A REVIEW:

Mechanisms involved in the entomopathogenesis of *Beauveria bassiana*

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The entomopathogenic fungus, *Beauveria bassiana,* is attracting increased attention as potential biological control agent against insect pests. Understanding mechanisms of fungal pathogenesis in insects will provide a rational basis for strain selection and improvement. The action of cytotoxins is suggested by cellular disruption prior to hyphae penetration. Behavioural symptoms such as partial or general paralysis, sluggishness and decreased irritability in mycosed insects are consistent with the action of neuromuscular toxins. There is strong evidence supporting the role of cuticle-degrading proteases (PR1 and PR2) as well as phospholipase B (PLB) in fungal pathogens and their correlations to virulence. Two PLB-encoding genes *(plb1* and *plb2,* 57% identity) and PR2-encoding genes *(try1* and *try2,* 22.4% identity) were detected in *Beauveria bassiana.* The structure similarity of TRY2 protease to insect enzymes might allow the fungal cells to evade host "non-self" recognition and thus might represent one important virulence determinant. PR1 is a serine protease that degrades rapidly cuticular proteins. Production of PR1 is transcriptionally modulated by carbon catabolite and nitrogen metabolite repression. The formation of PLB2 was not influenced by carbon or nitrogen sources. In poor media containing insect cuticles, the synthesis of PLB2 was prevalent. The detailed analysis of the role of putative pathogenic factors depends on the transformation-mediated site-specific disruption of the specific genes. Because of the presence of toxins, lipases and proteases released by the *Beauveria bassiana*, it can be exploited as an entomopathogen in the control of agricultural pests.

Insecticide resistance and the demand for reduced chemical inputs in agriculture have provided an impetus to the development of alternative forms of pest control. Biological control offers an attractive alternative or supplement to the use of chemical pesticides. Microbial biological control agents are naturally occurring organisms and perceived as being less damaging to the environment. Furthermore, their generally complex mode of action makes it unlikely that resistance could be developed to a bio-pesticide. Biological pest control agents include viruses, bacteria, fungi, and nematodes. The use of microorganisms as selective pesticides had some notable successes.

Entomopathogenic fungi:

Human appreciation of the fungi attacking insects is by no means limited to the modern concern in using them for the biological control of insect pests. Two millennia ago, the Chinese were aware of the mummification of silk worms and cicadas by species of *Cordyceps* and *Isaria,* and placed semiprecious and precious stone effigies of these insects in the mouth of their dead in an attempt to confer a similar degree of immortality (Kobayasi, 1977). The benevolence of fungi as microbial control agent was first brought to prominence in the legends of insect pathology in 325 B.C by Aristotle's *Historia animalium* describing diseases of honeybee. Naturalists and philosophers of succeeding generation alluded to the infections of honeybee, silkworm and other insects.

Entomopathogenic fungi were among the first organisms to be used for the biological control of pests. More than 700 species of fungi from around 90 genera are pathogenic to insects. Most are found within the deuteromycetes and entomophorales. Agostino Bassi established the germ theory of diseases in animals in the mid 1830s with his studies on *Beauveria bassiana* infections of silkworm larvae (Steinhaus, 1956). The fungus most frequently isolated from dead insects collected in the field is *B. bassiana*. The host range of the species is extensive and includes almost all the orders of insects (Narasimhan, 1970).

All the insects have their natural enemies, and parasites such as virus, bacteria nematode, protozoa and fungi. These natural enemies will

take a heavy toll occasionally, and prevents the epidemics insect pests of economic importance. A single female green fly can build up a population of half a million within a year if it is protected against natural enemies. The fact that insect population has remained fairly steady shows that natural control is effective and the mycologists by diligently regulating the process of multiplication of the fungal pathogens can achieve the purpose. The use of polyhedral virus in USA and UK for the contrail of lepidopteron larvae has been spectacular and the commercial production of polyhedral virus is already established. The discovery of spore forming *Bacillus thuriengiensis* for the control of lepidopteron and larvae of domestic flies has taken the advance and the bacterial insecticide production became an industry (Narasimhan, 1970). The fungi have remained not fully explored to this day as agent for biological control of insects, besides its ability to kill the insects immediately, one of the major advantages of *Beauveria* over conventional pesticides is its long-term effect on the host population. Reduced adult longevity and high mortality rate in larvae from these adults have been noted in insect populations exposed to *B.bassiana, B. brongniartii* as larvae and adults (Bajan *et al.*, 1996). Some insect-pathogenic fungi have restricted host-ranges, for example, *Aschersonia aleyrodis* infects only scale insects and whiteflies, while other fungal species have a wide host range, with individual isolates being more specific, for example, *Metarhizium anisopliae* and *Beauveria bassiana*. The commercially produced entomopathogenic fungi and their targeted hosts are shown in Table 1.

