Genetic Improvement of Biocontrol Agents for Sustainable Pest Management

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Abstract

Genetic improvement involves directed purposeful genetic alterations to enhance the efficacy of natural enemies for biological control. This may be achieved by conventional approaches as well as through recombinant DNA techniques. The conventional methods include strain selection, serial passage through hosts, mutation, conjugation, transduction, selective breeding, hybridisation, etc., whereas the genetic engineering approaches involve gene transfer utilising various methods. Entomophagous insects may be improved for climatic tolerance, sex ratio, host-finding ability, host preference, increased host range, increased pesticide resistance, etc. The main objectives in genetically altering microbes are to increase host range, virulence and persistence. The *cry* genes from *Bacillus thuringiensis* have been cloned and expressed in a wide variety of organisms (baculoviruses to cyanobacteria) as well as in plants in attempts to improve their delivery and efficacy against insect pests. Apart from *B. thuringiensis*, binary toxin from different *B. sphaericus* strains has been expressed in different hosts like *Escherichia coli*, non- or low-toxic *B. sphaericus* and crystal minus *Bt israelensis* as well as in *Caulobacter crescentus* or cyanobacteria *Anabaena* sp. Insect viruses, especially baculoviruses, are mostly specific viruses which can replicate only in hosts. The recombinant DNA technology has its current applications in inserting foreign genes into insect baculoviruses and achieving their rapid and efficient expression in the recipient host systems. Candidate genes for hyperexpression in the baculoviruses include those encoding insect-specific enzyme genes (juvenile hormone esterase gene), hormone genes

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K. Sahayaraj (ed.), *Basic and Applied Aspects of Biopesticides*,
DOI 10.1007/978-81-322-1877-7_15,

(eclosion hormone gene, diuretic hormone gene) and insect-specific foreign toxic genes (scorpion venom toxin genes, predatory mite toxin gene, predatory spider toxin gene, parasitic wasp venom gene and *Bt* δ-endotoxin genes). It is worthwhile to mention that genetic engineering of BCAs is potentially very promising and has led to the development of more effective entomopathogens with desired pathogenicity, virulence, broad host range and persistence, providing a valuable tool for sustainable pest management.

Keywords
Biological control • Entomopathogens • Genetic improvement

15.1 Introduction

The global population in 2011 has crossed the seven billion mark and is projected to increase to 9.6 billion in 2050. The population of developing countries would increase from 5.7 billion in 2011 to 8.3 billion in 2050 (PRB 2011). Obviously, escalating human population especially in developing countries necessitates augmentation in food, feed and fibres and that too from a limited agricultural land base. The average crop loss resulting from animals (mostly insects), diseases, viruses and weeds has been reported as 32.1 % of the potential production of all crops with a higher percentage of 40 % for potato and the lowest being 26 % for soybean (Oerke 2006). But losses are comparatively higher in tropical and subtropical areas. India loses about 30 % of its crops due to various insect pests and diseases each year resulting in estimated annual revenue losses of Rs. 100,000 crores (Anonymous 2009). Synthetic pesticides, as major agro-inputs and integral part of modern crop-management practices, have significantly contributed to the improved agricultural production in the country by minimising yield losses. However, the indiscriminate and broadly unscientific application of a number of different recalcitrant synthetic chemicals to control insect pests during the last four decades has led to many ecological backlashes. These include emergence of high levels of pesticide resistance in many pest species, environmental toxicity, fishery losses, ground water and surface water contamination, depletion of rhizosphere microflora, food safety hazards and human health concerns (Shetty and Sabitha 2009; Singh 2012). Besides growing public concern over potential health hazards of synthetic pesticides in the management of insect pests, a steep increase in cost of cultivation due to high cost of these chemicals is also major concern. Due to this, there is renewed interest in eco-friendly approaches to pest management, and rationale for biological control of insect pests is irrefutable (Koul 2009; Arora et al. 2012).

Biological control refers to the destruction or suppression of undesirable insect pests by the introduction, encouragement or artificial increase of biological control agents (BCAs) that include both macro- (predators, parasites, parasitoids) and microorganisms. This control based upon naturally derived pest control processes that are more easily biodegradable, more target specific and eco-friendly is argued to be preferable to conventional chemical pesticides. Natural (biological control) is constantly active in all world terrestrial ecosystems and keeps most of the potential arthropod pests under control. Most of the potential arthropod pests (95 %, 100,000 arthropod species) are under natural control; all other control methods used today are targeted at the remaining 5,000 arthropod pest species (Van-Lenteran 2008). The three basic strategies for the exploitation of biocontrol agents in insect pest management include importation or classical biological control, augmentation and conservation. Classical biological control involves intentional introduction of exotic usually co-evolved biocontrol agents to control a pest in an area where it has

been accidentally or speciously introduced. Conservation biological control refers to modification of the environment or existing practices to protect and enhance natural enemies or other organisms to reduce the effects of pests. Augmentation involves two approaches, viz. inoculative and inundative releases, designed to increase the population of biocontrol agents by mass multiplication and periodic releases. These approaches have different applications depending on the adaptability of natural enemy and environment in which they must operate (Eilenberg et al. 2001). Classical biological control is applied on 350 million hectares, which is about 8 % of land under culture, and has very high benefit-cost ratios of 20–500: 1. However, augmentative, commercial biological control is utilised only on 16 million hectares, which is 0.4 % of land under culture, and has a benefit-cost ratio of 2–5: 1, which is similar to or better than chemical pest control (Van-Lenteran 2008). The main reason for the limited success achieved with augmentative biological control is that biocontrol agents suffer from a number of important limitations, viz. narrow spectrum of activity, susceptibility to adverse environmental stress and slow speed of kill. However, recent advances in molecular biology techniques have offered opportunities to enter into a novel realm in overcoming these limitations through genetic improvement of biocontrol agents. It has broadened available techniques for genetic manipulation for diverse traits in species of interest (Atkinson et al. 2001; Kramer 2004).

