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Lecture#1

A Brief Introduction to Microbial Pesticide

Biopesticides fall into three major classes:

(1) **Microbial pesticides** which consist of bacteria, entomopathogenic fungi or viruses (and sometimes includes the metabolites that bacteria or fungi produce).
Entomopathogenic nematodes are also often classed as microbial pesticides, even though they are multi-cellular.
(2) **Biochemical pesticides or herbal pesticides** are naturally occurring substances that control (or monitor in the case of pheromones) pests and microbial diseases.
(3) **Plant-incorporated protectants (PIPs)** have genetic material from other species incorporated into their genetic material (*i.e.* GM crops). Their use is controversial, especially in many European countries.

Microbial pesticides

They come from naturally occurring or genetically altered bacteria, fungi, algae, viruses or protozoans. Microbial control agents can be effective and used as alternatives to chemical insecticides. A microbial toxin can be defined as a biological toxin material derived from a microorganism, such as a bacterium or fungus. Pathogenic effect of those microorganisms on the target pests are so species specific. The effect by microbial entomopathogens occurs by invasion through the integument or gut of the insect, followed by multiplication of the pathogen resulting in the death of the host, e.g., insects.
Studies have demonstrated that the pathogens produce insecticidal toxin important in pathogenesis. Most of the toxins produced by microbial pathogens which have been identified are peptides, but they vary greatly in terms of structure, toxicity and specificity.These microbial pesticides offer an alternative to chemical insecticides with increased target specificity and ecological safety so that they are used either uniqly or in combination with other pest management programmes.
One definition for integrated pest management (IPM) which is most relevant to this practice comes from Flint and van den Bosch [1981]: "It is a ecologically based pest control strategy that relies heavily on natural mortality factors and seeks out control tactics that disrupt these factors as little as possible. Ideally, an integrated pest management program considers all available pest control actions, including no action, and evaluates the potential interaction among various control tactics, cultural practices, weather, other pests, and the crop to be protected".

Examples*Bacillus thuringiensis*, a bacterial disease of Lepidoptera, Coleoptera and Diptera, is a well-known insecticide example. The toxin from *B. thuringiensis* (Bt toxin) has been incorporated directly into plants through the use of genetic engineering. The use of Bt Toxin is particularly controversial. Its manufacturers claim it has little effect on other organisms, and is more environmentally friendly than

synthetic pesticides. However, at least one scientific study has suggested that it may lead to slight histopathological changes on the liver and kidneys of mammals with Bt toxin in their diet.

Microorganism e.g., a bacterium, fungus, virus or protozoan as the active ingredient can control many different kinds of pests, although each separate active ingredient is relatively specific for its target pest. For example, there are fungi that control certain weeds, and other fungi that kill specific insects. One bacterial species like *Bacillus thuringiensis* may be more effectiv on *Aedes aegypti* while one another *B. sphaericus* strain can be effective on a different types of mosquito like *Culex quinquefasciatus*.

Other microbial control agents include products based on:

- entomopathogenic fungi (*e.g.Beauveria bassiana, Lecanicillium* spp., *Metarhizium* spp.),
- plant disease control agents: include *Trichoderma* spp. and *Ampelomyces quisqualis* (a hyper-parasite of grape powdery mildew); *Bacillus subtilis* is also used to control plant pathogens.[4]
- beneficial nematodes attacking insect (*e.g. Steinernema feltiae*) or slug (*e.g. Phasmarhabditis hermaphrodita*) pests
- entomopathogenic viruses (*e.g.*. *Cydia pomonella* granulovirus).
- weeds and rodents have also been controlled with microbial agents.

Various naturally occurring materials, including fungal and plant extracts, have been described as biopesticides. Products in this category include:

- Insect pheromones and other semiochemicals
- Fermentation products such as Spinosad (a macro-cyclic lactone)
- Chitosan: a plant in the presence of this product will naturally induce systemic resistance (ISR) to allow the plant to defend itself against disease, pathogens and pests.[9]
- Biopesticides may include natural plant-derived products, which include alkaloids, terpenoids, phenolics and other secondary chemicals. Certain vegetable oils such as canola oil are known to have pesticidal properties. Products based on extracts of plants such as garlic have now been registered in the EU and elsewhere.
- Naturally occurring minerals such as baking soda may also have pesticidal applications.

