Insect defence: its impact on microbial control of insect pests

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Insects are the earliest and most diverse taxon of animals accounting for more species than all other animals put together because of their high reproductive potential and varied niche. Though cellular reactions like phagocytosis, nodulation and encapsulation and humoral reaction through secretion of antibacterial proteins are known for fungus and bacterial invaders, little is known about insect defence against insect virus infection. This review provides a brief outline of the current state of knowledge on the various aspects of insect defence mechanisms through their innate, i.e. non-specific reactions, and how the various microbial control agents, viz. entomopathogenic fungi, bacteria, viruses and nematodes try to overcome the insect host defence. The need for basic studies on molecular characterization of major lepidopteran insect pests along with their associated pathogenic microbes has been stressed for better understanding and disarming of insect defence system for further exploitation of insect pathogens for microbial control of insect pests.

INSECTS are among the earliest and most diverse taxon of animals on the planet accounting for more species than all other animals combined. Insects are r-selective because of their short life span and high rate of reproduction. Further, insects are the most successful group of animals that exist in a myriad of environment where the potential for infection by microorganisms and parasites is great. Insects have also demonstrated considerable ability to develop resistance to conventional insecticides; and more than 645 species of insects and mites have already developed resistance to one or more chemicals (Figure 1). As part of a survival strategy, insects have evolved numerous effective resistance and defence mechanisms to most of the conventional chemical insecticides with possession of genes for high levels of oxidases, esterases, glutathione-s-transferases, ‘insensitive’ acetylcholinesterases (AChE), and nerve insensitivity to pyrethroids. Similarly, can an insect species susceptible to a pathogen become resistant to more microorganisms? Host specificity observed with many insect pathogens demonstrates that insect species are naturally resistant to these microorganisms. Some of the important lessons learnt from insect resistance to organochlorines, organophosphates, carbamates, pyrethroids and insect growth regulators from our past experience includes the following: (i) insect populations can and will evolve resistance to novel challenges that are initially devastating to them, (ii) life-history characteristics and the available supply of genetic variability make some species highly prone to evolve resistance, and (iii) there is no guarantee that a safe, effective, inexpensive insecticide, including biopesticides like Bacillus thuringiensis (Bt) cannot be misused so as to rapidly induce resistance to it, as it is evident from the Diamond back moth of cabbage, Plutella xylostella; Indian meal moth, Plodia interpunctella; American boll worm of cotton Heliothis virescens; Oriental boll worm of cotton, H. armigera; beet army worm, Spodoptera exigua, and tobacco caterpillar, S. litura. All have shown different degrees of resistance to Bt and insect viruses2,3 (Table 1). Indeed, insects that are susceptible to a pathogen can show resistance to various entomopathogens and try to resist infection through morphological, behavioural, developmental (like maturation immunity), physiological, nutritional, biochemical and molecular genetic mechanisms, etc. In order to appreciate the insect defence and its role in microbial control of insect pests by the conventional agricultural entomologists and applied insect pathologists, some preliminary information on different aspects of immunity, viz. passive and active defence mechanisms against foreign invaders in comparison with vertebrate immunity has been presented. How certain parasites and pathogens like fungi, bacteria, nematodes and viruses of insect pests evolved strategies for avoiding both the external barrier

![Figure 1](image-url)  
**Figure 1.** Development of resistance by insects and mites to insecticides.
Table 1. Insects showing resistance to Bt and insect viruses

<table>
<thead>
<tr>
<th>Species</th>
<th>Bt</th>
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<tbody>
<tr>
<td>Resistance to Bt</td>
<td></td>
</tr>
<tr>
<td>Plodia interpunctella</td>
<td>Btk</td>
</tr>
<tr>
<td>Planella xylostella</td>
<td>Btk</td>
</tr>
<tr>
<td>Heliothis virescens</td>
<td>Btk</td>
</tr>
<tr>
<td>Spodoptera litura</td>
<td>Btk</td>
</tr>
<tr>
<td>Spodoptera exigua</td>
<td>Btk</td>
</tr>
<tr>
<td>Spodoptera littoralis</td>
<td>Btk</td>
</tr>
<tr>
<td>Cadra cautella</td>
<td>Btk</td>
</tr>
<tr>
<td>Leptinotarsa decemlineata</td>
<td>Bin</td>
</tr>
<tr>
<td>Aedes aegypti</td>
<td>Bti</td>
</tr>
<tr>
<td>Culex quinquefasciatus</td>
<td>Bti</td>
</tr>
<tr>
<td>Musca domestica</td>
<td>Bti</td>
</tr>
<tr>
<td>Drosophila melanogaster</td>
<td>Bti</td>
</tr>
<tr>
<td>Resistance to insect viruses</td>
<td></td>
</tr>
<tr>
<td>Pieris brassicae</td>
<td>Granulovirus (GV)</td>
</tr>
<tr>
<td>Heliothis zea</td>
<td>Nucleopolyhedrovirus (NPV)</td>
</tr>
<tr>
<td>Mamestra brassicae</td>
<td>Cytoplasmic polyhedrovirus (CPV)</td>
</tr>
<tr>
<td>Bombyx mori</td>
<td>CPV</td>
</tr>
<tr>
<td>B. mori</td>
<td>NPV</td>
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<tr>
<td>B. mori</td>
<td>Infectious flacherivirus</td>
</tr>
<tr>
<td>B. mori</td>
<td>Densonucleovirus</td>
</tr>
<tr>
<td>Philornis opercula</td>
<td>GV</td>
</tr>
<tr>
<td>P. interpunctella</td>
<td>NPV</td>
</tr>
</tbody>
</table>

as well as to counteract the internal immune defence posed by the host insects, has also been discussed along with a cascade of events associated with their mode of action. Understanding of molecular genetics of both insect hosts and pathogens and molecular basis of the insect biochemical and cellular defence, including insect haematolgy has been stressed for the proper management of pests, especially using various biocontrol agents like parasites and pathogens, including Bt transgenic plants.

