.

Keywords: entomopathogenic fungi, phytopathogenic fungi, saprophytic fungi, protease production, carbohydrase production

Abbreviations: AFC, 7-amino-4-trifluoromethyl coumarin; EOMs, enzyme overlay membranes; NA, nitroanilide; PG, polygalacturonase; Pr1, subtilisin-like protease; Pr2, trypsin-like protease; Suc, succinyl; Z, benzoyl.

INTRODUCTION

Fungal pathogens have been recorded for virtually all groups of multicellular organisms. All classes of Eumycotina contain at least a few plant pathogens. Likewise, at least 90 genera and more than 700 species of fungi have been identified as insect pathogens (Roberts & Humber, 1981). For most fungal diseases, the genes and their products in both host and pathogen that limit host range and determine resistance or susceptibility are poorly understood. However, for a few model systems, molecular genetic approaches are beginning to identify microbial and host products that influence the progression of infection (Bowyer *et al*., 1995; Hensel & Holden, 1996; Knogge, 1996; Oliver & Osburn, 1995; Schafer, 1994).

The ability to infect insects must have evolved independently many times since pathogenic fungi are found in several divergent fungal groups. The huge variety of insects has given fungi countless opportunities to exploit them; the antiquity and subsequently extended co-evolution of such interrelationships being reflected in their present ubiquity and often high degree of sophistication (Cooke & Whipps, 1993). Obviously, given the many routes by which fungi have achieved their success as entomopathogens, different evolutionary pressures may have operated in taxonomically diverse groups. Nevertheless, to determine how pathogenicity evolved it might be useful to ask in what aspects does an entomopathogen differ from a saprophyte or plant pathogen. Fungi may have emerged onto the land as endophytes of plants (Lewis, 1987). Insect-pathogenic fungi could have evolved from these or from plant-pathogenic fungi derived from these via adaptations in extracellular hydrolytic enzymes so as to accommodate hydrolysis of proteinaceous insect cuticles (St Leger & Bidochka, 1996). Plant-pathogenic fungi produce a complement of extracellular enzymes that degrade carbohydrate plant tissues (Walton, 1994). The structure of these tissues is analogous to those found in insect cuticle, i.e. both are composite structures containing a fibrous material (chitin or cellulose) embedded in a matrix material (protein or pectic substances and hemicelluloses) (Carpita & Gibeaut, 1993; Charnley, 1984) and many of the plant pathogens possess structural and behavioural adaptations which are very similar to the entomopathogens, e.g. they produce an ordered sequence of infection structures, including appressoria. These observations strongly suggest that some of the underlying mechanisms of fungal pathogenesis may be similar in insects and plants. Since a vast majority of insects are herbivores, the opportunity for association of a plant-pathogenic fungus on an insect would be high. With the exception of the Entomophthorales (Zygomycetes), many fungi that infect plants and invertebrates fall within the same taxonomic groups. Species within the genus *Verticillium* include plant, human, nematode and insect pathogens (Segers *et al*., 1994; Schreiter *et al*., 1994; Amici *et al*., 1994; Cooper & Wood, 1975) and may represent a divergent genus that has evolved pathogenic specialization for various life forms. Aside from *Verticillium* spp. there is little overlap between plant and insect pathogens at the genus level in fungi (even those which, like *Verticillium*, are based on morphology of asexual stages) and virtually none at the species level. It follows that some of the characteristics needed by fungi to successfully establish disease in plants must be fundamentally different in some way from those needed to infect insects. These differences, when clearly delineated, will indicate probable key virulence characters for pathogens of the two host groups.

The present study comprises a comparative analysis of depolymerases produced by saprophytes and opportunists (*Aspergillus* spp. and *Neurospora crassa*) and pathogens of plants (*Nectria haematococca*, *Verticillium albo-atrum*, *Verticillium dahliae*), insects (*Verticillium lecanii*, *Beauveria bassiana*, *Metarhizium anisopliae*) and fungi (*Verticillium fungicola*).

METHODS

Organisms and growth. *A. nidulans* strain A4 and *Neu. crassa* strain 2489 were obtained from the Fungal Genetic Stock Center in the University of Kansas Medical Center, KS, USA. *A. fumigatus* strain 16424 was obtained from the American Type Culture Collection, Rockville, MD, USA. It was isolated from a human patient with aspergillosis. The entomopathogens *A. flavus* strain 3144 (host: gypsy moth, *Lymantria dispar*), *B. bassiana* strains 252 (host: Colorado potato beetle, *Leptinotarsa decemlineata*) and 1630 (host: *Calliphora* spp.), *M. anisopliae* strains 1080 (host: *Heliothis zea*) and 2575 (host: pecan weevil, *Curculio caryae*), *V. lecanii* strains 313 (single spore isolate of Vertelac), 1335 (host: *Malachius bipustulatus*) and 3709 (host: Homoptera, Aleyrodidae) and *V. fungicola* strain 2065 (host: egg mass, *Lymantria dispar*) were obtained from the USDA Entomopathogenic Fungus Collection, Ithaca, NY, USA. The mushroom pathogen *V. fungicola* strain VAMH 895 (cause of brown spot disease in mushrooms) was obtained from the University of Alberta Microfungus Collection and Herbarium, Canada. The plant pathogens *V. albo-atrum* strains 13-1, 26-1, 90-1 and 98-1 (host: alfalfa, *Medicago sativa*), *V. dahliae* (host: maple) were obtained from the teaching collection of the Department of Plant Pathology, Cornell University, Ithaca, NY, USA. *Nec. haematococca* (*Fusarium solanii*) strain T8 (host: pea plant) was obtained from Dr Olen Yoder, Department of Plant Pathology Cornell University, Ithaca, NY, USA.