Fungal species such as *M. anisopliae* and *B. bassiana* are well characterized in respect to pathogenicity to several insects and they have been used as agents for the biological control of agriculture pests worldwide. In Columbia, about 11 companies offer at least 16 products based on the entomopathogenic fungi *B. bassiana.* These products are used not only in the coffee crop but also in other crops such as cabbage, corn, bean, tomato, potato. They are also used to treat public disease vectors (e.g., flies and mosquitoes) (Florez, 2002).

Table 1 : Entomopathogenic fungi in commercial and experimental production

Fungus	Targets
Beauveria bassiana	Colorado potato beetle
	Coding moth
	European corn borer
	Pine caterpillar
Culicinomyces clavisporus	Mosquito larvae
Hirsutella thompsonii	Citrus rust mite
Metarhizium anisopliae	Spittle bug
	Sugarcane frog hopper
Nomuraea rileyi	Lepidopteran larvae
Verticillium lecanii	Aphids
	Coffee green bug
	Greenhouse whitefly
	Thrips

(adapted from Khachatourians, 1986)

Entomopathogenic deuteromycete *B. bassiana:*

The filamentous fungus, *Beauveria bassiana* belongs to a class of insect pathogenic deuteromycete (imperfect fungus). The different *Beauveria* strains are highly adapted to particular host insects. A broad range of *B. bassiana* species have been isolated from a variety of insects worldwide that are of medical or agricultural significance. An interesting feature of *Beauveria* is the high host specificity of many isolates. Hosts of medical importance include vectors for agents of tropical infectious diseases such as the tsetse fly (*Glossina morsitans morsitans),* the sand fly (*Phlebotomus)* that transmits *Leishmania* and the bugs of the genera *Triatoma* and *Rhodnius,* the vectors of chagas disease. Hosts of agricultural significance include the Colorado potato beetle, the codling moth and several genera of termites. Furthermore, the high level of persistence in the host population and in the environment provides long-term effects of the entomopathogenic fungi on pest suppression.

In China, *B. bassiana* is applied against the European corn borer, *Ostrinia mubilalis,* pine caterpillars, *Dendrolimus* spp. and green leafhoppers, *Nephotettix* spp. In the Soviet Union, *B. bassiana* is produced under the trade name Boverin for control of the Colorado potato beetle, *Leptinotarsa decemlineata* and the codling moth, *Laspeyresia pomonella*.

Life cycle of *B. bassiana:*

B. bassiana has a dimorphic mode of growth. In the absence of the specific insect host, *Beauveria* passes through an asexual vegetative life cycle that includes germination, filamentous growth and the formation of sympoduloconidia. In the presence of its host insect, *Beauveria* switches to the pathogenic life cycle. The conidiospores germinate on the surface of the cuticle and the germinated hyphal tubes penetrate the insect's integument directly. When having penetrated the cuticle, the fungus alters its growth morphology to a yeast-like phase and produces hyphal bodies, which circulate in the haemolymph and proliferate by budding. Following the

death of the host, fungal growth reverts back to the typical hyphal form (the saprotrophic stage). The ability to convert to the yeast-like phase may be a prerequisite for pathogenicity (Fig.1).