Insects vs vertebrate immunity

Compared to vertebrates, insects do not possess the ability to produce antibodies (immunoglobulins) and do not use immunoglobulin as recognition molecules in the classical sense, against foreign antigens and hence antigenic memory appears to be lacking, i.e. non-memory type. Further, they do not produce alpha/beta interferons (IFN-\(\alpha/\beta\)) in response to viral infections. Nevertheless, they are capable of ‘immune’ reactions, which appear to be predominantly cellular in nature. Several haemolymph-induced antibacterial proteins have been reported to be broad-spectrum in their action, which are produced in insects in response to the bacterial challenger, and of shorter duration in nature. This would suggest that analogy to the phenomenon of immunity in vertebrates may be inappropriate, and hence immunity in insects is different from immunity in vertebrates. Vertebrates have both innate and acquired or adaptive immunity with ‘immunological memory’, whereas invertebrates lack this immune system. Instead, insects possess innate immunity which is characterized by non-specific immune reactions against foreign materials. In general, insect immunity consists of cellular and humoral reactions. The defence mechanism in insects is classified into two broad groups. The first one is non-specific immunity, which consists of structural and passive barriers like cuticle, gut physicochemical properties, and peri-epithelial membrane (PM)\(^4\). The second one is specific immune system involving cellular and humoral immunity. Cellular reactions include phagocytosis, nodulation (haemocyte aggregation) and encapsulation, especially with reference to bacteria, fungi and protozoa, including nematode invaders. On the other hand, humoral reactions involve activation of prophenoloxidase cascade and induction of immune proteins such as lysozymes, lectins and anti-bacterial and anti-fungal proteins\(^5\).

Non-specific defence mechanisms

Morphological

Unlike vertebrates which have extensive exposure of epithelial cells to the external environment, insects have extensive protection of their epithelial tissue by the cuticular layer, which makes them less vulnerable to penetration by pathogens. The chitin-rich cuticle of the insect covers virtually the entire external surface, even extending through the foregut, hindgut and tracheal tubes, constituting the first line of passive defence in insects. Unlike that of the foregut and hindgut, the epithelium of the insect midgut does not have a cuticular lining\(^6\). However, in many insects the PM apparently functions to protect cells of the midgut from injury due to hard (or) sharp particles of food and from pathogens\(^7\) performing much the same function as mucus in the mammalian alimentary tract. The PM, which develops from specialized cells in the midgut along the columnar epithelia, consists of chitin, glucosaminoglycans, glycoproteins and proteins\(^8\). Further, the PM of larvae excludes particles greater than 20 nm, but it is efficient in allowing digestive enzymes to pass into the midgut and thus preventing bacteria and viruses from reaching the surrounding midgut epithelium.

Further, organ systems adjacent to the midgut are protected from viruses by their basal lamina, which forms a discrete layer of extracellular matrix that surrounds the organs and isolates them from the haemocoele. The basal lamina, which is composed of collagen, elastin, glucosaminoglycans and glycoproteins\(^9\) is similar to that of PM in serving as a filter to protect tissues from particles larger than 15 nm\(^10\). Also, basal lamina protects the tissues from viral particles by serving as an ionic barrier. The glucosaminoglycan molecules are negatively charged in the midgut environment and have been shown to repel negatively charged particles such as viruses\(^9\).
Physical resistance occurs when the nematode cannot penetrate the integument or the cocoon of a host insect. An insect resists entomopathogenic nematodes (EPNs) belonging to Steinernematids and Heterorhabditis infection through behavioural, physical, or physiological means. For example, *Romanomermis culicivorax* has difficulty in penetrating the integument of older mosquito larvae. Dauer juveniles of *Steinernema carpocapsae* cannot penetrate the silken cocoons of hymenopteran parasites. Spiralacular openings of insect respiratory system are the portals of entry for EPN, but sieve plates over the spiracles, especially in scarab larvae, may deny nematodes access through this entry point. Avoidance of nematodes due to the presence of the thick PM can act as a morphological defence against EPN.

**Behavioural**

Nearly all food digestion and nutrient absorption occurs in the midgut, where the typical pH ranges from 8 to 10.5 (ref. 12). The midgut and associate glands secrete digestive enzymes like proteases needed to break down food. The foregut and hindgut are dynamically and biochemically different from the midgut which is an endodermal derivative, unlike the foregut and hindgut which are ectodermal in origin, also, they do not secrete digestive enzymes and are at near neutral pH. Behavioural resistance occurs when the insect actively avoids or repels the nematode. Extremely active mosquito species had a lower prevalence of infection by the nemthiid *R. culicivorax* than less active ones. Scarab larvae may avoid infection by wiping nematodes away from the mouth. Younger instars of black fly larvae are resistant to infection by *S. carpocapsae* because the comparatively large nematode is excluded from the insect’s mouth. Aggressive behaviour (grooming with legs, mouthparts, and rasper) of *Papilio japonica* larvae when nematodes are present on the cuticle thereby removing and/or killing nematodes on the cuticle, has also been reported.

**Physiological**

Under defence mechanisms, the high gut pH, presence of protease, etc. are found to be detrimental to the infective juveniles of *Heterorhabditis bacteriophora*. Similarly, low gut pH, absence or low levels of protease activity, etc. in the insect system though do not play a key role in the resistant mechanism to bacterial pathogens like *Bt* and baculoviruses comprising nucleopolyhedrovirus (NPV) and granulovirus (GV); certainly have a role to play for their low susceptibility to bacterial and viral pathogens. Grasshoppers/locusts elevate their body temperatures higher than ambient through habitat selection and/or orientation to solar radiations called ‘basking’ by way of intercepting the solar radiation and raising internal thoracic temperature ranging from 38 to 42°C, thereby showing ‘behavioural fever’ response. Such a body temperature is predicted to inhibit fungal proliferation, thus giving the host immune system an edge in suppressing the fungus germination and growth which normally takes place from 25 to 30°C, thereby reducing the infection. Thus, thermoregulation by grasshoppers, *Melanoplus sanguinipes* has been shown to reduce mycosis caused by * Beauveria bassiana* and *Metarhizium anisopliae*. Physiological resistance to infection involves the destruction of the nematode by digestive enzymes in the alimentary tract of the insect and the melanization and encapsulation of the nematode within the hemocoel. Melanotic encapsulation of *S. carpocapsae* has been reported in larvae of several mosquito species. Although the nematode is encapsulated, majority of the larvae die of septicaemia caused by the bacterium, *Xenorhabdus nematophilus* which is mutually associated with this nematode.