15.2 Genetic Improvement of Arthropod Natural Enemies

Genetic improvement involves directed purposeful genetic alterations to enhance the efficacy of biocontrol agents for biological control. It may be achieved by artificial selection, hybridisation to achieve heterosis effects or use of recombinant DNA techniques. Biotechnological interventions can offer opportunity to improve beneficial arthropods for climatic tolerance, sex ratio, host-finding ability, host preference, increased host range, increased pesticide resistance, etc. There is tremendous scope for developing natural enemies with genes for resistance to pesticides and ability to withstand adverse weather conditions (Hoy 1992). However, genetic improvement of arthropod natural enemies has received little attention because of the concern that breeding and prolonged rearing under artificial condition would necessarily result in laboratory-adapted strains that would perform poorly in the field. Biotechnological approaches can be helpful in understanding the genetics and physiology of reproduction and control of sex ratio in natural enemies, which can be used to improve their rearing for biological control (Sharma 2009).

15.2.1 Climatic Tolerance

Genetic transformation can be exploited to augment tolerance to extreme hot or cold conditions in arthropod natural enemies. The superior strains of *Trichogramma chilonis* Ishii, an important egg parasitoid of lepidopterans have been developed by selection technique for adaptation to high as well as low temperature regime at National Bureau of Agricultural Important Insects (NBAII), Bangalore. The strain adapted to high temperature regime can be utilised in temperatures above 35 °C. This strain has been found to give increased longevity and parasitism at 36 °C and 60 % relative humidity. High temperature-tolerant strain of *T. chilonis* is useful against various insect pests of sugar cane, cotton and vegetable crops during hot months (Singh 2003). This strain has also proved superior in reducing the incidence of sugar cane stalk borer, *Chilo auricilius* (Dudgeon), and early shoot borer, *Chilo infuscatellus* (Snellen), as against local strain under Punjab conditions (Singh et al. 2007a, b). Similarly, low temperature-adapted strain, developed through selection for 30 generations under laboratory conditions at 18–24 °C, has shown better host searching ability and can be successfully exploited under low temperature agrosystem (Jalali et al. 2006a).

15.2.2 Tolerance to Insecticides

Insecticide tolerance is a key desirable trait for parasitoids and predators which can be genetically improved for use in integrated management programmes. Phytoseiid mite, *Metaseiulus occidentalis* (Nesbitt), the most important predator of spider mites in orchards and vineyards in the USA, has been improved through artificial selection to pesticide-resistant strain called COS strain (carbaryl-OP-sulphur-OP-resistant strain) and has also been documented in its field effectiveness. It acquired resistance to organophosphorus insecticides such as azinphos-methyl, diazinon and phosmet through selection in orchards and vineyards. Resistance to sulphur was also discovered in native California vineyard populations. To augment this naturally acquired resistance, *M. occidentalis* was artificially selected in the laboratory with permethrin and carbaryl and then selected to obtain multi-resistant strains. This laboratory-selected strain of *M. occidentalis* successfully met the criteria for a field release. The carbaryl-OP and permethrin-OP strains have also established in apple, pear and almond orchards in California, Oregon and Washington in the USA. The resistant strains were released in the field and multiplied, overwintered and survived pesticide application in the field. Mass rearing and commercial releases of this strain in California almond orchards have resulted in annual savings of about $ 21,56,000 to the almond growers alone (Hoy 1990). It has been reported that mortality of first instar of common green lacewing, *Chrysoperla carnea* (Stephens), selected in the laboratory for resistance to carbaryl decreased from 98 to 10–20 %. Further, tests using the oxidase inhibitor piperonyl butoxide and the esterase inhibitor phenyl saligenin cyclic phosphonate suggested that both oxidase and esterase enzymes contribute to the resistance (Grafton-Cardwelu and Hoy 1986). A parasitoid, *Aphytis melinus* DeBach, of the California red scale, *Aonidiella aurantii* (Maskell), has been selected successfully for resistance to carbaryl (Rosenheim and Hoy 1988). Similarly, *Trioxys pallidus* Haliday, an effective parasitoid of the walnut aphid, *Chromaphis juglandicola* Kaltenbach, has been selected for resistance to azinphos-methyl (Hoy and Cave 1991).

An endosulfan-resistant strain of *T. chilonis* termed as 'endogram' has been developed by sequentially exposing adult parasitoids to various concentrations of endosulfan (0.004–0.09 %) for 341 generations. The tolerant strain was found to parasitise 56 % of *Helicoverpa armigera* (Hübner) eggs immediately after insecticide spray as compared to 3 % by susceptible strain (Jalali et al. 2006b) in net house potted cotton plants. Endogram strain has been further improved for multiple resistance to monocrotophos and fenvalerate to obtain multiple insecticide resistance strain through selection process for over 72 generations. Field efficacy of this resistant strain has shown higher parasitism in *H. armigera* eggs on tomato by 260.4 % as compared to susceptible strain under sprayed conditions (Jalali and Venkatesan 2011). In Punjab, 11–13 releases of this multiple insecticide-resistant strain of *T. chilonis* at 1,50,000 per ha at weekly interval during July to October along with 6–9 insecticide applications have been reported to be effective for the control of cotton bollworms and resulted in increasing egg parasitism (Brar et al. 2007). Similarly, a strain of *Chrysoperla zastrowi arabica* Henry et al. (PTS-8) having tolerance to endosulfan, acephate and fenvalerate has been developed. PTS-8 acquires higher activity of detoxifying enzymes (esterase and glutathione S-transferase activity) as against susceptible population. It has been found to be effective against sucking pests of cotton under pesticide sprayed conditions (Venkatesan et al. 2011). These strains could be useful biocontrol agents for the suppression of insect pests in different crop ecosystems.