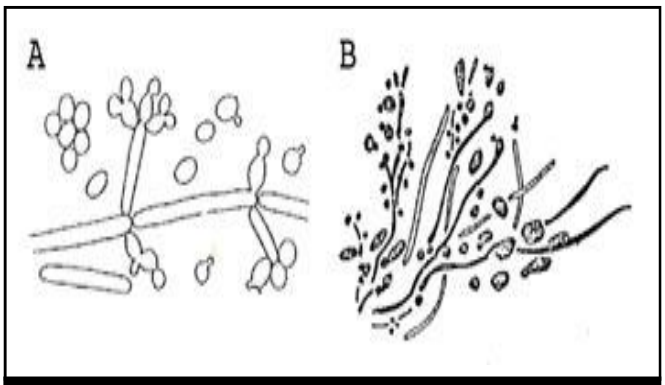

Fig.1 : Dimorphic growth mode of *B. bassiana*
(A) yeast like parasitic phase when infecting susceptible species
(B) Saprobic phase shows filamentous hypha

The infection process:

In contrast to bacteria and viruses that pass through the gut wall from contaminated food, fungi have a unique mode of infection. They reach the hamocoel through the cuticle or possibly through the mouthparts. Ingested fungal spores do not germinate in the gut and are voided in the faeces. The death of the insect results from a combination of factors: mechanical damage resulting from tissue invasion, depletion of nutrient resources and toxicosis (Fig. 2A, B and C).

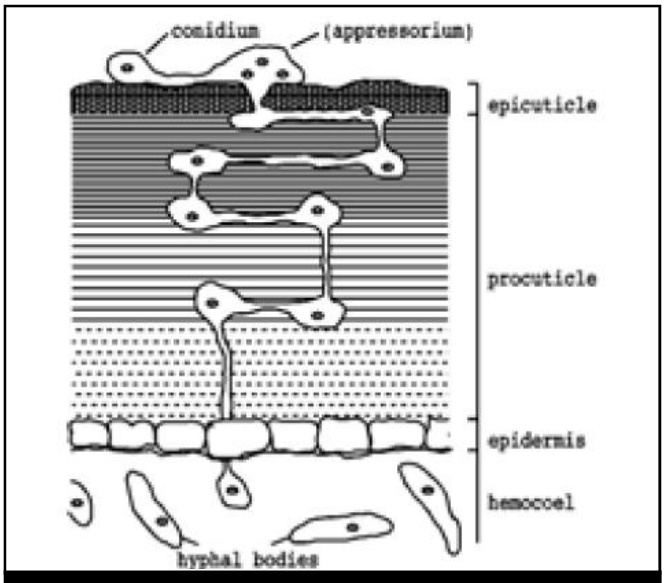

Fig. 2 (A):Structure of insect cuticle and mode of penetration

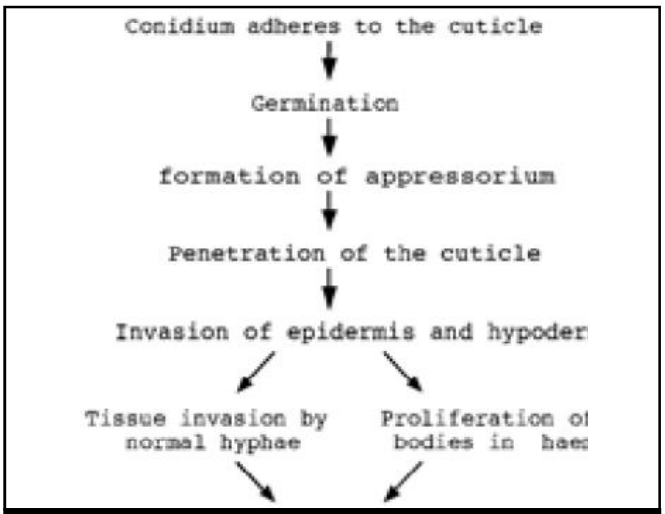

Fig. 2 (B) : Flow chart of infection steps

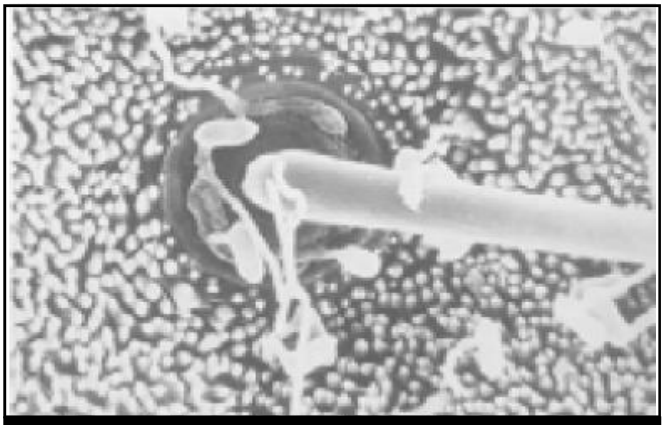

Fig. 2 (C) : Infection process of *B. bassiana.* According to Clarkson and Charnley (1996)
Scanning electron micrograph showing appressorium has formed at the base of the hair socket