Microbial Pesticide(Biopesticide), a contraction of 'biological pesticides', include several types of pest management intervention: through predatory, parasitic, or chemical relationships. The term has been associated historically with biological control - and by implication - the manipulation of living organisms. Regulatory positions can be influenced by public perceptions, thus:

- in the EU, biopesticides have been defined as "a form of pesticide based on micro-organisms or natural products".
- the US EPA states that they "include naturally occurring substances that control pests (biochemical pesticides), microorganisms that control pests (microbial pesticides), and pesticidal substances produced by plants containing added genetic material (plant-incorporated protectants) or PIPs".

They are typically created by growing and concentrating naturally occurring organisms and/or their metabolites including bacteria and other microbes, fungi, nematodes, proteins, *etc*. They are often considered to be important components of integrated pest management (IPM) programmes, and have received much practical attention as substitutes to synthetic chemical plant protection products (PPPs). *The Manual of Biocontrol Agents* (2009: formerly the *Biopesticide Manual*) gives a review of the available biological insecticide (and other biology-based control) products.

Microbial pesticides are some of the earliest developed and widely used biopesticides. Russia, Australia, the United States, Canada, Japan and other countries have done a lot of research on microbial pesticides. EPA indicates that more than 200 products are sold in the United States, compared to only 60 similar products available in the European Union. In Japan, 231 host-virus associations, 63 fungi, 38 protozoa, 15 bacteria and five nematodes had been reported (Table 1) (Kunimi, 2007). Until 2003, 168 viruses (1663 host-virus associations), 411 fungi, 1504 protozoa, 51 bacteria and 146 nematodes had been registered in the global insect pathogen database (Braxton et al., 2003), and 270 bacterial products, 22 fungal products, and 35 viral products were registered in China until 2008 (ICAMA, 2008). In total, at least 410 biopesticide production units had been established in India, while 130 in the private sector. Approximately 40 commercial mycoinsecticides available on Brazilian market were registered by 19 companies (Kabaluk et al., 2010). As of 2010, Canada had registered 32, 12 of which were bacterial species, 11 fungi, six nematodes, two viruses, and one protozoan based microbial pesticide. Microbial biopesticides represent less than 1% of the global market in agrochemical crop production (Hajek, 2004).For all crop types, bacterial biopesticides claim about 74% of the market; fungal biopesticides, about 10%; viral biopesticides, 5%; predator biopesticides, 8%; and “other” biopesticides, 3% (Thakore, 2006). However, only a few entomopathogens have been developed as biocontrol agents. *Trichoderma*, as a safety and promising microbial pesticides, has a better potential biocontrol and has been extensively studied. In general, the current literature indicated that *Trichoderma* sp. has been used mostly as biopesticide agent (Table 2) (Mausam et al., 2007). China, Russia, Belarus and to a lesser extent India and Thailand, had also become significant producers of *B.t.* products which are used extensively. Dror et al. (2009) reviewed the accumulating data in *B.t.* delta-endotoxin Cry1C research as a potential biopesticide in plants.

Advantages of microbial insecticides

Individual products differ in important ways, but the following list of beneficial characteristics applies to microbial insecticides in general.

• The organisms used in microbial insecticides are essentially nontoxic and nonpathogenic to wildlife, humans, and other organisms not closely related to the target pest. The safety offered by microbial insecticides is their greatest strength.
• The toxic action of microbial insecticides is often specific to a single group or species of insects, and this specificity means that most microbial insecticides do not directly affect beneficial insects (including predators or parasites of pests) in treated areas.
• If necessary, most microbial insecticides can be used in conjunction with synthetic chemical insecticides because in most cases the microbial product is not deactivated or damaged by residues of conventional insecticides. (Follow label directions concerning any limitations.)
• Because their residues present no hazards to humans or other animals, microbial insecticides can be applied even when a crop is almost ready for harvest.
• In some cases, the pathogenic microorganisms can become established in a pest population or its habitat and provide control during subsequent pest generations or seasons.
• They also enhance the root and plant growth by way of encouraging the beneficial soil microflora. By this way they take a part in the increase of the crop yield.

Disadvantages of microbial insecticides

Naturally there are also the limitations which are listed below, but do not prevent the successful use of microbial insecticides. These factors just provide users to choose effective microbial products and take necessary steps to achieve successful results.
• Because a single microbial insecticide is toxic to only a specific species or group of insects, each application may control only a portion of the pests present in a field and garden. If other types of pests are present in the treated area, they will survive and may continue to cause damage. Conventional insecticides are subject to similar limitations because they too are not equally effective against all pests. This is because of selectivity indeed and this negative aspect is often more noticeable for both general predators, chemicals and microbials. On the other hand predators and chemicals may be danger for other beneficial insects in threatened area.
• Heat, desiccation (drying out), or exposure to ultraviolet radiation reduces the effectiveness of several types of microbial insecticides. Consequently, proper timing and application procedures are especially important for some products.