Enzyme production on solid media. Enzyme activity was determined by the ability to produce zones of clearing in 1·2 % (w/v) agar media containing suitable substrates. Cellulase and xylanase activities were measured using minimal medium (MM) (0·3 % $NaNO_3$, 0·1 % KH_2PO_4, 0·05 % $MgSO_4$, pH 6) supplemented with 0·1 % yeast extract and 0·5 % CM-cellulose (medium viscosity) or birchwood xylan. For both media, clearing zones were visualized by staining with 1 mg Congo red ml^{-1} for 15 min, then destaining with 1 M NaCl (Teather & Wood, 1982). Polygalacturonase (PG) and pectin lyase were detected using the pectic screening media devised by Durrands & Cooper (1988) to study pectinase mutants of *V. albo-atrum*. PG detection medium contained non-methylated polygalacturonan (sodium polypectate) buffered at pH 5·0 (0·05 M sodium citrate) and was calcium free. In contrast, pectin lyase medium contained polymethylgalacturonan (pectin) buffered

at pH 8·0 (0·05 M HEPES) and contained calcium-rich agar. PG and PL activity were detected on these media by addition of 1 % (w/v) cetyltrimethylammonium bromide which precipitated undegraded substrate. Chitinase activity was detected using MM supplemented with 0·05 % yeast extract and 0·2 % (dry wt) colloidal chitin adjusted to pH 5·5. Plates were prepared in the manner described by Hankin & Anagnostakis (1975) with an underlay of 1·5 % agar and a 3 mm overlay of the chitin medium. Cutinase activity was detected using the polycaprolactone screening medium devised by Murphy *et al.* (1996). This contained MM (pH 6·0) supplemented with 0·1 % yeast extract and 0·5 % polycaprolactone, which formed a cloudy suspension so that clearing zones produced by secreted fungal enzymes could be measured directly.

Proteolytic activity was detected using MM solution (adjusted to pH 7 using NaOH) supplemented with 0·1 % yeast extract and 0·1 % elastin, 0·3 % porcine stomach mucin or 0·1 % hide protein azure. The test medium was prepared at double strength without agar and autoclaved separately. The components were cooled to about 50 °C before mixing.

Spores of each strain were point-inoculated onto agar surfaces and incubated at 25 °C for up to 7 d or until mycelial colonies reached 30 mm in diameter. *Neu. crassa* produced a thin net-like mycelium on the surface of the agar and a small diameter colony within the agar which produced a measurable halo of substrate degradation.

Preparation and analysis of culture filtrate. Cultures were inoculated with spores taken from 7- to 12-d-old agar plates or with standardized mycelial inocula (0·5 g wet wt per 10 ml) from 48 h Sabouraud dextrose cultures. The 250 ml conical flasks were incubated with shaking (100 r.p.m.) at 25 °C for up to 4 d in 20, 50 or 100 ml basal medium (containing, l^{-1}: 1 g KH_2PO_4, 0·5 g $MgSO_4$, 0·7 mg $Na_2B_4O_7.10H_2O$, 0·5 mg $(NH_4)_6Mo_7O_{24}.4H_2O$, 10·0 mg $Fe_2(SO_4)_3.6H_2O$, 0·3 mg $ZnSO_4.7H_2O$, adjusted to pH 6·0 with NaOH) supplemented with a carbon source at 0·5 %. Cultures were filtered through Whatman No. 1 filter paper and then through a 0·2 μm pore Millipore filter unit before being used for enzyme assays.

Electrophoresis. Analytical IEF on ultrathin polyacrylamide gels was performed in an IEF cell (Bio-Rad) using 1 % ampholytes (Bio-Lyte 3/10; Bio-Rad) as previously described (St Leger *et al.*, 1996a). For gelatin SDS-PAGE, electrophoresis was done in 11 % (w/v) polyacrylamide gels containing 0·2 % copolymerized gelatin (Lockwood *et al.*, 1987). After electrophoresis, the gels were incubated for 60 min (35 °C) in renaturation buffer [0·05 M Tris, pH 8, containing 2·5 % (v/v) Triton X-100; St Leger *et al.*, 1996a]. Protease activity against gelatin was detected by staining residual gelatin with Coomassie blue G-250. The molecular masses of the proteins were determined from a graph of log molecular mass versus migration distance of standard proteins (Bio-Rad).

To characterize protease isoforms, gels were overlaid with enzyme overlay membranes (EOMs) (St Leger *et al.*, 1994). These were nitrocellulose membranes previously impregnated with fluorogenic substrates Suc-Ala_2-Pro-Phe-AFC, Z-Val-Leu-Arg-AFC and Z-Gly-Gly-Arg-AFC (Enzyme Systems Products) using procedures described by Smith (1984) (Suc, succinyl; AFC, 7-amino-4-trifluoromethyl coumarin; Z, benzoyl). Gels were incubated at 37 °C and the appearance of fluorescent bands monitored by an ultraviolet lamp. The contact side of the membrane was visualized and photographed on thermal paper using an EagleEye II image capture system (Stratagene). The photographs were scanned using a flatbed scanner and Adobe Photoshop.