**Biochemical**

**Eicosanoids**: This is a collective term for all biologically active, oxygenated metabolites of arachidonic acid and two C₂₀ polyunsaturated fatty acids. Major groups of eicosanoids include prostaglandins, epoxy eicosanoid acids and various lipoxygenase products. Nodule formation is quantitatively the most important and specific cellular defence mechanism that depends upon eicosanoids. Inhibition of either total eicosanoid biosynthesis or of specific eicosanoid biosynthesis pathways by the pharmaceutical eicosanoid biosynthesis inhibitor, viz. ibuprofen, severely impaired the ability of the insects to clear bacterial populations from haemolymph by microaggregation and nodulation, and thereby increased larval mortality due to the infection in the case of *Manduca sexta*, *Agrosis ipsilon*, *Pseudaleata unipuncta* and *Bombix mori*; Though Jureenka *et al.* have shown the inhibition of total eicosanoid biosynthesis severely impaired the ability to form nodules in *B. mori*, Krishnan *et al.* have shown by their study on the role of DL-DOPA (3,4-dihydroxyphenylalanine) that eicosanoids may not be the sole mediators of nodulation reaction in *B. mori*, since nodule response is not completely ablated thereby suggesting the involvement of dopa/dopamine in steps leading to microaggregation, nodulation and melanization response of haemocytes.

**Superoxide dismutase**: Molecular oxygen is an essential element of life; yet as a result of incomplete reduction of oxygen to water, reactive oxygen species (ROS) are generated in all aerobes. Most ROS are generated by superoxide anions (O₂⁻), and are rapidly dismutated either non-enzymatically or enzymatically by the action, of superoxide dismutase (SOD) to hydrogen peroxide (H₂O₂) and oxygen (OH). In mammals, superoxide production by
leucocytes is common and essential for bacterial killing. Recently, Krishnan et al. have shown the superoxide dismutase activity in haemocytes and haemolymph of *B. mori* following bacterial infection. Hoover et al. have negatively correlated the presence of higher activity of foliar peroxidase in cotton with *Autographa californica* nucleopolyhedrovirus (AcMNPV)-induced mortality and with the production of free radicals, including highly damaging ROS such as H$_2$O$_2$ and OH; these radical species were strongly linked to inhibition of baculoviral disease because inhibition was markedly reversed in the presence of scavengers of these species.

**Biotechnological (molecular)**

Little is known about insect defence against virus infection, although insect haemocytes can provide cell-mediated immunity to bacterial pathogens through phagocytes and encapsulation. Neither cell-mediated nor humoral immunity has been demonstrated against virus infection in insects. ‘Apoptosis’ – distinctive type of programmed cell death – a phenomenon evolved as a primitive viral defence in certain vertebrate animals and invertebrates lacking humoral immunity to function as antiviral defence mechanisms, is gaining importance in cellular defence against viral infections. However, insect baculoviruses like NPV, GV and other DNA viruses of insects evolved methods apparently to bypass this defence phenomenon of apoptosis by directly blocking this response with possession of *p35* gene and the inhibitor of apoptosis (*iap*) gene, thereby promoting their own survival by suppressing apoptosis of host cells. The above findings lead to the future possibility of blocking apoptosis for increasing virulence of certain baculoviruses as well as for the determination of host range of certain baculoviruses and development of robust cells for *in vitro* multiplication of insect viruses and development of genetically improved parasitoids.

**Genetic**

Genetic resistance to bacterial and viral pathogens has been recorded against pestiferous insects. High levels of resistance to the δ-endotoxin of *Bt* subspecies *kurstaki* have been recorded for the Indian meal moth, *P. interpunctella*, a pest of stored-grain and cereal products. The resistant trait is incompletely autosomal, recessive, and several alleles or genes are believed to be involved, and resistance in this insect is linked to an alteration in toxin-membrane binding of the midgut cells.

Resistance of the silkworm to NPV, cytoplasmic polyhedrovirus (CPV) and infectious flacherie virus (IFV) is controlled by polygenes. The polygenes are supposed to be mainly concerned with defence mechanism of the midgut such as antiviral activity of gut juice, characteristic of peritrophic membrane, etc. On the other hand, non-susceptibility to densonucleosis is controlled by recessive (*nsd-1, nsd-2*) or dominant (*nid-1*) major genes. The major gene may cause a deficiency of an enzyme involved in viral multiplication or in the receptor synthesis within the midgut cell. Polygenic resistance can be introgressed into a silkworm variety by selection in a breeding programme of the silkworm variety. A hybrid of two strains usually shows high heterosis in polygenic resistance to viral diseases. The breeding procedure for non-susceptible variety to DNV is much easier because the mechanism of non-susceptibility is controlled by a single major gene. The gene can be introduced into the breeding programme or transferred to an existing superior variety by back crossing.

**Specific defence mechanisms/immunity**

**Cellular immunity**

In a cellular defence mechanism, unlike vertebrates which have red blood corpuscles and white blood corpuscles in a closed circulatory system, insects with open body cavity lack lymphocytes, the major source of vertebrate immunity to virus infection. But they have only free blood cells called haemocytes. Different types of blood cells have an important role in the protection of insects against invading microorganisms. Hence identification and classification of various types of insect blood cells based on the structure and function is important. Among the six major groups of insect haemocytes in recognizing the “self” (isografts) and non-self (allografts), plasmocytes and granulocytes are the major effector cells. They react to foreign invaders either by phagocytosing like microorganisms or nodulating and encapsulating objects too large to be individually engulfed, viz. metazoan parasite by way of haemocytes attaching and forming many layers which often become melanotic, thereby causing the death of the parasitoid through starvation and/or anoxia mechanism. Changes in total haemocytes during growth and development of healthy insects have been reported by a number of workers. Drastic reduction in the number of haemocytes during various microbial infections has also been reported by several workers. Infection by *B. bassiana* results in a gradual suppression of the phagocytic competence of circulating haemocytes and alteration in both total and differential haemocyte counts has been reported in the case of fungal, bacterial, viral and parasitic infection.