• Special formulation and storage procedures are necessary for some microbial pesticides. Although these procedures may complicate the production and distribution of certain products, storage requirements do not seriously limit the handling of microbial insecticides that are widely available. (Store all pesticides, including microbial insecticides, according to label directions.)
• Because several microbial insecticides are pest-specific, the potential market for these products may be limited. Their development, registration, and production costs cannot be spread over a wide range of pest control sales. Consequently, some products are not widely available or are relatively expensive (several insect viruses, for example).

Lecture#2

Relationship of Insect Resistance to Microbial Pesticide

(1)Viral pesticides

There are more than 1600 different viruses which infect 1100 species of insects and mites. A special group of viruses, called baculovirus, to which about 100 insect species are susceptible,

accounts for more than 10 percent of all insect pathogenic viruses. ***Baculoviridae*** is a family of viruses. Arthropods, lepidoptera, hymenoptera, diptera, and decapoda serve as natural hosts. There are currently 49 species in this family, divided among 4 genera.

Baculoviruses are known to infect invertebrates, with over 600 host species having been described. Immature (larval) forms of moth species are the most common hosts, but these viruses have also been found infecting sawflies, mosquitoes, and shrimp. Although baculoviruses are capable of entering mammalian cells in culture they are not known to be capable of replication in mammalian or other vertebrate animal cells.

Symbionts Commonly Provide Broad Spectrum Resistance to Viruses in Insects: A Comparative Analysis of Wolbachia Strains

In the last decade, bacterial symbionts have been shown to play an important role in protecting hosts against pathogens. *Wolbachia*, a widespread symbiont in arthropods, can protect*Drosophila* and mosquito species against viral infections. We have investigated antiviral protection in 19 *Wolbachia* strains originating from 16 *Drosophila* species after transfer into the same genotype of *Drosophila simulans*. We found that approximately half of the strains protected against two RNA viruses. Given that 40% of terrestrial arthropod species are estimated to harbour *Wolbachia*, as many as a fifth of all arthropods species may benefit from*Wolbachia*-mediated protection. The level of protection against two distantly related RNA viruses – DCV and FHV – was strongly genetically correlated, which suggests that there is a single mechanism of protection with broad specificity. Furthermore, *Wolbachia* is making flies resistant to viruses, as increases in survival can be largely explained by reductions in viral titer. Variation in the level of antiviral protection provided by different *Wolbachia* strains is strongly genetically correlated to the density of the bacteria strains in host tissues. We found no support for two previously proposed mechanisms of *Wolbachia*-mediated protection — activation of the immune system and upregulation of the methyltransferase *Dnmt2*. The large variation in *Wolbachia*'s antiviral properties highlights the need to carefully select *Wolbachia*strains introduced into mosquito populations to prevent the transmission of arboviruses. (Martinez *et al.,* **2014**)

Virus-derived genes for insect-resistant transgenic plants

Insect viruses have evolved to counter physiological barriers to infection presented by the host insect. For the Lepidoptera (butterflies and moths), these barriers include (1) the peritrophic membrane (PM) lining the gut, which presents a physical barrier to virus infection of the midgut epithelial cells, (2) the basement membrane (BM) that overlies the gut thereby restricting secondary infection of other tissues, and (3) the immune system of the host insect. Hence, insect viruses provide a resource for genes that disrupt host physiology in a specific manner, and these genes in turn serve as a resource both for the study of physiological processes, and for disruption of these processes for pest management purposes. There are several examples of the application of genes used by an insect virus to overcome the PM barrier for production of insect-resistant transgenic plants. There are other examples of intrahemocoelic effectors, such as BM-degrading proteases that can only be used with an appropriate system for delivery of the agent from the gut into the hemocoel (body cavity) of the insect pest. In this chapter, we describe (1) baculovirus- and entomopoxvirus-derived genes that alter the physiology of the host insect, (2) use of these and homologous genes for production of insect-resistant transgenic plants, (3) other viral genes that have potential for use in development of insect-resistant transgenic plants, and (4) the use of plant lectins for delivery of intrahemocoelic toxins from transgenic plants. Plant expression of polydnavirus-derived genes is described by Gill et al.