Adhesion and germination of conidia*:*

Attachment of a fungal spore to the cuticle surface of a susceptible host represents the initial event in the establishment of mycosis. For most entomopathogenic fungi, host location is a random event and attachment is a passive process with the aid of wind or water. It was found that dry spores of *B. bassiana* possess an outer layer composed of interwoven fascicles of hydrophobic rodlets. This rodlet layer appears lo be unique to the conidial stage and has not been detected on the vegetative cells. The adhesion of dry spores to the cuticle was suggested to be due to non-specific hydrophobic forces exerted by the rodlets (Boucias and Pendland, 1988). In addition, lectins, a kind of carbohydrate binding glycoproteins, have been detected on the conidial surface of *B. bassiana.* It was also suggested that lectins could

be involved in binding between conidia and the insect cuticle. The exact mechanisms responsible for the interaction between fungal spores and the cuticle remain to be determined (Latge and Monsigny, 1988).

After the pathogen reaches and adheres to the host surface, it proceeds with rapid germination and growth which are profoundly influenced by the availability of nutrients, oxygen, water, as well as pi I, and temperature, and by the effects of toxic host-surface compound. Generally, fungi with a broad host-range germinate in culture in response to a wide range of non-specific carbon and nitrogen sources. Entomopathogenic fungi with restricted host-range appear to have more specific requirements for germination (St Leger and Butt, 1989a).

Formation of an infection structure:

Entomopathogenic fungi invade their hosts by direct penetration of the host cuticle. The cuticle has two layers, the outer epicuticle and the procuticle. The epicuticle is a very complex thin structure that lacks chitin but contains phenol-stabilized proteins and is covered by a waxy layer containing fatly acids, lipids and sterols (Hackmann, 1984). The procuticle forms the majority of the cuticle and contains chitin fibrils embedded into a protein matrix together with lipids and quinones (Neville, 1984). Protein may account for up to 70% of the cuticle. In many areas of the cuticle the chitin is organized helically giving rise to a laminate structure.

In common with many Entomopathogenic fungi, *B. bassiana* conidia germinate on the host surface and differentiate an infection structure termed 'appressorium'. The appressorium represent an adaptation for concentrating physical and chemical energy over a very small area so that ingress may be achieved efficiently. Thus, formation of the appressorium plays a pivotal role in establishing a pathogenic interaction with the host. Appressorium formation may be influenced by host surface topography and biochemical investigations indicate the involvement of the intracellular second messengers Ca^{2+} and cyclic AMP (cAMP) in appressorium formation (St Leger and Roberts, 1991).

Penetration through the cuticle:

Pathogenic fungi need to penetrate through the cuticle into the insect body to obtain nutrients for their growth and reproduction. Entry into the host involves both enzymic degradation and mechanical pressure as evidenced by the physical separation of lamellae by penetrated hyphae. A range of extra cellular enzymes that can degrade the major components of insect cuticle, including chitinases, lipases, esterases and at least four different classes of proteases, have been suggested to function during the fungal pathogenesis. Among the first enzymes produced on the cuticle are endoproteases (termed PR1 and PR2) and aminopeptidases, coincident with the formation of appressoria. N-Acetylglucosaminidase is produced at a slow rate as compared to the proteolytic enzymes. Chitinase and lipase activities were not detected (St Leger and Butt, 1989b). Although the complex structure of the insect cuticle suggests that penetration would require the synergistic action of several different enzymes, much of the attention has focused on the cuticle-active endoproteases as a key factor in the process.