It will be desirable to select pesticide-resistant strains of natural enemies to overcome the problems caused by the use of pesticides (Dhaliwal and Arora 2006). However, concerns have been raised that insecticide resistance also may confer fitness disadvantages (Georghiou and Taylor 1977) that would reduce the stability of the trait within field populations in the absence of selection pressure. Carbaryl resistant strain of

C. carnea, when reared in the absence of carbaryl exhibited lower larval and pupal survival and produced fewer females than the colony from which it was derived. However, its fecundity was significantly higher and adult longevity slightly higher, which compensated in part for reduced survival of immature stages (Grafton-Cardwelu and Hoy 1986). Conversely, the COS strain of *M. occidentalis* exhibited life table parameters comparable to those of other strains, suggesting that the reproductive attributes of this predator were not altered as result of artificial laboratory selection (Bruce and Hoy 1990). Spollen and Hoy (1992) measured relative fitness components for a genetically improved strain of *Aphytis melinus* DeBach with increased resistance to carbaryl. Except for a difference in progeny sex ratio, developmental parameters were not significantly different between the resistant and susceptible strains. Baker et al. (1998) reported no fitness costs associated with malathion-resistant strain of the solitary parasitoid, *Anisopteromalus calandrae* (Howard), parasitising on immature rice weevils, *Sitophilus oryzae* (L.), in stored wheat.

In addition to selection, the resistance genes with potential importance have also been cloned through biotechnological interventions. These include cyclodiene resistance gene ($GABA_A$) from *Drosophila*, a cytochrome P450-B1 gene (*CYP6A2*) associated with DDT resistance in *Drosophila*, parathion hydrolase gene (*opd*) from *Pseudomonas diminuta* Leifson and Hugh and *Flavobacterium*, *β-tubulin* genes from *Neurospora crassa* (Draft) and *Septoria nodorum* (Berk.) conferring resistance to benomyl, acetylcholinesterase gene (Ace) from *Drosophila melanogaster* Meigen, glutathione S-transferase gene (*GST*) from *Musca domestica*, esterase B1 gene from *Culex* responsible for resistance to organophosphates (Atkinson et al. 2001) and a cytochrome P450-B1 gene (*CYP6F1*) from deltamethrin-resistant *Culex pipiens pallens* (Gong et al. 2005).

15.2.3 Tolerance to Multiple Traits

A strain of *T. chilonis* has been developed for tolerance to multiple traits known as MITT (multiple insecticide and temperature tolerant) strain for effective control of the pest even in harsh climatic conditions and under high insecticide pressure in different economically important crops. This strain has showed tolerance to three major groups of insecticides which include endosulfan (organochlorine), monocrotophos (organophosphate) and fenvalerate (synthetic pyrethroid) and to high temperature (32–38 °C) through selection for 81 generations. Increase in parasitisation from 35 to 90–95 % and decrease in mortality from 100 to 57–70% after 6 h of constant exposure to three insecticides and high temperature have been reported (Kumar et al. 2008).

15.2.4 Altered Biological Traits

Altering different biological traits such as enhancing fecundity, shortening developmental period, sex ratio, host or habitat preferences could enhance the effectiveness of biological control agents (Hoy 1976). Altering longevity of certain arthropods might be beneficial, and research on mechanisms of ageing may provide useful genes in the future (Sharma 2009). NBAII, Bangalore, has developed superior strains of *T. chilonis* such as Bio SC1 for graminaceous tissue borers, Bio H3 for *H. armigera* and Bio C1 for cotton bollworms. These strains are 60–100 % more prolific than the previously used strains. Hybridisation has also been attempted as a method for increasing vigour or fitness through heterosis. It was observed that the developmental rate, diapause attributes, sex ratio and parasitisation rate of gypsy moth parasite *Apanteles melanoscelus* (Ratzeburg) in the insectary were improved through hybridisation (Hoy 1975). However, limited evaluation conducted with hybrid strain did not indicate that it was more effective than parent strains under field conditions.

15.3 Genetic Improvement of Entomopathogenic Microbes

The entomopathogens have attained a special status in biopesticide umbrella and have the potential to provide economically viable and

environmentally safe alternative to chemical pesticides in many ecosystems. However, concerns related to their susceptibility to adverse environmental stress, less persistence under field conditions, limited host range, slow activity, deprived quality and limited storage stability have restricted their widespread use commercially. The main objectives in genetically altering microbes are to make them more effective by increasing host range, virulence and persistence, adapting to extreme temperature conditions and overcoming resistance. For the first time, cloning and expression of *Bacillus thuringiensis* (Berliner) *delta*-endotoxin gene in *Escherichia coli* Migula demonstrated the potential of genetic engineered technology for microbial control in the early 1980s. Based on selection, hybridisation, mutagenesis and DNA recombinant technology, microbial agents have now been genetically engineered for a number of traits.