(2)Bacterial biopesticides

Bacterial biopesticides are the most common and cheaper form of microbial pesticides. As an insecticide they are generally specific to individual species of moths and butterflies, as well as species of beetles, flies and mosquitoes.

Bacillus thuringiensis, BT *Bacillus thuringiensis* (Bt) is an aerobic, gram positive, spore forming soil bacterium that shows unusual ability to produce endogenous different kinds of crystals protein inclusions during its sporulation.
The *Bacillus* species, *Bacillus thuringiensis,* has developed many molecular mechanisms to produce pesticidal toxins; most of toxins are coded for by several *cry* genes. Since its discovery in 1901 as a microbial insecticide, *Bacillus thuringiensis* has been widely used to control insect pests important in agriculture, forestry and medicine.

The diversity of Bt resistance genes in species of Lepidoptera.

Although the mode of action of Cry1A toxins produced by Bacillus thuringiensis is fairly well understood, knowledge of the molecular mechanisms by which lepidopteran species have evolved resistance to them is still in its infancy. The most common type of resistance has been called "Mode 1" and is characterized by recessive inheritance, >500-fold resistance to and reduced binding by at least one Cry1A toxin, and negligible cross-resistance to Cry1C. In three lepidopteran species, Heliothis virescens, Pectinophora gossypiella, and Helicoverpa armigera, Mode 1 resistance is caused by mutations in a toxin-binding 12-cadherin-domain protein

expressed in the larval midgut. These mutations all interrupt the primary sequence of the protein and prevent its normal localization in the membrane, presumably removing a major toxic binding target of the Cry1A toxins. In Plutellaxylostella, however, Mode 1 resistance appears to be caused by a different genetic mechanism, as Cry1A resistance is unlinked to the cadherin gene. Mapping studies in H. virescens have detected an additional major Cry1A resistance gene, which on the basis of comparative linkage mapping is distinct from the one in P. xylostella. An additional resistance mechanism supported by genetic data involves a protoxin-processing protease in Plodiainterpunctella, and this is likely to be different from the genes mapped in Plutella and Heliothis. Thus, resistance to Cry1A toxins in species of Lepidoptera has a complex genetic basis, with at least four distinct, major resistance genes of which three are mapped in one or more species. The connection between resistance genes and the mechanisms they encode remains a challenging task to elucidate.

Bacterial Insecticide Resistance

By cultivating detoxifying bacteria in its gut, a pest called the bean bug can become instantly resistant to a common insecticide. Some Japanese scientists have found that the bean bug, a major pest of soybean crops, swallows bacteria that break down an insecticide chemical. The bacteria allow it to continue munching on treated crops will no ill effects, according to a study published today By Ed Yong | April 23, 2012.

Some *Burkholderia* strains can break down the insecticide fenitrothion for their own nourishment. In doing so, they render the chemical harmless to insects. These strains are normally so rare as to be undetectable, but Yoshitomo Kikuchi from the National Institute of Advanced Industrial Science and Technology in Japan found that they can increase rapidly in soils that are treated with fenitrothion, comprising some 80 percent of *Burkholderia* populations after just 1 month.

If bean bugs swallow these fenitrothion-degrading strains, they gain immediate resistance to the pesticide. In the lab, more than 70 percent of the bugs that ate fenitrothion-degrading *Burkholderia*survived a meal of seeds laced with the pesticide. Only 10 to 20 percent of bugs that ate normal *Burkholderia* strains could tolerate such exposures. “Now we are investigating how the symbionts confer insecticide resistance,” said Kikuchi.

B. thuringiensis

During 2009 and 2010, some Iowa fields showed severe injury to corn producing Bt toxin Cry3Bb1 by western corn rootworm. During 2011, mCry3A corn also displayed insect damage, including cross-resistance between these toxins. Resistance persisted and spread in Iowa. Bt corn that targets western corn rootworm does not produce a high dose of Bt toxin, and displays less resistance than that seen in a high-dose Bt crop.

Products such as Capture LFR (containing the pyrethroid Bifenthrin) and SmartChoice (containing a pyrethroid and an organophosphate) have been increasingly used to complement Bt crops that farmers find alone to be unable to prevent insect-driven injury. Multiple studies have found the practice to be either ineffective or to accelerate the development of resistant strains.