**Humoral immunity**

Humoral reactions require several hours for their full expression and involve induced synthesis of several families of characterized antibacterial proteins like cecropins,
attacins, dipterocins and defensins, including recent identification of 'haemolin' which belongs to the immunoglobulin superfamily. These proteins are small and strongly basic, and comprise three major forms: A, B and D. They exhibit a broad spectrum of antibacterial activity against both Gram-negative and Gram-positive bacteria by adopting alpha-helical structure on interaction with bacterial membranes resulting in the formation of ion channels. This observation prompted scientists to propose the idea of using the genes cloned from insects to enhance bacterial disease resistance in plants. The detergent properties of their antibacterial proteins disrupt cell membranes of the invading bacteria. Insects also synthesise lysozymes which enzymatically attack bacteria by hydrolysing their peptidoglycan cell walls. Humoral reaction involves synthesis and release of several antibacterial (immune) proteins. The antibacterial nature of the gut contents and partial characterization of haemolymph bacterial proteins has been reported. These insect antibacterials are the best characterized invertebrate antibacterial factors and they have counterparts in mammals. The role of these proteins in non-self recognition as well as acting against both prokaryotic and eukaryotic cells, including human infectious parasites has been well studied.

The phenoloxidase pathway has been demonstrated in insects. The function of the pathway is to synthesise phenoloxidase, an enzyme which is responsible for the melanization and encapsulation of pathogens. For example, in the case of B. mori, prophenol oxidase, the proenzyme is released by exocytosis and is converted to its activated form by serine protease. Activated phenoloxidase adheres to foreign particles due to the 'sticky' nature and generates cytotoxic quinoid compounds which kill the intruders.

Application of insect defence in insect pest management

Parasitoids

One approach in understanding variation in insect vulnerability to parasitoids is to study characteristics of host species that provide protection against the search, attack or development of parasitoids. These host traits can be grouped mostly into morphological, behavioural and physiological defences.

Morphological and behavioural: Weseloh observed that long hairs and vigorous movements of older larvae of Lymantria dispar by means of head jerking prevented oviposition behaviour by Cotesia melanoscelsa. Cabbage looper, Trichoplusia ni was observed to regurgitate on a tachinid egg, Vorta ruralis, thereby dislodging it. Further, some hymenopteran idiobionts kill or permanently paralyse the host before oviposition in contrast to koinobionts, e.g. Bracon hebetor paralysing Coreya cephalonica.

Physiological: Physiological mechanisms include the sequestration of host plant allelochemicals and encapsulation by means of polydnavirus (PDV) present in calyx fluids of certain female parasitoids belonging to Ichneumonids and Braconids. Parasites in general, in their habitual host, are able to escape potential lethal defence response posed by the host. Recently, it has been shown that females of certain species of endo parasitic wasps belonging to hymenopteran families, Ichneumonidae and Braconidae, produce particles containing double-stranded, circular, multipartite DNA virus called 'Bracovirus' and 'Ichnovirus' respectively and PDV in the ovarian calyx tissue of the female wasp which enable the parasitoid to initially circumvent host defence. These viruses cause host immune suppression (the equivalent of an insect AIDS-like virus) allowing the parasitoids to mature, without incurring a host immune response. The PDV triggers apoptosis of host haemocytes, thus causing the host to be immuno-suppressed during the initial stages of parasite infection. The possibility of using PDVs for abrogating the host defence mechanism by 'molecular mimicry' and later by way of changing the haemolymph and the mobility of haemocytes to encapsulate, thereby increasing the efficiency of both homologous and heterologous parasites by way of cross protection thereby making even non-habitual host to become parasitised, has been postulated. Further, future possibility of laboratory breeding of certain parasites for genetic improvement in functions required for successful parasitism, has also been indicated. For example in the case of tobacco horn worm, M. sexta which is semipermisive to A. california AcMNPV, co-infection of M. sexta larvae with PDV from a braconid parasitoid, Cotesia congregata which produces 33 kDa PDV-encoded early glycoprotein EPI, increases susceptibility to fatal infection by AcMNPV.

Entomopathogens

Entomopathogenic fungus: Insect mycopathogens such as B. bassiana and M. anisopliae undergo in vivo development cycle which includes the following stages: (i) adhesion of conidia to the host cuticle, (ii) germ tube formation, (iii) penetration of host cuticle, (iv) vegetative growth within the host, and (v) production of externally borne conidiospores (Box 1). Basic studies on the in vivo development of insect mycopathogens like white muscardine B. bassiana, green muscardine M. anisopliae, Nomuraea rileyi and Verticillium lecanii, have addressed the 'determinants' responsible for attachment, germination and cuticular penetration. At present, little is known about the survival and development of insect mycopathogens.
within the host insect. Insect mycopathogens may overcome the internal defence response by utilizing one or more of the following strategies. Either the fungal cells developing within the insect may possess an outer coat, which is neutral to circulating haemocytes or they are effectively masked by host proteins or by producing immuno-modulating substances which suppress the cellular defence system; thereby the fungal cells may be tolerant to the humoral and cellular defence system of the insects. In the case of Spodoptera exigua infection by B. bassiana results in a gradual suppression of the phagocytic competence of circulating haemocytes, especially granular haemocytes, and alteration in total and differential haemocyte counts.\footnote{35}

**Entomopathogenic bacteria:** The toxin pathway of Bt Cry proteins involves several steps (Box 2). Upon ingestion by a susceptible insect, the insoluble bipyramidal crystalline is solubilized in the gut which has a high alkaline pH of more than 8, and the protoxins are released. These protoxins are then processed by midgut proteases which typically cleave the 130 kDa protein into about 500 amino acids from the C-terminus and 28 amino acids from the N-terminus into protease-resistant core fragment of 55–70 kDa protease-resistant active toxins, which pass through the PM and bind to the specific receptors located in the brush border membrane vesicles (BBMV) of the columnar midgut cells of the target tissue.\footnote{54} Binding is followed by irreversible insertion of the toxins into the apical microvilli membrane of the epithelial midgut columnar cell. The interaction of the toxin with those receptors which have been characterized as aminopeptidase-N (120–180 kDa glycoproteins), triggers the formation of anionic channel (pore formation) which disrupts the osmotic equilibrium maintained by the cells by pumping ions into the extracellular medium. Perforation in the columnar cell apical membrane renders the cell volume regulation mechanism ineffective. Accordingly, the cell swells and ultimately bursts by a process known as colloid-osmotic lysis.\footnote{55,56} This leads to the disruption of gut integrity and finally the intoxicated insect stops feeding and death of the insect results due to anorexia (cessation of feeding or starvation) or septicemia.\footnote{57}