15.3.1 Entomopathogenic Bacteria

Most of the insect pathogenic bacteria occur in the orders Bacillales (Bacillaceae, Paenibacillaceae), Lactobacillales (Enterococcaceae, Streptococcaceae), Enterobacteriales (Enterobacteriaceae), Pseudomonadales (Pseudomonadaceae), Neisserales (Neisseriaceae), Rickettsiales (Rickettsiaceae, Anaplasmataceae) and Entomoplasmatales (Spiroplasmataceae) (Jurat-Fuentes and Jackson 2012). Of these, the pathogen that has received special attention and attained considerable success is the Gram-positive spore-former and crystalliferous *B. thuringiensis* which accounts for nearly 90 % of the sale of bioinsecticides. Other bacterial pathogens do exist, but only few have been successful pest control agents such as *Lysinibacillus sphaericus*, *B. cereus*, *Paenibacillus popilliae*, *P. lentimorbus*, *Xenorhabdus* spp., *Photorhabdus* spp. and *Serratia entomophila* enumerating their growing commercial importance.

15.3.1.1 *Bacillus thuringiensis*

Bacillus thuringiensis (*Bt*) is a motile, Gram-positive spore-forming phylloplane and soil-inhabiting bacterium and, in addition to endospores, produces a proteinaceous parasporal crystal in the sporangium at the time of sporulation. Due to its remarkable activity against a wide range of nefarious insect pests, *Bt* has been extensively studied and excellent reviews are available on its insecticidal properties (Baum 1998; Glare and O'Callaghan 2000; Nester et al. 2002; Sanchis 2011; Jurat-Fuentes and Jackson 2012). The ability to identify and clone *Bt* cry genes and the characterisation of the specific activities of individual cry proteins, as well as the availability of recombinant DNA technology, led to the development of new strategies for improving the exploitation of *Bt* for increasing its entomopathogenic potential. The first step towards improving *Bt* strains naturally involved the isolation of new strains with new or higher insecticidal activity against targeted insect pests. Until the 1970s, it was generally accepted that lepidopteran insects (moths and butterflies) were the only targets of *Bt*. In 1976, Goldberg and Margalit reported that a new *Bt* subspecies found in the Negev Desert, called *israelensis* (or *Bti*), killed mosquito and black fly larvae; both are from the order Diptera. This was the first documented case of a *Bt* strain killing an insect other than a caterpillar (Goldberg and Margalit 1977). The dipteran-active *Bt* subsp. *israelensis* was used extensively for vector control, particularly of black flies and mosquitoes, providing both medical and environmental benefits. In 1983, another new subspecies of *Bt*, subsp. *morrissoni* var. *tenebrionis*, was isolated (Krieg et al. 1983). This isolate, discovered in Germany, had excellent activity against the larvae of certain coleopteran species, and enhanced commercial development of this organism as a bioinsecticide. More recently, *Bt* crystal proteins were screened for activity against the free-living larval stages of nematode pests that infect animals and plants, and some of them were identified with significant activity in inhibiting larval development, thus demonstrating that the phylum Nematoda was also a target of *Bt* crystal proteins (Wei et al. 2003). To date, several thousand natural strains have been isolated from various geographical areas and from different sources, including grain dust, soil, insects and plants (Martin and Travers 1989; Smith and Couche 1991). These

Table 15.1 Commercial genetically engineered *Bt* products for pest control

Bt strain	Trade name	Company	Target insects
Kurstaki recipient, *aizawai* donor	Condor, Cutglass (transconjugant)	Ecogen	Lepidoptera
Aizawai recipient, *kurstaki* donor	Agree, Design, Turex (transconjugant)	Thermo Trilogy	Lepidoptera (*Bt*-resistant *Plutella xylostella*)
Bt kurstaki ED 7841	CRYMAX	Ecogen	Lepidoptera
ED7826	Leptinox (recombinant)	Ecogen	Lepidoptera
EQ7673	Raven (recombinant)	Ecogen	Lepidoptera, Coleoptera
delta-endotoxin encapsulated in *P. fluorescens*	MVO, MATCH, M-Trak (Cell cap®)	Mycogen	Lepidoptera, Coleoptera
Bt israelensis	Acrobe	Cyanamid	Diptera
Bt israelensis	Skeetal	Novo-Nordisk	Mosquito
Bt israelensis	Teknar, Teknar HPD	Zoecon	Black flies, mosquito
Bt israelensis	Vectobac G, Gnatrol, Bactimos	Abbott	Mosquitoes, black flies
Bt israelensis 187, CS-8	MieJueLing Preparation	Huazhong Agri. University	Diptera

Source: modified after Sharma (2009)

isolates have been classified into about 85 serotypes based on biochemical properties and flagellar antigens or H-antigens (de Barjac and Frachon 1990; Lecadet et al. 1999; Jurat-Fuentes and Jackson 2012), producing several hundred crystal proteins that are active against most orders of insects (>575 species) and some other invertebrates and recently, leukemic cells (Ohba et al. 2009). A full listing and nomenclature of *B. thuringiensis* toxins is maintained by *Bt* toxin Nomenclature Committee headed by Neil Crickmore at www.lifesci.sussex.ac.uk/home/Neil_Crickmore/*Bt*. It is also important to note that most *Bt* strains produce more than one type of crystal protein that can act in combination (Sanchis 2011). Detailed information on bioassays and range of activity for specific cry toxins is available in the *Bt* specificity database (www.glfc.cfs.nrcan.gc.ca/bacillus/bt search.cfm).