(3)Entomopathogenic fung

Entomopathogenic fungi are important natural regulators of insect populations and have potential as mycoinsecticide agents against diverse insect pests in agriculture. These fungi infect their hosts by penetrating through the cuticle, gaining access to the hemolymph,producing toxins, and grow by utilizing nutrients present in the haemocoel to avoid insectimmune responses. In addition,entomopathogenic fungi may indirectly affect certain natural enemies when feeding on prey that have been sprayed (contaminated prey). For example, larvae of the mealybug destroyer, *Cryptolaemus montrouzieri* were killed (50% mortality) after consuming mealybugs that had been sprayed with *Beauveria bassiana.*

Examples

(1)The commercial mycoinsecticide 'Boverin' based on *B. bassiana* with reduced doses of trichlorophon have been used to suppress the second-generation outbreaks of *Cydia pomonella* L.Anderson *et al.* (1989) detected higher insect mortality when *B. bassiana* and sublethal concentrations of insecticides were applied to control Colorado potato beetle (*Leptinotarsa decemlineata*), attributing higher rates of synergism between two agents.
(2)The use of the insect-pathogenic fungus *Metarhizium anisopliae* against adult *Aedes aegypti* and *Aedes albopictus* mosquitoes has also been reportedThe life span of fungus-contaminated mosquitoes of both species was significantly reduced compared to uninfected mosquitoes. The results indicated that both mosquito species are highly susceptible to infection with this entomopathogen.

Can insects develop resistance to insect pathogenic fungi?

Microevolutionary adaptations and mechanisms of fungal pathogen resistance were explored in a melanic population of the Greater wax moth, Galleria mellonella. Under constant selective pressure from the insect pathogenic fungus Beauveria bassiana, 25(th) generation larvae exhibited significantly enhanced resistance, which was specific to this pathogen and not to another insect pathogenic fungus, Metarhizium anisopliae. Defense and stress management strategies of selected (resistant) and non-selected (susceptible) insect lines were compared to uncover mechanisms underpinning resistance, and the possible cost of those survival strategies. We hypothesize that the insects developed a transgenerationally primed resistance to the fungus B. bassiana, a costly trait that was achieved not by compromising life-history traits but rather by prioritizing and re-allocating pathogen-species-specific augmentations to integumental front-line defenses that are most likely to be encountered by invading fungi. Specifically during B. bassiana infection, systemic immune defenses are suppressed in favour of a more limited but targeted repertoire of enhanced responses in the cuticle and epidermis of the integument (e.g. expression of the fungal enzyme inhibitor IMPI, and cuticular phenoloxidase activity). A range of putative stress-management factors (e.g. antioxidants) is also activated during the specific response of selected insects to B. bassiana but not M. anisopliae. This too occurs primarily in the integument, and probably contributes to antifungal defense and/or helps ameliorate the damage inflicted by the fungus or the host's own immune responses.

Lecture#3

Co-evolution of pathogen and its insect host

Coevolution can be defined as a series of synchronous mutual evolutionary changes in interacting species which act as agents of natural selection for each other.

Progress in understanding molecular mechanisms behind coevolution is limited by the availability of suited models because reciprocal adaptations during evolutionary processes are difficult to trace and to reconstruct at the genetic level. Pathogens and their hosts provide powerful models to investigate coevolution characterized by reciprocal changes in genetic composition of interacting species which have a close ecological relationship.

Insects are the most successful group of organisms on earth regarding species diversity, and their abilities in managing symbiotic bacteria or fungi and defending against pathogenic ones play a predominant role in the outcome of the evolutionary competition with microbes. In this I focus on parasitic fungi and their insect hosts as a model system for exploring coevolutionary mechanisms because it provides informative examples for interactions between molecules mediating either virulence of fungal pathogens or resistance of the host, which are characterized by reciprocal adaptations.

Example

The greater wax *Galleria mellonella* has emerged as powerful model hosts both for studying co-evolution between entomopathogenic bacteria and fungi host with their host insects,and as heterologous hosts for human pathogenic bacteria and fungi.*G. mellonella* combines a number of advantages when used as an alternative host for human pathogens. Firstly, the low overall costs of breeding large numbers facilitate its use as an inexpensive whole-animal high throughput infection system. Secondly,*G. mellonella* can be adapted in the laboratory to 37°C which is important because human pathogens are adapted to the physiological temperature of its host. Thirdly, *G. mellonella* can be used as an insect model to mimic oral infections both with human or insect pathogens. *G. mellonella* provides also a model to study host-pathogen co-evolution, particularly in regard of entomopathogenic fungi such as *Beauveria bassiana* and *Metarhizium anisopliae* which are world-wide used to control pest and vector insects. These fungi produce a defined spectrum of molecules considered as virulence factors such as proteolytic enzymes and secondary metabolites which have been designated as fungal toxins.The antifungal immunity of *G. mellonella* has been intensively studied during past decade and resulted in identification of an array of defense molecules which can either directly kill parasitic fungi (antifungal peptides) or inactivate their virulence factors (inhibitors of fungal proteinases or proteins detoxifyingfungal toxins).