Preparation of plant cell walls and insect cuticle substrates. Clean samples of cuticle from fifth instar *Manduca sexta* larvae were prepared as described previously (St Leger *et al.*, 1987a).

Cell walls were prepared from the stems of 8-week-old alfalfa (*M. sativa*) seedlings by stripping off the leaves and extracting the comminuted stems with organic solvents (York *et al.*, 1985).

Enzyme assays. Protease activity versus *M. sexta* cuticle, cuticle proteins, hide protein azure, subtilisin-like protease (Pr1) activity (versus Suc-Ala_2-Pro-Phe-NA) and trypsin-like protease (Pr2) activity (versus Z-Phe-Val-Arg-NA) were determined as described previously for *V. lecanii* and other entomopathogens (St Leger *et al.*, 1987b, c) (NA, nitroanilide).

Polysaccharidase activities were assayed according to Riou *et al.* (1991) using the pH optima reported by Cooper & Wood (1975) for enzymes from *V. albo-atrum*. Liberated reducing sugars were estimated with dinitrosalicylic reagent (Miller, 1959) using D-glucose as standard. Assays were repeated at pH 4, 6 and 8 in cultures containing no detectable activity at the reported pH optima.

Assays were performed in duplicate. All results are representative of at least two similar experiments using different enzyme preparations.

Materials. Reagents were procured as follows: Suc-Ala_2-Pro-Phe-CH_2Cl and EOM components were from Enzyme Systems Products; polycaprolactone LPS-60 HP was from Union Carbide Chemicals and Plastics Company. Other enzyme substrates (including the polysaccharides) and inhibitors were from Sigma.

RESULTS

Plate clearing assays

Table 1 shows that saprophytes (*A. nidulans*, *Neu. crassa*) and opportunistic pathogens (*A. fumigatus* and *A. flavus*) secrete activities against a broad spectrum of protein and carbohydrate substrates. In contrast, we observed consistent patterns for the occurrence of extracellular enzymes distinguishing plant pathogens from insect pathogens. Plant pathogens were all able to degrade pectic substances, cellulose and xylan, as well as the cutinase substrate. By contrast, *V. fungicola* and the entomopathogens did not produce clearing zones in xylan or pectic substances, indicating that if xylanase, pectinases or PGs are secreted, they were at a level below the sensitivity of the assay. Isolates of *M. anisopliae* and *B. bassiana* did not produce detectable levels of cellulase. However, large clearing zones in cellulose medium formed around all strains of *Verticillium* spp., irrespective of their host. *B. bassiana* also produced clearing zones in polycaprolactone, but this plastic may also be a substrate of some non-cutinase esterases (Murphy *et al.*, 1996).

All strains degraded hide protein azure, but only *Neu. crassa*, *Aspergillus* spp., *V. fungicola* and the entomopathogens secreted proteases capable of degrading elastin or mucin. Likewise, none of the plant pathogens produced detectable levels of secreted chitinase.

Table 1. Enzyme production by different species of fungi

Plates were point-inoculated and incubated for up to 7 d or until mycelia reached 30 mm in diameter. Enzyme activities were calculated as an index of the total diameter of the colony plus the clear zone around it divided by the diameter of the colony. When the value is >1·0 it indicates that the isolate is releasing the enzyme into the medium. When the value is <1·0 it indicates partial clearing of the substrate under the colony. TR, partial clearing was observed under the centre of mature (>7-d-old) cultures >50 mm in diameter.

Substrate	*A. fumigatus*	*A. flavus*	*A. nidulans*	*Neu. crassa*	*Nec. haematococca*	*V. dahliae*	*V. albo-atrum*			
							13-1	26-1	90-1	98-1
Protein										
Hide protein azure	1·3	1·3	1·3	1·2	1·4	1·2	1·4	1·2	1·5	1·3
Elastin	1·0	1·2	1·1	1·0	<1	TR	TR	TR	TR	TR
Mucin	1·0	1·1	1·0	1·2	<1	TR	TR	TR	TR	TR
Carbohydrate										
Chitin	1·1	1·1	1·1	1·0	0·0	0·0	0·0	0·0	0·0	0·0
Cellulose	1·2	1·2	1·3	1·4	1·4	1·7	1·9	1·8	1·7	1·7
Pectin	1·1	1·1	1·2	1·1	1·2	1·3	1·4	1·4	1·3	1·3
Sodium polypectate	1·0	1·0	1·2	1·2	1·3	3·5	3·7	3·5	3·9	3·4
Xylan	1·2	1·3	1·2	1·2	1·3	1·1	1·3	1·3	1·0	1·3
Cutinase substrate	1·0	1·4	1·1	1·0	1·2	1·1	1·1	1·1	1·1	1·1