Amongst the critical milestones was the discovery that the genes coding for the toxin crystals were located on transmissible plasmids enabling exchange of genetic information between *Bt* strains (Gonzáles et al. 1982). This opened up the way to manipulation of genes, including transfer between *Bt* strains. Conjugation was used to develop strains with optimised activity against a given insect pest or strains with a broadened toxicity spectrum (Sanchis 2000). *Bt* toxin genes have been cloned and expressed in a wide variety of organisms (baculoviruses to cyanobacteria) as well as in plants in attempts to improve their delivery and efficacy against insect pests. Several commercial products and novel strains of *Bt* have been developed through biotechnological intervention (Table 15.1). Breeding strategies involving both conventional (conjugation, transduction, electroporation, classical mutation) and genetic engineering methods have been employed for *B. thuringiensis* improvement. Recombinant DNA technology offers the possibility of transferring specific genes from one cell to another giving rise to a new array of new products with desired characteristics. An important delivery system was the encapsulation of the *cry* genes in a non-pathogenic *Pseudomonas fluorescens* (Flugge). This approach has been used to produce two commercial products: MVP for controlling lepidopterans and M-Trak for controlling coleopterans in which bacterial cells are killed by means of a physical chemical process after

fermentation and the toxins remained enclosed in the cell wall of the dead microorganisms as crystalline inclusions. These two products were registered by US Environmental Protection Agency in 1991. The process results in an active, stable biotoxin encapsulated within nonviable cells. The microencapsulation of *Bt* crystal, i.e. biopacking by *P. fluorescens* cell wall, thus protects the endotoxin from environmental factors. This process significantly increased the efficacy of the Cry proteins, increasing their persistence in the environment by protecting them against degradation and inactivation by UV irradiation (Gaertner et al. 1993).

The development of transformation protocols (Bone and Ellar 1989) and the availability of *Bt* shuttle vectors with multiple cloning sites (Baum et al. 1990; Arantis and Lereclus 1991) have greatly advanced the potential to produce improved strains. The use of vectors containing *Bt* replication origins and promoter sequences to derive expression of the introduced toxin gene (Gamel and Piot 1992; Sanchis et al. 1996), and site-specific recombination systems (Baum et al. 1996), has allowed elimination of foreign DNA sequences in the generated strains to result in a non-transgenic product (Jurat-Fuentes and Jackson 2012).

Improvement in Host Range: The efforts to improve insecticidal activity have been based on the transfer of *Bt* genes into nonhomologous isolates of *Bt*, a means of combining *delta*-endotoxins to produce either additive or synergistic effects and thus expand the host range. A self transmissible *cry1A* plasmid was transferred via conjugation from *Bt aizawai* strain to *Bt kurstaki* to design the commercial product Condor® (EG2348) to target lepidopteran pests as it combined the insecticidal properties of both the parents. An *rDNA* modified variant (ECX9399) of EG2348, the active ingredient in the bioinsecticide Condor®, has also been developed, which showed superiority to its parent strain against fall armyworm *Spodoptera frugiperda* (Smith) on corn (All et al. 1994). Similarly, two commercial products Agree® and Design® were developed by the transfer of cry1 plasmid from *Btk* to *Bta* strains. A unique combination of coleopteran- and lepidopteran-active insecticidal crystal proteins Cry 1Ac and Cry 3A was achieved by conjugation of *Btk* with *Bt tenebrionis*. This commercial product Foil® had an expanded host range to control Colorado potato beetle *Leptinotarsa decemlineata* (Say) in potatoes and European corn borer *Ostrinia nubilalis* (Hübner) in the corn (Carlton and Burke 1993; Baum 1998). The cloned genes from *Bt tenebrionis* were introduced into *Bt israelensis*, thus increasing host spectrum. The resulting organism demonstrated activity against mosquitoes and beetles as expected, but also showed unexpected additional activity against *Pieris brassicae* Linnaeus, a property which neither parent strain possessed (Crickmore et al. 1990).

A new type of biocide GCSC-*Bt*A based on 'Germany-China Scientific Cooperation' research has been developed by conjugation of *delta*-endotoxin from *B. thuringiensis* with abamectin, a toxin of *Streptomyces avermitilis* (ex Burg et al.). The conjugated GCSC-*Bt*A biocide had a broader host spectrum and a faster killing speed than either the *Bt* crystal or abamectin alone for the control of agricultural pests (Liu and Sengonca 2003). GCSC-*Bt*A has been recommended for use in the integrated pest control programmes in the vegetable fields as it not only proved to be highly effective against *Plutella xylostella* (Linnaeus), *Myzus persicae* (Sulzer), *Phyllotreta vittata* Fabricius, *Tetranychus cinnabarinus* (Boisd.), *Frankliniella occidentalis* (Pergande), *Aphis fabae* Scopoli and *Cameraria ohridella* (Deschka & Dimić) but also displayed safety to some predators (Zhu and Sengonca 2006).