Analysis of the interactions between fungal virulence factors and *G. mellonella* defense molecules provides novel insights into mechanisms behind host-pathogen coevolution, particularly in those driving evolution of virulence or immune defense strategies. Host innate immunity relies on both cellular and humoral mechanisms. The latter is based on a variety of molecules used to arrest development of and kill invading pathogens among which antimicrobial peptides and peptide families such as the defensins are evolutionarily conserved.The diversity of constitutively expressed or induced host peptides exhibiting antibacterial and/or antifungal activity determines the resistance of a particular host against a broad spectrum of potentially pathogenic microbes. Owing to their short generation times and small genomes, pathogens exhibit a high capacity to genetically generate virulence factors. Consequently, the ability of higher organism to successfully compete with pathogens in an evolutionary arms race depends on their sophisticated mechanisms allowing reciprocal diversification of defense molecules. This paradigm has recently attracted many researches to investigate the molecular mechanisms providing evolutionary diversification of antimicrobial peptides conferring immunity in a variety of host model organisms including, for example, insects such as termites and the dipteran species Drosophila and Anopheles, as well as in nematodes and mammals. In this, I will first briefly outline the current knowledge about antifungal peptides in insects, emphasizing those of the *G. mellonella*. In response to diversifying host defense molecules, fungal pathogens have evolved mechanisms mediating either suppression or avoidance of host immune responses.Another strategy of pathogens to overcome the host immune system targets its defense molecules directly. Pathogen-associated proteinases capable of digesting host defense proteins and peptides are essential during pathogenesis and operate as virulence factors.

Consequently, a subsequent chapter addresses the role of fungal proteinases during pathogenesis, particularly, in degradation of host defense molecules in insects. Host counter-adaptation to proteinases produced by pathogens or parasites to inactivate host defense molecules has led to the evolution of corresponding proteinase inhibitors which have become constituents of the host innate immune system. Microbial proteinases and immunityrelated proteinase inhibitors can be considered as favorite models to analyze evolutionary arms races in an antagonist system at molecular level because their intimate interactions at the frontline between pathogens and their hosts are characterized by rapid reciprocal adaptations.Emphasizing coevolutionary insights, I will briefly outline in the last chapter biological functions of proteinases associated with fungal pathogens and immunity-related proteinase inhibitors of their host insects.

Antifungal Peptides and Proteins of Insects

The antifungal defense of insects against fungal pathogens relies on both cellular and humoral components.The cellular defense encompasses phagocytosis of fungal cells entering the insect body by immune-competent hemocytes circulating in the hemocoel, and, if the number of fungal cells is too large or fungal mycelia are too large to be engulfed, multicellular encapsulation. The latter is a complex process in which pathogens or parasites are separated from the body by a multilayered sheet of different hemocytes types whose orchestrated action results in formation of a black capsule.Phenoloxidase-mediated formation of chemically inert melanin around the entrapped microbes or parasites arrests exchange of molecules between the host and the pathogen.However, the humoral part of insect immunity against parasitic fungi is based on peptides or proteins which can either kill fungi directly or which neutralize toxic molecules released thereof.Accordingly, I will first address insect antifungal proteins and then peptides with