	V. lecanii			*V. fungicola*		*B. bassiana*		*M. anisopliae*	
	313	1335	3709	VAMH 895	2065	252	1630	1080	2575
Protein									
Hide protein azure	1·3	1·3	1·4	1·4	1·5	1·7	1·6	1·5	1·6
Elastin	1·1	1·1	1·1	1·2	1·2	1·1	1·2	1·1	1·1
Mucin	1·5	1·4	1·6	1·5	1·4	1·2	1·3	1·3	1·3
Carbohydrate									
Chitin	1·1	1·1	1·1	1·2	1·2	1·1	1·0	1·2	1·1
Cellulose	1·9	1·5	1·7	1·8	1·4	0·0	0·0	0·0	0·0
Pectin	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0
Sodium polypectate	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0
Xylan	TR	TR	TR	TR	TR	0·0	0·0	0·0	0·0
Cutinase substrate	0·0	0·0	0·0	0·0	0·0	1·2	1·1	TR	TR

Effect of the carbohydrate growth substrate on polysaccharidase activities

The ability of *V. lecanii* isolate 313 and *V. albo-atrum* isolate 13-1 to metabolize the cell wall components of plants was evaluated in liquid cultures (Tables 2 and 3). As expected from studies by Cooper & Wood (1975), *V. albo-atrum* was able to utilize pectic substances, cellulose and xylan as sole carbon sources and to secrete enzymes that convert cellulosic, hemicellulosic and pectic polysaccharides to assimilable simple sugars. Maximal activities were generally obtained in cultures grown on the appropriate structure-related polysaccharide (Table 2). By contrast, we observed only marginal growth of *V. lecanii* on individual carbohydrates with little or no activities against polysaccharides when assayed at the pH optima of enzymes from *V. albo-atrum* (Cooper & Wood, 1975) and at pH 4, 6 or 8. The biomasses produced in these cultures were much lower than those obtained on glucose media (Table 3). In comparison to enzyme production on single carbohydrates, both *V. lecanii* and *V. albo-atrum* showed greatly enhanced enzyme production on alfalfa cell walls. Pectinase activities produced by *V. albo-atrum* against pectin and polygalacturonic acid were, respectively 4 and 44 times higher in cell wall media than in media containing only polygalacturonic acid or pectin. Cell walls were the only carbohydrate substrate that induced production of a polysaccharidase (PG) by *V. lecanii*. However, this enzyme was also produced in insect cuticle media, indicating that increases in enzyme activity can be unrelated to the carbon source.

Identification of *Verticillium* spp. proteinases

Seven bands with a pI range 6·8–9·6 could be distinguished on IEF gels when protease activity in collagen-grown cultures of *V. lecanii* strain 1335 was detected with the Pr1 subtilisin substrate Suc-Ala_2-Pro-

Table 2. Protease and polysaccharidase activities of extracellular preparations from *V. albo-atrum* strain 13-1 grown on a range of carbon sources for 4 d

Cultures (50 ml) were inoculated with 2×10^6 spores.

Carbohydrate growth substrate	Sugar released (μg ml^{-1} h^{-1})*				*p*NA released (nmol ml^{-1} min^{-1})*		Biomass (mg dry wt)†
	Pectin	PGA‡	Xylan	CMC§	Suc-Ala$_2$-Pro-Phe-NA	Z-Phe-Val-Arg-NA	
Alfalfa cell walls	473 ± 54	1549 ± 126	110 ± 21	34 ± 8	10 ± 3	44 ± 11	NM
M. sexta cuticle	10	43	0	0	14 ± 3	94 ± 11	NM
Pectin	86 ± 17	35 ± 6	4 ± 1	3 ± 0	0	3 ± 0	36 ± 3·7
PGA‡	57 ± 9	79 ± 14	3 ± 0	18 ± 2	0	2 ± 0	28 ± 4·6
Xylan	5 ± 2	12 ± 3	33 ± 9	7 ± 1	0	1 ± 0	34 ± 4·1
CMC§	2 ± 0	4 ± 0	8 ± 1	8 ± 2	1 ± 0	0	14 ± 2
Crystalline cellulose	2 ± 1	2 ± 0	3 ± 1	12 ± 2	1 ± 0	1 ± 0	NM
Glucose	2 ± 0	2 ± 0	0	0	0	1 ± 0	43 ± 3·4

* Enzyme type: pectinase (pectin); PG (PGA); xylanase (xylan); cellulase (CMC); Pr1 (Suc-Ala$_2$-Pro-Phe-NA); Pr2 (Z-Phe-Val-Arg-NA).

† NM, not measured

‡ PGA, sodium polygalacturonate.

CMC§, CM-cellulose.

Table 3. Protease and polysaccharidase activities of extracellular preparations from *V. lecanii* strain 313 grown on a range of carbon sources for 4 d

Cultures (50 ml) were inoculated with 2×10^6 spores. Abbreviations are defined in the legend to Table 2.