Improvement in Photostability and Activity: The use of *B. thuringiensis* as insecticide is limited in field applications because of the rapid inactivation of toxins and spores after exposure to sunlight (Pusztai et al. 1991). Ultraviolet light is mainly responsible for this inactivation because of its impact on cells by direct DNA damage (e.g. pyrimidine dimers, cross-linking with proteins) or by producing reactive oxygen-derived free radicals (Zhang et al. 2008). *B. thuringiensis* mutants producing melanin from *Btk* and other strains obtained by inducing mutagenesis utilising

ethyl methanesulphonate (EMS) were found to be more resistant to UV irradiation (Saxena et al. 2002) and also showed highly insecticidal activities against potato tuber moth, *Phthorimaea operculella* (Zeller) (Mohamed et al. 2010). Melanin is a natural product, easily biodegradable in nature, and has been found to be excellent UV-protective agent (Liu et al. 1993). *Bt tenebrionis* strain NB-125, a mutant strain NB-176, was developed by gamma irradiation which contains two to three copies of Cry 3A. The commercial product Novodor® (Abbott) based on this mutant strain was able to produce large thromboid crystals composed primarily of Cry 3A toxin thus enhancing the field activity (Gurtler and Peterson 1991).

Improvement in Activity Through Alternate Delivery System: The insecticidal activity of *B. thuringiensis* has also been improved by delivering the toxins to target insects through alternate means of delivery. *Bt* genes have been cloned and expressed in endophytic, epiphytic and/or aquatic bacteria as well as eukaryotic plants to improve their delivery and residual activity. The rationale for using live endophytic or epiphytic bacteria as hosts is to prolong the persistence of Cry proteins in the field by using a host that can propagate itself at the site of feeding and continue to produce crystal protein (Sharma 2009).

Endophytic Delivery of *Bt* Gene: Crop Genetics International (CGI) transferred *cry 1Ac* gene from *Btk* ND-73 into *Clavibacter xyli* var. *cynodontis* (Cxc), an endophytic bacterium, that colonises the vascular system of various plants including maize. The transgenic Cxc Incide® infects the internal tissues of growing plants, and gene introduced into this bacterium encoded a protein toxic to the larvae of the European corn borer (Lampel et al. 1994). Endophytic isolates of *B. cereus* have been used as hosts for *cry 2A* gene, and *B. megaterium* isolates that persist in phyllosphere have been used as host for *cry 1A* genes (Mahaffee et al. 1994; Bora et al. 1994). Plant colonising bacteria such as *P. fluorescens*, *P. cepacia* Burkholder, *Rhizobium leguminosarum* Jordan and *Azospirillum* spp. have also been used to produce and deliver *Bt* proteins (Udayasuriyan et al. 1995). Two endophytes, *Rhizobium meliloti* Dangeard and *R. leguminosarum* (Frank) (nodule-forming bacteria), were introduced with δ-endotoxin *cry 3A* gene from *Bt tenebrionis* for the control of two coleopteran insects, *Sitona hispidulus* (Fabricius) and *S. lineatus* (Linnaeus), respectively (Bezdicek et al. 1991). A strain RSI identified as *Bacillus* sp. has been isolated, which is capable of colonising in cotton leaves. It has been found to be an excellent coloniser of cotton phyllosphere. *Cry 1Aa* gene of *Bt* was introduced into it by conjugal transfer. It has been shown that the resultant transconjugant colonises cotton plants for prolonged period and also protects the plant from *H. armigera* attack for more than 30 days (Narayanan 2002).

Epiphytic Delivery of *Bt* Genes: The major problem with *Bt* toxins is that they are not stable in soil, i.e. their residual activity is very low. Thus microbes, which normally occur in close association with roots of the crop plants, can be used for engineering these bacteria to produce the appropriate *Bt* toxin(s). A cloned *Bt* endotoxin gene (*cry 1Ab*) has been expressed in the corn root colonising Gram-negative bacterium, *P. fluorescens*, to deliver lepidopteran-active toxin beneath the soil surface for root-feeding insects. The recombinant bacteria were subsequently killed by heat and iodine, and thus the pseudomonad cell protected the *Bt* protein from environmental degradation, thus providing longer residual activity. The symbiotic relationship between pigeon pea, *Cajanus indica* Adans and nitrogen-fixing bacterium *Bradyrhizobium* has also been utilised for improving delivery system for soil insects. Nambiar et al. (1990) expressed dipteran-active *cry 4D* gene from *Bt israelensis* into *Bradyrhizobium* spp. that nodulate pigeon pea. The transfer of plasmid by conjugative mobilisation into this species provided protection against root nodule damage by *Rivellia angulata* (Hendel) on pigeon peas. Similarly, cloned genes from coleopteran active *Bt tenebrionis* have been transferred to *R. leguminosarum* (nodule-forming bacteria) by conjugation. Pea and white clover plants showed reduced root and nodule damage by larvae of

Sitona lepidus Gyllen when inoculated with toxin gene containing *Rhizobium* strains (Skot et al. 1990).

Aquatic Delivery of *Bt* Genes: The major limitation of *Bt israelensis* used for mosquito control is the rapid sedimentation of the spores and insecticidal crystals as most of the larvae feed near the water surface. So alternate delivery system has been sought for increasing their persistence in aquatic environment by expressing the *Bt* toxin genes in the hosts such as *Caulobacter crescentus* Poindexter, cyanobacteria, *Agmenellum quadruplicatum* (Menegh.) and *Synechococcus* spp. that are found predominantly in regions at or close to the water surface where larvae of many mosquito species feed. *Cry 4B* toxin gene from *Bt israelensis* was cloned into the broad host range plasmid Prk 248 and was expressed in *C. crescentus* CB15 (a motile ubiquitous bacterium living in upper layer of aquatic habitat). The recombinant *Caulobacter* can provide the potential for prolonged mosquito control (Thanabalu et al. 1992).