less than 10 kDa before I focus on insect-derived molecules involved in defense against fungal virulence factors such as toxic proteinases. Innate immune response of *G. mellonella* encompasses the expression of lysozyme. Its activity against gram-positive bacteria has been attributed to its ability to degrade cell wall peptidoglycan by hydrolysis of the β-1-4 linkages between N-acetylglucosamine and N-acetylmuramic acid residues. Besides moderate activity against gram-negative bacteria, *G. mellonella* lysozyme was also shown to exhibit antifungal activity in vitro,4 similar to that of human lysozyme against the pathogenic yeasts *Candida albicans* and *Coccidioides immitis*. The effect of commercially available hen-egg-white lysozyme on virulent or non-virulent strains of *M. anisopliae* is illustrated in Figure 1. A recent analysis of the *G. mellonella* immunity-related transcriptome resulted in identification of four lysozyme homologues and an additional i-type lysozyme whose precise functions in antifungal immunity remain to be elucidated (Vogel, et al. unpublished). To date, more than a thousand peptides and proteins exhibiting antimicrobial activity have been found in living organisms ranging from bacteria to humans.The gene-encoded antifungal peptides and proteins of insects share unifying themes with those of other animals and plants, for example, they have retained their membrane-active efficacy despite the presence of highly mutable target microorganisms. The spanning of conserved motifs among particular polypeptides involved in antimicrobial defense across the phylogenetic continuum affirms their ancient role in coevolutionary relationships between hosts and pathogens.However, analysis of immunity-related genes and pathways in mosquitoes shows that evolutionary dynamics differs among functional gene categories. Genes involved in immunity-related recognition and signal transduction are rather conservative when compared with rapidly evolving genes encoding effectors involved in killing of pathogens. Antimicrobial peptides (AMPs) diversify not by sequence divergence but rather by gene duplication and creation of new families.Naturally occurring polymorphisms of AMPs seems to drive the variability in immune-competence among natural insect populations.

AMPs are typically cationic and consist often of less than 100 and mostly between 12 and 50 amino acid residues.Despite the low similarity at the amino acid sequence level the great majority of AMPs from insects can be categorized into one of three structural classes: (1) linear alpha-helical peptides free of cysteine residues, (2) peptides adopting a beta-sheet globular structure stabilised by intramolecular disulfide bridges, (3) peptides with unusual bias in certain amino acids such as proline and/or glycin. Insect AMPs such as those found the greater wax moth *G. mellonella* can exhibit antibacterial and/or antifungal activity. The number of strictly antifungal peptides is rather low when compared with antibacterial ones. Two cysteine-rich peptides which exclusively inhibit growth of filamentous fungi have been isolated from *G. mellonella*, the defensin-like antifungal peptide and gallerimycin.Confirming the postulated contribution of gallerimycinto antifungal defense in insects, its transgenic expression has been determined to confer resistance to fungal diseases even in crops.Comparison of defensin sequences from arthorpods and mollusks revealed that all exons and introns, aside from the exon encoding the mature peptide, differ widely in number size and sequence. This variability implicates that the exon encoding the mature peptide was modified by exon-shuffling and integrated down-stream of unrelated leader sequences during evolution.

Fungal Proteinases as Virulence Factors

The proteinaceous exoskeleton of insects forms an efficient primary physical barrier against most microbes. Viruses and bacteria infect their host insects usually upon up-take with the food via the

alimentary channel while only parasitic fungi can directly penetrate the cuticle using a set of enzymes among which proteinases have been recognized as virulence factors. The substrate specificity and the controlled expression of invasive proteinases predict that adaptation to varying host ranges drives diversification and functional shifts of these enzymes.
M. anisopliae produces at least three distinct types of proteinases during growth on insect cuticle: the subtilisin-like serine proteinase Pr1 (EC3.4), the trypsin-like serine proteinase Pr2 and a metalloproteinase.Trypsins and the subtilisins belong to distinct super-families of serine proteinases which independently evolved similar catalytic mechanisms. The function of these fungal proteinases during pathogenesis can be expanded beyond facilitating penetration of the exoskeleton to include utilization of host proteins for nutrition, suppression of host cellular defense,and degradation of host defense molecules.

Host Inhibitors of Microbial Proteinases

Insect hemolymph contains relatively high concentrations of serine proteinase inhibitors belonging to Kunitz, Kazal, Serpin and α-macroglobolin families among which some have been recognized to function as effectors in innate immunity by inhibition of pathogen-associated proteinases.Our previous efforts in identification and characterization
of immunity-related peptides from *G. mellonella* larvae resulted in the discovery of a number of novel peptidic inhibitors of pathogen-associated proteinases which are simultaneously induced and secreted within the hemolymph during innate immune responses along with antimicrobial
peptides. Three heat-stable serine proteinase inhibitors (ISPI-, ISPI-2 and ISPI-3) have been purified from hemolymph whose molecular masses ranged between 6.3 and 9.2 kDa. The determined N-terminal amino-acid sequences provide evidence that one belongs to the Kunitz and another to the Kazal family of proteinase inhibitors while the third shared no sequence similarity with known peptides.58 Inhibitors of the Kunitz and Kazal families are widespread in the hemolymph of arthropods and are likely involved in protecting host from microbial proteinases while also functioning in regulation of endogenous proteinases. Kazal and Kunitz type inhibitors share intra-domain disulfide cross-links determined by six conserved cystein residues.
Interestingly, all three serine proteinase inhibitors purified from immunized *G. mellonella* larvae were found to inhibit the trypsin-like proteinase (Pr2) produced by *M. anisopliae*, and ISPI-3 was also active against the chymoelastase secreted by this fungus.These findings and our observation that inducible proteinase inhibitors in the hemolymph of *G. mellonella* inhibit both fungal proteases and fungal development in the host lend some credit to our hypothesis that low molecular mass proteinase inhibitors participate in antifungal defense in *G. mellonella* by inactiviation of secreted fungal proteinases thereby preventing host defense molecules from degradation. However, these findings provide a new framework to reassess the coevolution between pathogen-associated proteinases and host proteinase inhibitors because their interactions are more complex than previously thought and must be interpreted in the context with host proteinases and pathogen-associated substrates which are also involved in the molecular arms race.