Carbohydrate growth substrate	Sugar released (μg ml^{-1} h^{-1})				*p*NA released (nmol ml^{-1} min^{-1})		Biomass (mg dry wt)
	Pectin	PGA	Xylan	CMC	Suc-Ala$_2$-Pro-Phe-NA	Z-Phe-Val-Arg-NA	
Alfalfa cell walls	0	35 ± 7	0	0	16 ± 3	12 ± 2	NM
M. sexta cuticle	0	31 ± 8	0	0	83 ± 7	74 ± 3	NM
Pectin	0	0	0	0	2 ± 0	5 ± 1	3 ± 1
PGA	0	6 ± 3	0	0	16 ± 3	10 ± 3	14 ± 2
Xylan	0	0	0	0	0	1 ± 0	4 ± 0
CMC	0	0	0	0	5 ± 1	1 ± 0	2 ± 0
Crystalline cellulose	0	0	0	0	5 ± 1	3 ± 1	NM
Glucose	0	0	0	0	18 ± 2	9 ± 2	52 ± 4·3

Phe-AFC (Fig. 1a). Lower levels and a reduced complexity of bands were produced by other strains (Fig. 1a). However, basic and/or neutral bands were also produced by *V. albo-atrum* and *V. dahliae* The major trypsin-like Pr2 enzymes produced by *Verticillium* spp. detected with Z-Val-Leu-Arg-AFC were very basic (pI >10) and diffused into the region of the electrode (Fig. 1b). Additional acidic bands (pI ca 4·5) were also detected. The highest producer was *V. albo-atrum*.

The data from the IEF gels suggest that trypsin-like enzymes are the major proteases produced by plant-pathogenic *Verticillium* spp. To confirm this, culture filtrates were assayed against a range of substrates previously used to detect subtilisins and trypsins in insect pathogens (St Leger *et al*., 1987b, c) (Table 4). *V. lecanii* and *V. fungicola* cleaved the subtilisin substrate Suc-Ala$_2$-Pro-Phe-NA faster than Z-Phe-Val-Arg-NA and possessed no activity against Z-Val-Gly-Arg-NA or Z-Pro-Phe-Arg-NA, indicating that the trypsins of these species require specific substrate sequences. In contrast, the plant pathogens hydrolysed all three trypsin substrates and cleaved the most susceptible of these (Z-Val-Gly-Arg-NA) at least tenfold faster than Suc-Ala$_2$-Pro-Phe-NA (Table 4).

Regulation of proteases

The production of proteases by *V. albo-atrum* and *V. lecanii* was compared on potential protease substrates

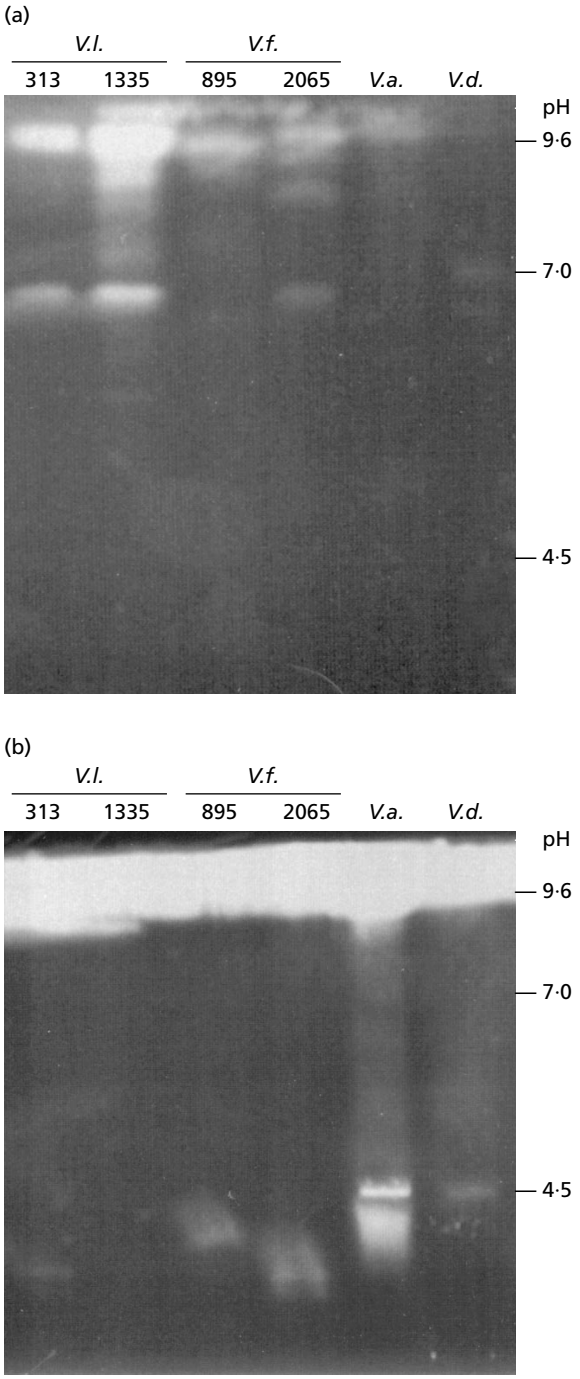

Fig. 1. Analytical (polyacrylamide) IEF (pH 3–10) of proteinases produced by *V. lecanii* (*V. l.*), *V. fungicola* (*V. f.*), *V. albo-atrum* (*V. a.*) and *V. dahliae* (*V. d.*) grown for 72 h in 1% collagen medium. Zymograms were prepared with EOMs containing (a) Suc-Ala_2-Pro-Phe-AFC and (b) Z-Val-Leu-Arg-AFC for isoforms of subtilisin-like proteases (Pr1) and trypsin-like proteases (Pr2), respectively. The membrane/gel sandwiches were incubated at 37 °C for 10 min.