Any interference with the cascade of events associated with the mode of action helps the insects to survive and thereby develop resistance (Table 2). Generally, pathogenic effect of Gram-positive, spore-forming and crystallicferous bacteria, Bt is determined by the activity of the spore to pass through the gut wall, which is lined by the PM. Increased pathogenicity of Bt fed along with boric acid to Spodoptera litura was reported by Govindarajan et al.,\footnote{59} demonstrating the destruction of the protective layer of the PM, thereby facilitating the invasion by the pathogen. Further, the role of certain proteolytic immune inhibitors by the Gram-negative, non-spore-forming and non-crystalliferous potential pathogens like Serratia marcescens as well as by Bt by way of proteolytic digestion of certain insect antibacterial proteins like cecropins and attacins for their successful invasion and infection has been elucidated.\footnote{60}

**Mechanisms of resistance to Bt**

**P. interpunctella**

Reduced binding of Bt toxin to the brush border membrane of the midgut epithelium has been identified as a primary mechanism of resistance in Indian meal moth, P. interpunctella with a 50-fold reduction in binding which was correlated with a > 100-fold reduction in toxicity to cry IAab. Both midgut pH and altered proteolytic processing were not the major mechanism of resistance.\footnote{27}
Table 2. Major steps of mode of action of Bt and the nature of interference

<table>
<thead>
<tr>
<th>Step affected</th>
<th>Nature of interference</th>
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</thead>
<tbody>
<tr>
<td>Solubilization</td>
<td>Failure</td>
</tr>
<tr>
<td>Activation</td>
<td>Defective proteolysis</td>
</tr>
<tr>
<td>Activation</td>
<td>Over proteolysis</td>
</tr>
<tr>
<td>Access to target</td>
<td>Competitive inhibition binding sink</td>
</tr>
<tr>
<td>Target binding</td>
<td>Change to primary structure of target</td>
</tr>
<tr>
<td>Target binding</td>
<td>Change to target modification</td>
</tr>
<tr>
<td>Pore formation</td>
<td>Hindered</td>
</tr>
<tr>
<td>Pore function</td>
<td>Plugged</td>
</tr>
<tr>
<td>Loss of midgut epithelium</td>
<td>Physiological coping</td>
</tr>
<tr>
<td>Self dosing</td>
<td>Behavioural changes</td>
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</table>

P. xylostella

As in the case of P. interpunctella reduced binding of Bt toxin has been identified as a primary mechanism of resistance with > 200-fold resistance to cry 1Ab in the case of diamondback moth of cabbage, P. xylostella. Both altered proteolytic processing and increased behavioural avoidance in consumption and movement patterns were not a key or important resistant mechanisms in P. xylostella.61

H. virescens

Though the most common mechanism of resistance involves changes in binding affinity of toxin receptors in the insect midgut membrane, this has not been the case with H. virescens. Somewhat different results have been obtained with a laboratory-selected resistant strain of H. virescens. The BBMV from the resistant strain showed only a 2–4-fold decrease in binding affinity for cry1Ab and cry1Ac, but an increase of 4–6-fold in the number of binding sites for these two Bt toxins, thereby indicating that additional factors must be responsible for the 20–70-fold level of resistance exhibited by this strain. Recently, Forcada et al.62 have demonstrated an important role of midgut protease in Bt resistance via reduced protoxin activation and increased toxin degradation.

Resistant management in Bt transgenics

Even before the commercial release of Bt transgenic crops, significant levels of Bt resistance have been attained in some insect species, either in the field or in the laboratory. These findings warn us that resistance management cannot be ignored at any stage in the deployment of transgenic Bt products. Understanding the mechanism of resistance will provide strategies to prevent or delay resistance and hence prolong the usefulness of Bt insecticidal crystal proteins as environmentally safe insecticides. In recent years, concerns have been raised about the development of resistance in insects to Bt crystal proteins of genetically engineered plants. The following are the various strategies.

Gene pyramiding (stacking)

This is based on the presumption that there is an almost unlimited number of different Bt toxins available in nature and that resistance can be managed using these in various mixture, mosaic, rotational or sequential systems. Recently, Chakraborti et al.63 have reported the synergism of cry1Ac with cry1F toxin by way of lowering EC50 of cry1Ac toxin to 13 times due to the presence of cry1F, thereby suggesting that the toxins cry1Ac and cry1F can be expressed together in transgenic crop plants for future effective control of H. armigera and also for resistance management strategy. Further, Monsanto has developed a two-gene product named Bollgard II, in which the second Bt gene cry2Ab2 has been incorporated along with cry1Ac. Cry2A proteins are leading candidates because they lack immunological cross-reactivity with the Cry1Ac proteins, allowing the Cry2A protein to be followed independently when stacked or bioengineered into bollgard cotton. This will have an additive effect of both the Bt protein with an expanded host range in order to control the fall armyworm, Spodoptera frugiperda and beet armyworm, S. exigua and tobacco caterpillar S. litura (which was shown to be resistant to commercial formulation of Bt even before the Bt crystal toxic protein genes are sequenced and cloned64, along with false American bollworm, Helicoverpa armigera64), thereby checking the spread of resistant insects. However caution has to be exercised in future research since already extensive cross-resistance among different Bt toxins has been reported in the case of P. xylostella and in the laboratory populations of P. unipuncta.

Refugia

Facilitating the survival of susceptible insects by way of growing non-transgenic plants along with transgenic plants in a definite ratio is one of the best theoretical approaches to slow resistance development. This potentially delays the development of insect resistance to Bt crops by providing susceptible insects for mating with resistant insects, thus they mix with genes.65 This theoretical strategy has got experimental support from the study by Tabashnik et al.65, who has reported rapid reversal of up to 2800-fold resistance to Bt in P. xylostella in the absence of exposure to ICPs, which was associated with restoration of ICP binding and increased biotic fitness. Thus, the provision of periods/refugia during which insects are not exposed to Bt is a promising management option.
Toxin dose acquisition

High dose of Bt, which consistently kills heterozygotes along with untreated refuges as a potential means of managing resistance development in transgenic plants, was advocated. This approach maintains constitutive and continuous exposures of Bt toxins in transgenic plants, which is sufficient to kill the heterozygotes in a population82.