Coevolution Between Fungal Proteinases and Host Proteinase Inhibitors

The parasitic life has arisen and also lost multiple times in many independent lines of fungal evolution. Interestingly, there is also evidence for interkingdom host-jumping by parasitic fungi from plants to insects.Adaptation of fungal populations to different hosts has been suggested to drive sympatric divergence of parasitic fungi. In parasite-host associations, speciation in the host can lead to speciation of the parasite,but this is obviously not the case in entomopathogenic fungi infecting a broad host rage such as *B. bassiana* and *M. anisopliae*. The evolution of a parasitic life style depends on the availability of enzymatic virulence factors which mediate utilization of the host as a source of nutrients and which can degrade its defense molecules. Particularly, genes encoding fungal proteinases should provide a good model to study adaptive evolution of multigene families and its impact on speciation.For example, evidence has been elaborated that multiplication of an ancestral proteinase gene seems to precede species differentiation in parasitic fungi.Because pathogen virulence is thought to coevolve as a result of reciprocal selection with its host, it can be postulated that positive selection exists for the evolution of novel proteinases or proteinase isoforms which are not inactivated by proteinase inhibitors of the host.
This hypothesis regarding coevolution between proteinases and proteinase inhibitors in an antagonist system can be described as possible scenarios.
Thermolysin is also a potent activator of the enzyme cascade that controls phenoloxidase activity which catalyzes the formation of melanin. Hemolymph coagulation resulting in hemolymph clots is a first response to wounding in insects and phenoloxidase has been shown to shape the clot's physical properties by crosslinking of proteins and melanization. Entrapping of bacteria within the clots alone is not sufficient to kill them and requires bactericidal compounds among which some occur as intermediates during synthesis of melanin.Because melanization of entrapped bacterial or fungal cells represents an efficient defense mechanism in insects, adapted pathogens should avoid excessive melanization caused by their secreted microbial metalloproteinases.
Indeed, as a counter-adaptation to efficient mechanisms mediating both sensing of microbial metalloproteinases and activation of immune responses against their producers, entomopathogenic bacteria and fungi tightly regulate the activity of thermolysin. The mature enzyme can be inhibited by its propeptide. A fine-tuned timing of enzyme production has been documented by analysis of gene expression during germination, pathogenesis and conidiogenesis and of the parasitic fungus *M. anisopliae*.Secretion of thermolysin in order to degrade extracellular matrix proteins and to colonize host tissues occurs in a later stage of mycosis when host hemocytes undergo apoptosis initiated by released destruxins. At this phase of infection, the insect hosts occur moribund and are not able to mount an immune response sufficient to protect them from death.These findings add to our knowledge about molecular mechanisms that pathogens and parasites use in evasion of host immunity. Pathogenic mechanisms that manipulate host immunity or escape from host defense are sensitive to parasite fitness and thus dominate as causes of parasite virulence.Theory predicts that adaptation of *M. anisopliae* and other pathogens to a broad host range should be accompanied by rapid diversification of genes involved in an arms race with multiple hosts, while adaptation to particular host species should promote loss of genes lacking selection pressure because they are not required for infection of a limited host range. Comparative genomic analyses of *M. anisopliae* strains with either broad or narrow host ranges have recently confirmedthis hypothesis.However, identifying coevolving

partners from paralogous gene families remains to be elucidated and recent bioinformatic tools will help in the near future to more precisely reconstruct coevolution between pathogen-associated proteinases and host proteinase inhibitors.