(Table 5). *V. albo-atrum* produced most Pr1 and Pr2 activities on medium supplemented with collagen. *M. sexta* cuticle was also a good inducer of proteases. Low levels of protease were produced in the presence of plant cell walls. A similar pattern of protease induction by protein substrates was observed with *V. lecanii*, except that the highest levels were produced on the cuticle substrate. Analysis of *V. albo-atrum* protease composition by gelatin SDS-PAGE revealed that two major isoforms were produced in each medium (Fig. 2a). Both isoforms had activity against the trypsin substrates Z-Val-Leu-Arg-AFC (Fig. 2b) and Z-Gly-Gly-Arg-AFC (Fig. 2c) but lacked activity against an EOM containing Suc-Ala_2-Pro-Phe-NA (data not shown).

Effect of inhibitors on protein degradation

The relative contributions of Pr1- and Pr2-type enzymes from *V. lecanii* and *V. albo-atrum* to degradation of hide protein azure and *M. sexta* cuticle were assessed with specific inhibitors for each enzyme. The Pr1 inhibitor Suc-Ala_2-Pro-Phe-CH_2Cl (St Leger *et al.*, 1994) inhibited degradation of cuticle and hide protein azure by ca 90% in culture filtrates of *V. lecanii* containing both Pr1 and Pr2 (Table 6). The Pr2 inhibitor leupeptin (St Leger *et al.*, 1996a) did not significantly inhibit protein degradation. In contrast, in culture filtrates of *V. albo-atrum*, Suc-Ala_2-Pro-Phe-CH_2Cl was more effective at inhibiting cuticle degradation (66%) than leupeptin (30%) but less effective at inhibiting degradation of hide protein azure (44% vs 75%).

DISCUSSION

The saprophytes, including *Aspergillus* spp., produced the broadest spectrum of protein and polysaccharide-hydrolysing enzymes, which could be useful in the complete hydrolysis of complex living and non-living organic substrates and implies the greatest genetic and biochemical versatility. This versatility may not result from specific adaptation but rather reflect maintenance of the ability to exploit resources which temporarily become available. This is consistent with the 'alert' opportunistic saprophyte/pathogen life style of these fungi as exemplified by *A. flavus* which contains strains pathogenic to plants, insects and mammals (Cotty *et al.*, 1990).

Aspergillus spp. generally have a broad host range but low virulence, and usually require weakened hosts for colonization (Raper & Fennell, 1965). The other disease-causing fungi included in this study are all facultative saprophytes (true pathogens that can survive outside their hosts) that have specific genetic interactions with their hosts. Most are highly virulent on only a limited number of species. To be successful, most plant and insect pathogens breach the outer integument of their hosts. It is likely that in the evolution towards pathogenicity, an ancestral saprophytic fungus needed first to gain attributes enabling it to live on numerous plant species before refining those traits and/or developing additional devices to increase virulence on individual host species (Knogge, 1996). Because the enzymes required to gain entry into plant hosts (various poly-

Table 4. Extracellular proteases in 4-d-old culture filtrates from isolates of four *Verticillium* spp. grown in 1% (w/v) collagen

Cultures (20 ml) were inoculated with 7×10^5 spores. Filtrates were assayed at 23 °C in 10 mM Tris/HCl, pH 8, 4% (v/v) dimethyl sulfoxide with 0·06 mM substrate. Activities are expressed as a percentage of maximum activity. Absolute values (nmol min^{-1} ml^{-1}) corresponding to 100% are given in parentheses at the bottom of each column.

Substrate	Activity (%) secreted by isolate						
	V. lecanii		*V. fungicola*		*V. albo-atrum*		*V. dahliae*
	313	**1335**	**895**	**2065**	**13-1**	**90-1**	
Subtilisin							
Suc-Ala_2-Pro-Phe-NA	100	100	100	100	9	7	5
Trypsin							
Z-Phe-Val-Arg-NA	54	65	36	72	83	86	67
Z-Val-Gly-Arg-NA	0	0	0	0	100	100	100
Z-Pro-Phe-Arg-NA	0	0	0	0	12	17	8
	(95)	(210)	(64)	(101)	(216)	(291)	(54)

Table 5. Effect of different media supplements on Pr1 (vs Suc-Ala_2-Pro-Phe-NA) and Pr2 (vs Z-Phe-Val-Arg-NA) activities in culture filtrates of *V. lecanii* and *V. albo-atrum*

Mycelial inocula from Sabouraud dextrose cultures was incubated for 24 h in MM supplemented with the potential inducer at 1% (w/v).

Supplement	*V. albo-atrum*		*V. lecanii*	
	Pr1	**Pr2**	**Pr1**	**Pr2**
MM	0·0	11·4 ± 1·8	0·1 ± 0	0·0
Collagen	32·5	345·0 ± 27·7	83·1 ± 12	55·4 ± 8·0
M. sexta cuticle	16·2 ± 2·3	296·7 ± 35·5	143·7 ± 19	37·7 ± 6·4
Elastin	5·3 ± 0·7	117·6 ± 2·2	26·5 ± 2·3	8·2 ± 1·1
Alfalfa cell walls	1·5 ± 0·1	100·5 ± 16·5	4·0 ± 0·2	6·8 ± 1