Insertion of *Bt* δ-Endotoxin into Eukaryote Plants: Remarkable progress based on recombinant DNA technology has been made over the past last three decades for developing crops with novel genes for resistance to insects, plant pathogens and herbicides. Development of insect-resistant transgenic crops has mainly focused on the integration of bacterial genes encoding for the production of toxic proteins, especially from *B. thuringiensis*. To date, *Bt* genes have subjugated the commercial scene very influentially for the last 15 years, at least in cotton and corn crops. More than 150 cry toxins have been cloned and tested for their toxicity to various insect pests belonging to different orders (Crickmore et al. 2011). Several *Bt* genes encoding cry toxins have been introduced either alone or stacked with other *Bt* genes or with herbicide resistance genes in different crops for imparting resistance to different insect pests. The crops in which *Bt* genes have been inserted for producing insect-resistant transgenic crops include cotton, maize, brinjal, rice, sorghum, tomato, cabbage, cauliflower, sugar cane, chickpea, alfalfa, broccoli and poplar (Table 15.2).

15.3.1.2 *Lysinibacillus sphaericus*

Lysinibacillus sphaericus (syn. *Bacillus sphaericus* Meyer and Neide) is a highly heterogeneous species that contains both saprophytic and pathogenic species. It has attracted attention due to its pathogenicity to several mosquito species. A defining feature of this bacterium is the production of a spherical spore that is located in a terminal position within the swollen sporangium (Jurat-Fuentes and Jackson 2012). *L. sphaericus* spores are less sensitive to inactivation by UV radiation than other *Bacillus* spp. spores owing to high concentrations of small acid-soluble protein and DNA repair systems (Myasnik et al. 2001). The cloning and characterisation of binary toxin genes have been done from many *B. sphaericus* strains with different level of toxicity. Besides these, the *mtx* genes have also been detected in some toxic *B. sphaericus* strains. With the aim of expanding host range, increasing virulence and overcoming resistant colonies, the binary toxin from different strains has been expressed in different hosts like *E. coli*, non- or low-toxic *B. sphaericus* and crystal minus *Bt israelensis* as well as *C. crescentus* or cyanobacteria *Anabaena* sp. which naturally occurs in every aquatic habitat at or close to water surface.

Expanding Host Range: Cry 11A from *Bt israelensis* and Cry 11Ba from *Bt jegathesan* introduced, separately and in combination, into the chromosome of *B. sphaericus* 2297 by in vivo recombination resulted in recombinant strains toxic to *Aedes aegypti* (Linnaeus) larvae to which the parental strain was not toxic (Servant et al. 1999). It also overcame the resistance of *Culex pipiens* Linnaeus and *C. quinquefasciatus* Say to *B. sphaericus* strain 2297 partially. It has been reported that recombinant *E. coli* strain expressing a plasmid encoding a combination of mosquito-larvicidal genes from *Bt israelensis* (Cry 4A, Cry 4B and Cry 11A) and binary toxin genes from *B. sphaericus* exhibited broad range larvicidal activity against all *Aedes*, *Culex* and *Anopheles* larvae (Tanapongpipat et al. 2003). Recombinant *Bt israelensis* IPS-82/*B. sphaericus* 2362 showed high potency against 4th instars of *C. quinquefasciatus* and also improved efficacy

Table 15.2 Insect transgenic crops having *Bt genes* from *Bacillus thuringiensis*

Crop	*Bt* gene	Target pests
Cotton	*cry 1Ac, cry 1Ab/cry 1Ac, cry 1Ac + cry2Ab, cry1C*	*Helicoverpa armigera*, *Pectinophora gossypiella*, *Earias* spp., *Heliothis virescens*, *H. zea*, *Trichoplusia ni*, *Spodoptera* spp.
Rice	*cry 1 Ab, cry 1 Ac, cry 1Ab/cry1Ac, cry2a*	*Chilo suppressalis*, *Cnaphalocrocis medinalis*, *Scirpophaga incertulas*
Corn	*cry 1Ab, cry 9C, cry 3Bb, cry 1 F, cry 34Ab1/ cry 35 Ab1, cry 1 Ab + cry 3Bb, cry 1 F + cry 34Ab1/cry 35 Ab1*	*Ostrinia nubilalis*, *Chilo partellus*, *Busseola fusca*, *H. zea*, *Diatraea grandiosella*, *D. saccharalis*, *S. frugiperda*, *Diabrotica undecimpunctata howardi*, *D. virgifera virgifera*
Poplar	*cry 1Aa*	*Lymantria dispar*
Soybean	*cry 1Ac*	*H. virescens*, *H zea*
Sorghum	*cry 1Ac*	*C. partellus*
Sugar cane	*cry 1Ab*	*D. Saccharalis*
Groundnut	*cry 1Ac*	*Elasmopalpus lignosellus*
Chickpea	*cry 1Ac*	*H. armigera*
Tobacco	*cry 3, cry 2a5, cry 1Aa, cry 1Ab, cry 1Ac*	*H. virescens*, *Manduca sexta*, *H. armigera*, *H. zea*, *Leptinotarsa decemlineata*
Potato	*cry 3, cry 3a, cry 3b, cry 2a5, cry 1Ab, cry 1Ac9, cry 5*	*Leptinotarsa decemlineata*, *Phthorimaea operculella*
Tomato	*cry 1Ab, cry 1Ac*	*M. Sexta*, *H. armigera*
Brinjal	*cry 1Ac, cry 3b*	*Leucinodes orbonalis*, *Leptinotarsa decemlineata*
Chinese cabbage	*cry 1Ab, cry 1Ac*	*Plutella xylostella*
Broccoli	*cry 1C*	*P. xylostella*, *T. ni*, *Pieris rapae*
Alfalfa	*cry 1C*	*S. littoralis*
Canola	*cry 1C*	*H. zea*, *S. exigua*

Source: Modified after Sharma (2009), Dhaliwal and Koul (2010) and Gujar and Dhillon (2011)

against larvae of *C. tarsalis* than *Bt israelensis* IPS-82 and *B. sphaericus* 2362 alone (Park et al. 2005). Moreover, it also suppressed resistance to *B. sphaericus* 2362 in *C. quinquefasciatus*.