Production of toxins:

There is considerable circumstantial evidence from deuteromycete pathogens for the involvement of fungal toxins in host death. The action of cytotoxins is suggested by cellular disruption prior to hyphae penetration. Behavioural symptoms such as partial or general paralysis, sluggishness and decreased irritability in mycosed insects are consistent with the action of neuromuscular toxins. *B. bassiana* and *M. anisopliae* produced significant amounts of toxic compounds within their hosts. For example, the toxins Beauvericin, Beauverolides, Bassianolide and Isarolides have been isolated from *B. bassiana* infected hosts (Hamill and Sullivan, 1969; Elsworth and Grove, 1977); toxins Destruxins (DTXs) and Cylochalasins have been isolated from *M. anisopliae* infected hosts. The toxins have shown to have diverse effects on various insect tissues. DTX depolarizes the *Lepidopteron* muscle membrane by activating calcium channels. In addition, function of insect hemocytes can be inhibited by DTX (Bradfisch and Harmer, 1990). Presumably, there are still many toxins that remain to be isolated from parasitized insects and except DTXs, their relevance to pathogenicity remains to be established.

Host defense systems:

In order to prevent invasion by fungi, insects have evolved various defense mechanisms. The defensive arsenal of insects contains both passive structural barriers, such as the cuticle, and a cascade of active responses to pathogens that gain access to the hemocoel. This active response includes melanization, cellular reactions, humoral reaction to recognize the non-self pathogen, and production of protease inhibitors.

Melanization:

The oxidation of phenolic compounds to dihydroxyphenylalanine, typified by the production of

brown or black melanic pigments, is a common feature of the response of many insects to fungal infection. Melanin may partially shield cuticle from enzymatic attack or may be toxic to fungi. However, such protection is incomplete. The investigation from St Leger and Cooper (1988) indicates that melanization is primarily an effective defense against weak or slow growing pathogens, but is ineffective against more virulent fungi.

Cellular reactions:

Once the cuticle and epidermis have been breached, the invading fungus is faced with the defense systems of the haemolymph. The responses to mycopathogens within the hamocoel include phagocytosis, encapsulation and nodulation. However, the effect on fungal elements is uncertain. With the arbitrary injection method, Bidochka and Khachatourians (1987) found that hemocytes of the migratory grasshopper, *M.sanguinipes,* encapsulate viable conidia of *B. bassiana,* however they fail to suppress conidial germination within the nodule. It was suggested that the production of toxins and extracellular proteases by *B. bassiana* could trigger the evadation of encapsulation.

Humoral reactions:

In response to fungal challenge, insects elicit an acquired humoral "immunity" to subsequent infection. Recognition of "non-self is critical to the initiation of the hemocytic defense reaction and this selective response in insects depends on a specific chemical recognition on part of the hemocytes. Scrum and hemocyte cell membrane-bound lectins have been found in many insects. They could play a role in immune defense reactions since they agglutinate pathogens as well as fungi (Mello and Nigam, 1999). Thus, insect serum agglutinin may function as opsonic mediating the enhanced attachment of granulocytes to the hyphal bodies (Pendland and Heath, 1988).

Production of protease inhibitors:

Host-produced protease inhibitors, which inhibit cuticle-degrading enzyme activities of pathogens, may contribute to insect defense systems. Such compounds have been isolated from the serum of *Anticarsia gemmatalis* larvae, which were resistant to infection by *Nomuraea rileyi* (Boucias and Pendland, 1987).

Characterization of insect cuticle-degrading enzymes from *B. bassiana:*

Mechanism of cuticle degradation by endoproteases:

Metarhizium anisopliae is by far the best-studied entomopathogenic fungus and several virulence factors involved in the disease process have been identified (Clarkson and Charnley, 1996). A subtilisin-like protease, termed PR1 has been cloned and characterized (St Leger and Frank, 1992). PR1 is synthesized as a large precursor containing an 18-amino acid signal peptide and an 89-amino acid propeptide. The mature protein (28.6 kDa) contains 281 amino acid residues. The sequence shows considerable similarities with other enzymes of the subtilisin subclass of serine endoproteases. In particular, the serine, histidine and aspartate residues that comprise the active site of these proteases are conserved in PR1. PR1 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 recognizing at least five subsite residues. This relative nonspecificity accounts for its activity against a range of proteins.