saccharidases) are also produced by saprophytes, they are unlikely to represent the tools specifically developed by fungi to implement pathogenicity (Knogge, 1996). This does not preclude adaptation of enzyme production to the specific needs of a pathogen on a particular host plant, as shown by the contrasting pattern of enzyme production by pathogens with dicotyledonous and monocotyledonous hosts that reflects a disparity between the primary wall structures of these hosts (Cooper *et al.*, 1988). Overall, our results suggest that specialized pathogens isolated from either plant or insect hosts are less versatile than the opportunists and saprophytes, with patterns of enzyme production corresponding to the protein and carbohydrate contents of plant or insect integuments. That entomopathogenic strains of three species produce little or no polysaccharidase activity may indicate adaptation to an ecological niche where these enzymes are not required. It is of interest that *V. lecanii* produced cellulase activity on solid medium, but not in liquid culture, where low levels of PG activity were detected. Thus genes encoding these carbohydrases are still present in *V. lecanii* but they are not expressed in as wide a range of environmental conditions as compared to *V. albo-atrum*. This presumably reflects adaptation of the biosynthetic regulation of key enzymes to the specific needs of different *Verticillium* spp. on their particular hosts. Due to the redundancy of the encoding genes, gene disruption experiments have not produced evidence that plant cell-wall-degrading enzymes are required for pathogenicity (Mendgen *et al.*, 1996). Likewise, several proteases and chitinases act synergistically during pathogen degradation of insect cuticle (St Leger *et al.*, 1996a, b). It is therefore possible to rationalize differences between insect and plant pathogens assuming that depolymerases are one of the interactive phenomena in a complex multifactorial

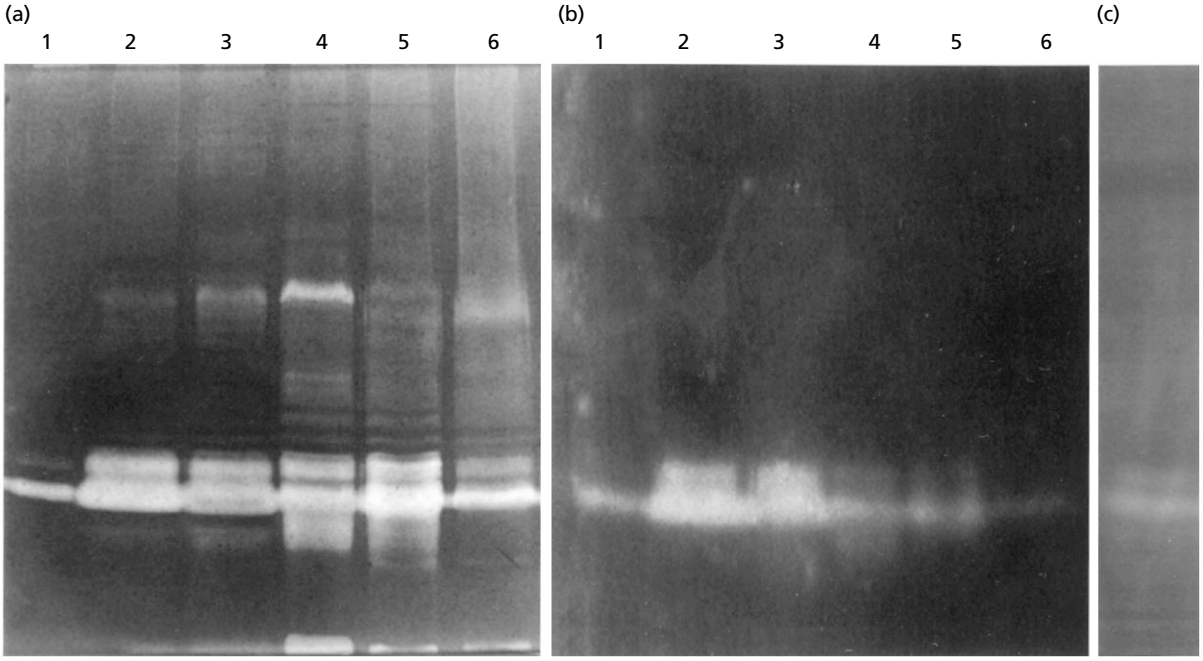

Fig. 2. SDS-PAGE analysis of proteinases produced by mycelial inocula of *V. albo-atrum* incubated for 24 h in basal medium (lanes 1) supplemented with collagen (2), *M. sexta* cuticle (3), elastin (4), alfalfa cell walls (5) and pectin (6). (a) Gelatin SDS-PAGE analysis. Cleared regions of the gel represent proteinase digestion of gelatin copolymerized with acrylamide. (b) Zymogram prepared with an EOM containing Z-Val-Leu-Arg-AFC. (c) Zymogram of lane 2 prepared with an EOM containing Z-Gly-Gly-Arg-AFC.

process enabling the pathogen to overcome host barriers. Consequently, pathogens of plants and animals differ by polygenic factors, that is, by genes of individually small effect that determine the extent of colonization of plants and insects. These enzymes considered separately are directly involved in, but are probably not essential for pathogenicity.