Targeted delivery

Since continuous and constitutive expression of Bt toxic genes may result in selection pressure, tissue-specific (use of stem, root, boll, pod or seed) and stage-specific promoters along with chemical sprays like salicylic acid to induce gene expression at will, aid in delaying the resistant development in insect pests83.

Second generation toxic genes

From bacteria: There are other insecticidal proteins from bacteria, plant and animals which are being utilized in developing plant transgenics. Recently, Estruch et al.84 characterized the novel insecticidal proteins produced by certain Bt isolates in logarithmic stage of bacterial growth called vegetative insecticidal proteins (VIPs). Sequences encoding for a VIP have been cloned and the protein has been expressed in E. coli. Further, VIP has been successfully expressed in monocots and dicot plants85.

From mutualistic bacteria: Photorhabdus luminescens that dwells inside the gut of entomopathogenic nematode belonging to the family Heterorhabditis favoured the synthesis of high molecular weight protein complexes toxic to insects ranging from lepidoptera, coleoptera to dictyoptera. Recently, Bowen et al.86 have characterized four insecticidal toxins from the bacterium P. luminescense encoded by toxin complex loci ica, tcb, tcc, and tcd, representing the second generation of insecticidal trans-genes that will complement the novel Bt α-endotoxin in future (Table 3).

From plants

Secondary metabolites from plants: Plants are known to release localized and systemic signals in response to wounding by phytophagous insects. Systemic induction of resistance implies the production of a signal at the site of primary wounding or infection, the signal being transported to the other parts of the plant where it induces defence mechanisms. Among the myriad of secondary metabolites, gallic acid and salicylic acid, which are hydroxy benzoic acids produced by shikmatic acid pathway by plants, are known to play an important role in insect-plant interactions and have considerable practical application in the area of biotechnology of crop protection87.

Salicylic acid: This has been known to be a feeding deterrent for the phytophagous soybean loopers, Pseudaphisica includens88. Survival and adult emergence of H. armigera also varied greatly with concentration tested89.

Gallic acid: Apart from antibiotic effect, synergistic interactions involving gallic acid have been observed to increase the effect of Bt endotoxin on H. armigera90.

Proteinase inhibitors: Plants have a wide array of defence proteins, including the proteinaceous proteinase inhibitors (PIs) and lectins induced in response to insect attack. PIs inhibit the gut proteinase of the insect which adversely affects the protein digestion in the gut and forces the insect to synthesize alternative proteases to compensate for the inhibited activity. This leads to deficiency of essential amino acids and exhibits physiological stress on the insect, leading to growth retardation. This mechanism of action minimizes the possibility of developing resistance in the insect and reduces crop damage. A direct proof of the protective role of PIs against insect herbivores was provided by Hilder et al.91, who showed that the transgenic tobacco plants expressing cowpea trypsin inhibitor (T1) were resistant to the tobacco budworm, Heliothis virescens.

Lectins: These are proteins having affinity for specific carbohydrate moieties. They bind to glycoprotein in the peritropic matrix lining of the insect midgut to disrupt the digestive processes and nutrient assimilation. A lectin from snowdrop (Galanthus nivalis), when expressed in transgenic tobacco and potato, has been found to be toxic to aphids92.

α(1,α) amylase inhibitors: The common bean (Phaseolus vulgaris) contains a family of seed-related proteins
called α-amylase inhibitors (A1). A1 forms a complex with certain insect amylases and is supposed to play a role in plant defence against insects. The introdution and expression of the bean alpha A1 in pea confers resistance to bruchid beetles. For example, bruchids such as Zabrotes can feed on plants producing A1 because they possess a serine proteinase able to cleave some kind of A1. It is therefore difficult to evaluate the long-term benefits of the expression of these genes in plants.

**Insect chitinase**: Chitin is an insoluble structural polysaccharide that occurs in the exoskeleton and gut lining of insects. It is believed to protect the insect against water loss and abrasive agents. Dissolution of chitin by chitinase (either from insects themselves or from fungi) is known to perforate peritrophic matrix and exoskeleton and make insects vulnerable to attack by different pathogens. Expression of cDNA for chitinase obtained from the tobacco horn worm, Manduca sexta in tobacco plants offered partial protection against H. virescens.

**Bt toxic gene as an attractive candidate for transgenics**

Most natural plant defences against insects such as alkaloids, tannins and terpenes are products of complex metabolic processes. These multi-enzyme pathways would be difficult to engineer into new plant species when compared to Bt endotoxin, which is comparatively straightforward to introduce into plants. There are non-Bt proteins which interfere with nutritional needs vis-à-vis development of the insect, e.g. PLs and amylase inhibitors and chitinase. In spite of small size, abundance and stability of the PL insects have proven to be flexible enough to alter the proteinase composition in their midgut to overcome the inhibitor produced by transgenic plants. So also the case with chitinase, though its target is the PM, which is the main internal defence barrier; continuous regeneration of PM by certain insects scored this with limited success. Similarly, expression of four gene complexes from P. luminescens may pose complications for their expressions in plant cells. With regard to lectins having insecticidal property due to their ability to stoichiometrically bind to glycosylate proteins of the insect midgut, chronic exposure to relatively high amounts is needed for insecticidal activity, whereas in the case of Bt, LC₅₀ ranges from only 50 to 500 ng/ml of diet. Hence, Bt endotoxins are particularly attractive candidates for genetic engineering. Bt toxins are extraordinarily lethal to certain pests, e.g. based on quantities used in agricultural applications, molecules of Bt toxin are 80,000 times more potent than organophosphates and 300 times more potent than pyrethroids. Moreover, Bt causes rapid cessation of pest feeding (called anorexiant effect) thereby limiting further crop damage.