By immunogold electron microscopy, it was shown that PR1 is secreted by the appressorium and penetrating hyphae within the cuticle. For *M. anisopliae,* PR1 appears to be a pathogenicity determinant by virtue of its ability to extensively degrade the cuticle and its production at high levels by the pathogen in situ during infection (St Leger and Cooper, 1987). Furthermore, addition of multiple copies of *pr1* under the control of a constitutive promoter increases the virulence of the transformants (St Leger and Joshi, 1996). The mechanism of cuticle degradation by PR1 in *M. anisopliae* was suggested as follows: (1) PR1 is adsorbed to the cuticle via nonspecific electrostatic forces; (2) the active site comes into contact with any part of the accessible cuticle protein chains and under appropriate conditions, e.g., temperature, splits susceptible peptide bonds thus releasing cuticle proteins; (3) solubilized proteins are further degraded until a chain length of around 5 is obtained (St Leger and Cooper, 1987). The progression of knowledge regarding the major pathogen protease, PR1, followed a course that may serve as a model for research on other entomopathogenic fungi. However, the characterization of PR1 aided in unraveling additional factors that contribute to pathogenicity. In addition to PR1. another endoprotease, trypsin-like protease (PR2), has also been characterized from *M. anisopliae,* but the role of PR2 is not clear (St Leger and Joshi, 1996).

Function of phospholipases in cuticle penetration:

Since lipids represent major chemical constituents of the insect cuticle, enzymes capable of hydrolyzing these compounds, such as phospholipases, could be expected

to be involved in the cuticle disruption processes that occur during host invasion. Phospholipases are a heterogeneous group or enzymes that arc able to hydrolyze one or more ester linkages in glycerophospholipids. The action of phospholipases can result in the destabilization of membranes, cell lysis and release of lipid second messengers (Ghannoum, 2000). These enzymes are categorized according to the location of the ester link that is cleaved. Although phospholipase B (PLB) refers to an enzyme (hat can remove both *sn*-1 and *sn*-2 fatty acids, this enzyme also has lysophospholipase-transacylase activity.

Extracellular phospholipases have been implicated as pathogenicity factors for bacteria, rickettsiae and protozoa. The type of phospholipase involved in virulence varies with the organism. For example, *C. perfringens* (Alape-Giron and Flores-Diaz, 2000) secretes a phospholipase C (PLC), whereas *T. gondii* secretes a phospholipase A (PLA). The importance of these enzymes, especially PLB, for virulence has so far only been verified in medically important fungi. PLB was secreted by different clinically important fungal species such as *Candida albicans* (Mukherjee and Seshan, 2001), *Aspergillus fumigatus* (Burch and Robson, 1996.) and *Cryplococcus neoformans* (Cox and McDade, 2001). The role of PLB in the pathogenicity of entomopathogenic fungi remains to be determined, even in the best-studied species *M. anisopliae.*

Production of proteases by *B. bassiana:*

Insect pathogenic fungi produce an array of enzymes capable of degrading protein, chitin, and lipid components of the insect cuticle (St Leger and Cooper, 1986a). However, a convincing role in pathogenesis has been established only for the serine protease PR1. The activity of PR1 is a prerequisite for successful penetration of the host by *M. anisopliae* (St Leger and Cooper, 1988).

From the cDNA sequence and protein structure, it has been shown that PR1 resembles the powerful serine endoprotease proteinase K, but PR1 is far more effective than proteinase K in degrading insect cuticle, indicative of pathogenic specialization. The higher activity of PR1 could come from the positively charged residues (Hisl7, Arand18 and Arg20) located on the surface of the protease PR1, that are absent from Proteinase K. The presence of this charged domain might allow electrostatic interactions with negatively charged groups in the insect cuticle (St Leger and Frank, 1992).

The study of PR1 from *M. anisopliae* has proven that PR1 expression is regulated by both carbon catabolite and nitrogen metabolite repression and is specifically induced by cuticular proteins after carbon and nitrogen starvation. The level of PR1 was induced tenfold within 24h of contact with cuticle and the regulation was found to be at the transcriptional level (Paterson and Charnley, 1994). Data from this work show that although mycelium growing in complete medium containing carbon and nitrogen sources can synthesize different proteins, the production of PR1 is simultaneously repressed by high levels of nutrients (e.g., sucrose). Under these conditions expression of PR1 was not detectable al both transcriptional and translational level. In *B bassiana,* the production of PR 1 seems to be regulated in a similar way as in *M. anisopliae.* PR] was found to be exclusively expressed under the cuticle-induced conditions, as judged by immunoblot analysis using an anti-PRl antiserum.

Western Blot analysts detected a protein band migrating slightly slower than PR1, but its identity is not determined. Several possibilities can be considered: it may arise from protein degradation, cross reaction of the antibody possibly related to the infectious process as suggested by St Leger and Butt (1989) or posttranslational modification.