A large number of proteases are needed for complete digestion of the insect cuticle complex (St Leger, 1995). Nevertheless, the broad-spectrum subtilisin-like Pr1 protease is the major protein produced by *M. anisopliae* during infection processes and a comparison of the overall rates of hydrolysis suggests that Pr1 has a much greater ability than the trypsin-like Pr2 to degrade cuticle (St Leger *et al*., 1996a). In addition Pr1, unlike Pr2, is highly toxic to insects (St Leger *et al*., 1996c). Subtilisin-related proteases may therefore have a greater potential for pathogenicity to insects and possibly also for scavenging extracellular proteins, their most likely biological role in saprophytes (Gunkle & Gassen, 1989). However, as there are no reports of trypsins being secreted by saprophytes, trypsin-related enzymes presumably possess a specific role in pathogenicity. In our previous study on the proteases of *V. lecanii*, we suggested that the very narrow specificities of their Pr2 enzymes, cleaving at the C-terminal of Phe-Val-Arg, may be required for selective extracellular proteolytic activation and inactivation processes (St Leger *et al*., 1987c). In contrast to pathogens with insect hosts, only low levels of subtilisins accumulated in cultures of *V. albo-atrum* and *V. dahliae*. Apparently during adaptation of *Verticillium* spp. to the plant wall the functions of a broad-spectrum protease were assumed by trypsins. The broader specificity of these enzymes as compared with the enzymes from entomopathogens is presumably responsible for their activity against hide protein azure and the leupeptin-sensitive hydrolysis of insect cuticle (Table 6). The profile of proteases produced by other plant-pathogenic fungi has not been thoroughly investigated. Subtilisin and trypsin activities were identified by gene cloning in *Cochliobolus carbonum* (Murphy & Walton, 1996). Disruption of the trypsin gene reduced total proteolytic activity versus azocasein by up to 45%, but pathogenicity was not affected, indicating that trypsin by itself is not required for pathogenicity. A trypsin from *Fusarium oxysporum* has also been characterized (Rypniewski *et al*., 1993). Like the plant-pathogenic *Verticillium* spp., *C. carbonum* produced most total protease on collagen medium, a hydroxyproline-rich glycoprotein resembling structural proteins in plant cell walls (Murphy & Walton, 1996). This suggests that trypsins are substrate-induced to degrade wall structural proteins. This may not be a pathogenic specialization, as collagen is also a good inducer of *Aspergillus* spp. proteases (St Leger, unpublished data). Insect cuticle stimulated protease production by both *V. lecanii* and *V. albo-atrum*, suggesting

Table 6. Relative cuticle-degrading and anilidolytic activities of Pr1 and Pr2 in cultures of *V. albo-atrum* and *V. lecanii* grown on collagen for 2 d

Culture filtrates were incubated with leupeptin (25 μg ml^{-1}) or Suc-Ala_2-Pro-Phe-CH_2Cl (0·1 mM) for 30 min before assaying with *M. sexta* cuticle hide protein azure or specific anilide substrates for Pr1 (Suc-Ala_2-Pro-Phe-NA) or Pr2 (Z-Phe-Val-Arg-NA). Controls contained autoclaved enzyme.

Substrates and inhibitors	Relative activity of Pr1 and Pr2 produced by:	
	V. albo-atrum	*V. lecanii*
Suc-Ala_2-Pro-Phe-NA*	9±2	95±7
+Leupeptin	9±3	95±8
+Suc-Ala_2-Pro-Phe-CH_2Cl	1±0	5±1
Z-Phe-Val-Arg-NA*	122±5	68±5
+Leupeptin	12±0	9±1
+Suc-Ala_2-Pro-Phe-CH_2Cl	128±6	73±8
Hide protein azure†	14·3±1·1	7·5±0·6
+Leupeptin	3·5±0·2	7·8±0·7
+Suc-Ala_2-Pro-Phe-CH_2Cl	6·3±0·5	0·7±0·1
M. sexta cuticle‡	12±1·3	9·5±0·24
+Leupeptin	8·4±0·6	8·9±0·46
+Suc-Ala_2-Pro-Phe-CH_2Cl	5·3±0·3	1·3±0·08

* Assays against *p*NA substrates were performed at 23 °C in 10 mM Tris/HCl, pH 8, 4% (v/v) dimethyl sulfoxide and 0·6 mM substrate. Activities were expressed as μmol NA min^{-1} ml^{-1}.

† Activity against hide protein azure was determined at 23 °C in 10 mM Tris/HCl, pH 8, containing 2·0 mg cuticle ml^{-1}. Following incubation for 15 min, A_{595} was measured. Activities were expressed as μg trypsin equivalents (St Leger *et al*., 1986a).

‡ Activity against *M. sexta* cuticle was determined at 23 °C in 10 mM Tris/HCl, pH 8, containing 2·5 mg cuticle ml^{-1}. Following incubation for 1 h, A_{280} of trichloroacetic-acid-soluble products was measured. Activities were expressed as μmol tyrosine equivalents min^{-1} ml^{-1} (St Leger *et al*., 1992).

that adaptation of *Verticillium* spp. to the insect cuticle did not require substantial changes in the regulatory controls of protease gene expression.

An interesting contrast to this strategy is provided by the extracellular metalloproteases of the obligate plant pathogen *Uromyces viciae-fabae*, which specifically degrade fibrous, hydroxyproline-rich proteins (Rauscher *et al*., 1995). These enzymes are regulated in a differentiation-specific manner, i.e. their synthesis requires fungal morphogenesis but not the presence of substrate (Rauscher *et al*., 1995). The refined specificity of these proteases, and their mode of regulation reflects a further degree of specialization achieved by obligate pathogens, as compared to facultative saprophytes such as *Cochliobolus* spp., *Verticillium* spp. and *F. oxysporum*.

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