**Insect viruses**

Insect baculoviruses have a genome of double-stranded, covalently closed circular DNA of approximately 100-130 kilobase pairs (kbp). This is packed into rod-shaped nucleocapsids, which are enveloped by a lipid–protein membrane to form the virion. Based on the structural criteria, the family of Baculoviridae is divided into three subgroups. Sub-group A consists of nucleopolyhedroviruses in which virions are occluded with an intra-nuclear paracrystalline matrix formed by the single viral-encoded polyhedrin protein called polyhedrin. The polyhedral protein has a molecular weight of 29 kDa and is highly conserved among different baculoviruses. These occlusions called polyhedra are bigger (0.5–5 microns) in size with silicaceous framework which protects the virus from environmental hazards such as UV rays and desiccation. They have a unique bi-phasic life cycle (differing from most of the other DNA animal virus groups) which involves the temporarily regulated expression of two morphologically and functionally different viruses, i.e. they differ in their protein composition, morphology and tissue tropism and the roles in the viral life cycle. But genetically similar viral phenotypes are found, viz. budded virus or extracellular virus in the first phase and occluded virus in the later phase of the infection. The primary and natural infection starts when the insect ingests polyhedra-contaminated plants. The occlusions or polyhedra dissolve in the alkaline environment of the insect gut juices and by some enzymatic degradation, release the virions, i.e. polyhedral-derived virus (PDV) that invades and replicates in the midgut epithelial cells, especially in the columnar epithelial cells of the insect midgut by fusion with microvilli. They are responsible for the horizontal transmission from insect to insect under field condition (Box 3).

The study on the response of insects towards viral invaders has not been extensive and little has been known regarding insect antiviral immunity. Four virus diseases of the silkworm are known: nucleopolyhedrovirus (NPV), cytoplasmic polyhedrovirus (CPV), infectious flacherie (IFV), and densovirus (DNV). The NPV infects various tissues and multiplies in the nucleus forming occlusion bodies called polyhedra, which occlude virus particles. CPV infects the midgut epithelium and multiplies in the cytoplasm of columnar cells forming occlusion bodies which occlude the virus particles. IFV infects the midgut epithelium and multiplies in the cytoplasm of goblet cell without forming occlusion bodies. The DNV infects the midgut epithelium and multiplies in the nucleus of columnar cells. As early as 1936, the digestive juice of Bombyx mori has been known to have antiviral properties. Recently, advances have been made which demonstrate viral resistance and the ability of an insect to clear viral pathogens. Baculovirus resistance has been observed in inbred insect populations in the laboratory, but
whether it will occur extensively to compromise the application of these viruses against field populations remains to be seen. Few field studies have addressed this problem. After a granulosis virus epizootic in *Eucosma griseana* populations, there was an increase in the LD$_{50}$ and in the regression slope for this insect the year after the epizootic, which suggested that the epizootic affected the susceptible population and the residual populations did not show true resistance. Resistance to baculoviruses has been observed in field populations of *Spodoptera fragiperda*. At the beginning of the season, the larvae are susceptible to the NPV, but later in the season, there is a trend towards reduced susceptibility and increased heterogeneity after exposure to the virus.

As early as 1959, Drake and McEven reported phagocytosis of polyhedral occlusion bodies of NPV of *T. ni*. Similarly, Wittig has reported substantial percentage of haemocytes (up to 20% of total circulating blood cells) taking part in phagocytizing the polyhedral bodies of NPV of *P. unipuncta*. Generally, insect haemocytes are thought to play a central role in baculovirus pathogenesis by amplifying virus and disseminating infection. However, haemocytes have been implicated in countering disease progression by way of encapsulating and melanizing virus-infected treacheal elements instead of amplifying them.

**Developmental resistance**

Developmental resistance to NPV infection typically increases with the larval age in the case of *H. armigera*. Traditionally, this developmental resistance has been attributed to an increase in biomass. Recently, it was shown that active sloughing of the midgut may play a more important role in the developmental resistance than the increased biomass. The strongest evidence that accelerated midgut cell sloughing is involved in the decreased incidence of polyhedrosis virus disease in insect fed with cotton, was generated by the addition of stilbene optical brighteners such as M2R, which block sloughing of infected primary target cells in the midgut, thereby countering developmental resistance and increasing mortality. Further in the case of *H. zeae*, which is a highly refractory host to AcMNPV, Washburn et al. allowed an ichneumonid parasite, *Cameloletis sonorensis* before they orally inoculated them with AcMNPV – hsp 70/ lacZ. Then they subsequently compared lacZ expression in these caterpillars with unparasitized control insects, thereby indicating the cellular immune response. The above study is the first of its kind from the insects that implicates the haemocyte encapsulation response in the clearance of viral pathogen and also suggests a possible strategy whereby the baculoviruses can be genetically manipulated to become a more efficacious biopesticide by way of increasing functional host range of baculovirus.

**Molecular mechanism of resistance**

Insect DNA viruses have evolved methods to bypass the defence mechanism posed by insect cells either by way of blocking the cellular apoptosis as a part of their invasion strategy or by evolving unusual strategy to circumvent apoptosis, as follows:

**Occluded baculovirus:** Insect baculoviruses like NPV, GV and other DNA viruses of insects evolved methods apparently to bypass the defence phenomenon of apoptosis by directly blocking this response with the possession of *p35* and *iap* genes, thereby monitoring their own survival by suppressing apoptosis of host cells.

**PDV:** Recently, Strand and Pech, while studying the mechanism underlying the immune suppression of *Pseudoplasia includens* for the parasitization of *Microplitis demolitor*, found that M. demolitor PDV induced apoptosis in granular cells with characteristic condensation of chromatin, cell-surface blebbing and fragmentation of DNA into a 200 bp ladder. Thus, the M. demolitor PDV promotes its own survival by inducing apoptosis of host immune cells which would otherwise kill the developing M. demolitor egg.
Ascoviruses: These are a new group of viruses that cause a chronic but a fatal disease in lepidopteran larvae. As the disease advances, the host cell is divided into membrane bound vesicles or ‘sacs’, containing a large number of virions, formed by cleavage of infected host cells that accumulate in the haemolymph, imparting an opaque white colour. The process of vesicle formation and the nature of vesicles themselves strongly resemble apoptosis and apoptotic bodies. These viruses are vectored by parasitoid wasps during oviposition, and the vesicle possibly represents one infectious form of the virus. Thus, ascovirus may have developed an unusual strategy for circumventing apoptosis using a part of its replication pathway.