Trypsin-Iike serine proteases:

Another major endoprotease produced in vitro on insect cuticle is the trypsin-like serine protease PR2. The expression of this enzyme has been detected in *M. anisopliae.* The PR2 protease has been purified and its regulation has been investigated in both *M. anisopliae* and *B. bassiana* (Cole and Charnley, 1993 and St Leger and Cooper, 1987). But biochemical characterization of PR2 has only been performed in *M. anisopliae.* Two isoforms of PR2 have been identified from *M. anisopliae;* however, the unique, specific functions of PR2 in pathogenesis have not yet been elucidated. St Leger and Joshi (1996) determined the location of PR2, using immunogold labeling, to be at the *M. anisopliae* cell wall during growth through insect cuticles. This indicates that the PR2 proteins might have a role in degrading extracellular proteins as a complement to PR1 and other enzymes. PR1 is produced as a pro-enzyme, requiring processing before it is active. Thus, PR2 could also play a role in the activation of PR1.

Cloning data from this work showed that *B. bassiana* possesses two genes encoding PR2-like serine proteases of the trypsin family. The sequences of these two genes, designated *tryl* and *try2,* have been determined. Interestingly, the predicted amino acid sequence of *tryl* showed highest homology to the trypsin-like proteases from fungi, whereas the predicted TRY2 protein showed highest homology to the trypsin-like

proteases from insects. Considering the experimental conditions and previous results obtained under the same conditions, the possibility of contamination was ruled out. Similar observations have been made regarding trypsin-like proteases from the bacterium *Streptomyces griseus* that showed close homology to mammalian trypsin-like proteases. It was hypothesized that a gene transfer might have been taken place from a mammal to a bacterium (Olafson and Jurasek, 1975). However, this suggestion could later be ruled out using phylogenetic analysis of sequence data accumulated from different organisms (Rypniewski and Perrakis, 1994). The structure similarity of TRY2 protease from *B. bassiana* to insects enzymes might allow the fungal cells to evade host "non-self" recognition and thus might represents one of the important virulence determinants.

Like PR1, the trypsin-like protease PR2 is also controlled by multiple regulatory mechanisms that include carbon and nitrogen metabolite repression/ derepression as well as induction by a range of proteins. Based on the model system of *M. anisopliae,* St Leger and Durrands (1988) found that the regulation of PR1 and PR2 is not identical; although PR1 was not detected under carbon/ nitrogen repression conditions; low levels of PR2 were produced. It was found that the soluble protein bovine scrum albumin (BSA) represses production of PR1, and at the same time stimulates synthesis of PR2 in poor medium. In addition, PR2 production could be more lightly regulated by nitrogen than carbon availability (Paterson and Charnley, 1993). This type of protein regulation is not uncommon. There are examples of fungal proteases, which are induced by any protein substrate, as observed in *N. crassa* (Drucker, 1975) and *Candida spp.* (Ross and De Bernardis, 1990). These proteases are produced when the fungus is starved for cither carbon, nitrogen or sulphur sources. Some fungal proteases appear it) be regulated by derepression alone, for example, in *Schizophyllm commune* (Willick and Morosoli, 1984) and many *Aspergillus* species (Hanzi and Shimizu, 1993).

Expression of phospholipase B in *B. bassiana:*

Two PLB-encoding genes were cloned from *B. bassiana* and their sequences were determined. They displayed 57% identity at the amino acid level. Three PLB-encoding genes in *S. cerevisiae* (Witt and Mertsching, 1984) and two PLB homologues in *C. albicans* (Takahashi and Banno, 1991) have been isolated. The multiple PLB homologues from these fungi display high homology to each other, but the significance of this genetic redundancy is unclear.

Potential N-glycosylation sites were identified in both PLB1 and PLB2. All characterized fungal phospholipases have been glycosylated. Chen and Wright (2000) found that deglycosylalion of the protein resulted in almost total loss of enzyme activity. This indicates that N-linked carbohydrates are important for the catalytic function of the protein. The immuno blot assay in this work with an antibody against PLB2 produced a broad band corresponding to a mass of around 70 kDa. The slightly heterogeneous migration of PLB2 upon SDS-PAGE separation has also been observed in *C. neoformans* (Chen and Wright, 2000) and other fungal PLBs (Oishi and Morimoto, 1999). It is supposedly due to heterogeneity of the carbohydrate moiety linked to the enzyme.

A hydrophobic carboxy-terminal region was identified in PLBs from non-pathogenic fungi, such *as S. cerevisiae, S. pombe, P. notatum* (Caro and Tettelin, 1997; Gerber and Kodukula, 1992). These COOH-terminal regions have been hypothesized to contain conserved sequence motif that is a signal for the addition of a glycosylphosphatidylinositol (GPI) anchor. Proteins modified with a GPT anchor may be transiently tethered to the plasma membrane or ultimately cross-linked to the insoluble glucan component of the cell wall (Lu and Montijn, 1995). Release of proteins associated with the plasma membrane would require the action of a GPI-specific phospholipase. Therefore, the GPI anchor may serve to regulate the release of the enzyme to the surroundings. The absence of a GPI anchor in *B. bassiana* PLBs that is also observed in the *C. albicans* PLBs, could cause the PLBs from these organisms to be secreted directly thereby enhance the virulence of pathogenic fungi.

The high amounts of PLB2 synthesized from *B. bassiana* grown on insect cuticle suggests that this enzyme is likely to be involved in the early steps of host invasion Unlike PR1, production of PLB was not tightly regulated by carbon/ nitrogen repression mechanisms. The transcription of PLB mRNA was detected by RT-PCR in both mycelial samples harvested from rich medium and cuticle-induced medium Although PLB2 synthesis is highest in the mycelium grown in cuticle induced medium, the enzyme is also detected in cultures grown on rich medium. Immunoblot analysis and this unspecific banding maybe because the synthesized antisera were not pure enough detected some unspecific bands.

Function of cuticle-degrading enzymes during fungal infection processes:

The high-level production of proteases during infection process implies an important function for these enzymes. Aside from the proteolytic degradation of the cuticular

barrier, other possible roles include the utilization of host proteins for nutrition, the destruction of antifungal proteins of the host, and the release of amino acids for amine production, which could elevate the pH to produce better growth conditions. Other effects may be indirect, for example, the proteolytic activation of toxin precursors.

The results obtained from this work are consistent with the idea that a major function of the extracellular proteases is to make nutrients available from the cuticle. The pathogenic process involving enzyme production and penetration of host cuticle occurs only when it is necessary to establish a nutritional relationship with the host. It is probable that the synthesis of proteolytic enzymes and phospholipases occurring in cultures containing host cuticle may also occur when the pathogens are infecting living insects.

A highly reliable method categorically demonstrates the involvement of a protein in pathogenicity through genetically engineered mutants. The multiplicity of cuticle degrading enzymes provides a major challenge with respect to establish which particular enzyme has which function in adapting to a new environment or in pathogenicity. The exact role of individual cuticle-degrading enzymes in pathogenicity could be assessed by disruption of multiple genes in combination with site-directed mutagenesis experiments. An increased understanding of these virulence factors could ultimately lead lo the generation of genetically engineered strains more suited for insect pest control.

Aspects of using entomopathogenic *Beauveria bassiana* as bio-control agent:

The advantages of using *Beauveria bassiana* as insecticides are:

– Their high degree of specificity for pest control. *Beauveria bassiana* can be used to control harmful insect pests without affecting beneficial insect predators and non-harmful parasites.

– The absence of effects on mammals and thus the reduction of the hazards normally encountered with insecticide applications, such as pollution of the environment.

– The lack of problems caused to insect resistance and prolonged pest control

– A high potential for further development by biotechnological research.

– High persistence in the environment provides long-term effects of entomopathogenic fungi on pest suppression.

However, there are also a number of constraints on the use of *Beauveria bassiana* as insecticide:

– 2-3 weeks are required to kill the insects whereas chemical insecticides may need only 2-3 hours.

– Application needs to coincide with high relative humidity, low pest numbers and a fungicide free period.

– Due to the high specificity, additional control agents are needed for other pests.

– Their production is relatively expensive and the short shelf-life of spores necessitates cold storage.

– The persistence and efficacy of entomopathogenic *Beauveria bassiana* in the host population varies among different insects species, thus insect-specific application techniques need to be optimized to retain long-term impacts.

– A potential risk to immune-depressive people.

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