Non-occluded virus: The non-occluded baculoviruses (NOB) do not produce occlusion bodies at any stage in their reproductive cycle. The type species of NOB is the Heliothis Hz-1. NOBs like Hz-1 have evolved yet another strategy unlike that of other occluded baculoviruses, ascovirus or PDV to respond to cellular apoptosis. In a typical wild baculovirus infection, polyhedron occlusion bodies are made and are usually visible during the late phase of infection which initiates between 18 and 24 h post infection. On the other hand, AcMNPV mutant vAcAnh induces apoptosis around 9–12 h after infection. Whereas in the case of non-occluded Hz-1, it is able to complete its replication rapidly by about 12 h post infection, before apoptosis occurs. Thus, it is able to circumvent apoptosis efficiently.

Granulovirus – enhancer

Enhancers, which are metalloproteinase, are found in certain granulovirus occlusion bodies and have the ability to enhance the infection of some NPVs by way of degrading insect intestinal mucin (comparable to vertebrate mucin), a major protein constituent of the internal anatomical barrier, viz. PM.

Application: The ability of the T. ni GV enhancer, as well as S. litura GV enhancer to enhance the NPVs of A. californica and Mythimna separata respectively, to several folds has been demonstrated.

Entomopoxvirus – fusolin

Fusolin, which are proteins that are found in certain entomopox viruses (EPVs), have the ability to enhance NPV infection through enhanced fusion of polyhedron-derived virus with the microvillus membrane. Further, fusolin proteins that are found in spindles of entomopox virus have the ability to enhance NPV infection due to the greater number of NPV virions reaching the microvilli of the midgut susceptible to NPV, since the fusolin proteins present in the spindles lead to the disintegration of the PM, which in turn acts as a barrier against NPVs, has also been suggested as a mechanism of enhancement of EPV spindles towards NPVs.

Mechanism: Fusolin shows a distant similarity to bacterial chitin-binding protein. Chitin being a component of the PM, the disintegration of the PM may possibly be due to the disruption by the proteins of the formation of the PM by binding the chitin instead of chitin binding PM proteins in the midgut, thereby making more NPVs to reach the microvilli easily and become fused to the microvilli due to fusolin.

Application: Recently, the ability of fusolin protein found in the entomopoxvirus of Anomala cuprea and H. armigera to enhance the NPVs of B. mori and M. separata respectively, to several hundred and thousand folds, has been shown.

Entomopathogenic nematodes

The mode of action of entomopathogenic nematodes along with their associated mutualistic bacterium has been depicted (Box 4). Several behavioural defence mechanisms exhibited by certain host insects like Papilio japonica by way of aggressiveness, grooming with legs and mouthparts against entomopathogenic nematodes, have been already discussed. The production of certain immune inhibitors by the mutualistic bacteria of Xenorhabdus and Photorhabdus present in certain Steinernematids and Heterorhabditids of entomopathogenic nematodes to destroy certain antibacterial proteins like cecropins and attacins, thereby keeping the cadaver free from purification by saprophytic microbes, has been shown to be an effective strategy developed by symbiotic microbes against insect defence mechanism.
Conclusion

In eukaryotes, the genome projects of various species have been vigorously pushed forward. Genome sequences have been completed in *Escherichia coli*, *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Oryza sativa*, *Arabidopsis thaliana* and *Drosophila melanogaster* and those of humans, *Homo sapiens*, including some of the human pathogens like *Mycobacterium tuberculosis* (Table 4). On the other hand, development of the genetics of lepidopteran insects to which most of the crop pests belong, has lagged far behind, except *B. mori* which again is an useful insect. Though the genome of the silkworm is well studied (530 million base pairs long and having 28 chromosomes), not much work has been carried out on other lepidopteran crop pests. However, nearly eleven baculovirus genomes have been characterized (Table 5). Further, the crop gene pool may be under direct human control, but the pest gene pool is not so. It is less widely appreciated, however, that the application of molecular genetics to the pest species themselves will be just as important as *Bt* products and baculoviruses, or as a matter of fact, any other insect pathogens to fulfill their promise. So, a better understanding of the current knowledge of insect molecular genetics and molecular basis of the insect biochemical and cellular and humoral defence mechanism pave the way for the proper management of the pests, especially using various biocontrol agents like parasitoids and pathogens after understanding their strategy in by-passing the insect defence. Future possibilities are plenty by way of cloning and expressing some of the antibacterial proteins under insect baculovirus expression system not only for developing effective biocontrol agents but also for the control of certain insect vectors which carry parasites of certain human diseases by way of transforming the insects themselves.

So, a better understanding of insect haematology and molecular basis of the insect biochemical and cellular defence agents invading parasites and pathogens is important for better management and efficient utilization of various entomopathogens. In the case of insect viruses, as it is evident from the above discussion, the cellular defensive strategy, namely apoptosis by the host cell and insect DNA viral offensive strategy posed by the virus with the possession of *p35* gene, which inhibit apoptosis, are co-evolved. Hence an understanding of molecular genetics of both host and pathogen is important so as to know which genes play what role in which tissue of which species. Thus, discussing insect defences both at cellular and organism levels will provide information necessary to control or modify host range properties of certain insect viruses in future.

Finally, the striking similarities on a cellular and biochemical level in insect haemolymph clotting/coagulation and vertebrate blood clotting indicate these processes are evolutionarily conserved. Recent findings have demonstrated that insects and humans share homologous molecules in controlling non-self recognition and the expression of immune proteins, especially to bacterial invaders as it is evident from the control of Chagas disease (Malaya disease) with antibacterial proteins like cecropins from insect origin. The recent development of transgenic rice plant carrying *B. mori* cecropin B gene fused with signal peptide sequence of chitinase showed strong resistance to *Xanthomonas oryzae pv oryzae* and *Pseudomonas syringae pv oryzae*, which are the casual agents for bacterial leaf blight diseases of rice. Recently, Chang et al. have improved *A. californica* NPV by way of generating a recombinant baculovirus that produces occlusion bodies incorporating the *Bt* insecticidal cry1Ac toxic protein, thereby increasing the speed of action and pathogenicity by dual mode of action. Thus, the understanding of insect immunity will assist in further exploitation of various insect pathogens. (The more we understand insect immunity, the more we will understand how our own immune system has evolved and how it functions, leaving the investigations of the immune response of insects that assist in further exploitation of various insect pathogens.) Hence, the need of the hour is ‘back to basics’ approach to initiate more studies on lepidopteran pests along with their associated entomopathogenic microbes.

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