Enhanced Virulence: Isolation of two *B. cereus* strains, Ae10 and Cx5, from mosquito larval guts and transformation with a recombinant plasmid, pBS373, harbouring binary toxin genes from *B. sphaericus* 2297 showed very high toxicity against *C. quinquefasciatus* larvae (Luxananil et al. 2003). Recombinant *Bt israelensis* Bti IPS-82/*B. sphaericus* BsB showed high potency against fourth instars of *C. quinquefasciatus*, being 21-fold as potent as *Bt israelensis* and 32-fold as potent as *B. sphaericus* alone (Park et al. 2005). The expression of chitinase gene *chi Ac* from *B. thuringiensis* in *B. sphaericus* 2297 using binary toxin promoter yielded a recombinant strain that was 4,297-fold more toxic than strain 2297 against *C. quinquefasciatus* thereby synergising the toxicity of binary toxin and may be useful in managing resistance to *B. sphaericus* (Cai et al. 2007).

Caulobacter crescentus Poindexter naturally occurs in every aquatic habitat and is found predominantly in regions at or close to the water surface. The *bin* gene from strain 2297 and *mtx 1* gene from SSII-1 were separately linked with the broad host range plasmid pRK248 and then electroporated in *C. crescentus* CB 15. The resulting recombinants expressing binary toxin were very active to *Culex*. sp. with LC_{50} value of 2×10^5 cells/ml, which was similar to the toxicity of natural strain SSII-1 (Zhimming 2002). Like *Caulobacter*, various species of cyanobacteria are widely found near the water surface in both freshwater and saltwater environments. The effective expression of binary toxin in a single-cell cyanobacterium

Anabaena sp. 7120 resulted in recombinants with very high toxicity to targets and might be a potential agent for mosquito control with the persistence of one month (Xu et al. 2000).

Overcoming Resistant Colonies: Digestion of total genomic DNA from *B. sphaericus* LP1-G; ligation of purified products into vector pUc18 and then transformation in competent *E. coli* resulted in recombinant *E. coli* (E-UL68) which showed toxicity against both susceptible and two resistant colonies having the same level of toxicity as that of wild strain Lp1-G (Shi et al. 2003). The introduction of the mosquitocidal toxin gene *mtx 1* from *B. sphaericus* strain SSII-1 into an acrystalliferous strain of *B. thuringiensis* individually had moderate toxicity to binary toxin-susceptible and toxin-resistant *C. quinquefasciatus*; however, in combination with cytolytic protein gene *cyt1Aa* from *Bt israelensis*, the activity of *mtx 1* to target mosquito larvae enhanced, suggesting a synergism between Cyt 1Aa and mtx1 toxins (Zhang et al. 2006). The conjugal transfer of toxin-encoding megaplasmid from *Bt israelensis* to *B. sphaericus* produced the transconjugant bacteria that were significantly more toxic to *Aedes* sp. and were able to overcome resistance to *B. sphaericus* in resistant colony of *C. quinquefasciatus* (Gammon et al. 2006).

15.3.1.3 *Bacillus cereus*

Most strains of *Bacillus cereus* Frankland & Frankland consistently associated with insects are saprophytic or symbiotic bacteria occupying the digestive tracts. While genetically highly similar to *Bt*, *B. cereus* does not produce parasporal crystalline toxins, which limit its virulence against insect hosts (Jurat-Fuentes and Jackson 2012). However, *B. cereus* strains have been found to be adequate hosts for expression of binary toxin genes from *B. sphaericus* as well as *cry4B* gene from *Bt israelensis* resulting in higher larvicide efficiency along with preventing mosquito population for obtaining resistance against these strains at early stages. Transformation of recombinant plasmid pBS373 harbouring binary toxin genes from *B. sphaericus* 2297 into two *B. cereus* strains, Ae10 and cx5, isolated from mosquito larval gut revealed the production and presence of 51 kDa toxin protein in both strains, and these two recombinant strains showed very high toxicity against *C. quinquefasciatus* larvae (Luxananil et al. 2003). A recombinant sphingomyelinase C, purified toxin, isolated from larvae of *Myrmeleon bore* (Tjeder) expressed in *E. coli* was as potent as the native protein in killing the German cockroaches, *Blattella germanica* (Linnaeus) (Nishiwaki et al. 2004).

15.3.1.4 *Xenorhabdus*

Xenorhabdus nematophila (Poinar and Thomas), a facultative anaerobic bacterium, is engaged in mutualistic relationship with specific soil nematode *Steinernema* sp. Lee et al. (2004) identified and cloned a novel toxin gene (*tccC1*/*xptB1*) from *X. nematophila* strain isolated from Korea-specific entomophagous nematode *Steinernema glaseri* (Steiner) and expressed in *E. coli*, and the recombinant toxin protein caused a rapid mortality (80 % death within 2 days) of wax moth, *Galleria mellonella* (L.) larvae.

15.3.1.5 *Serratia entomophila*

Serratia entomophila (Grimont et al.), a non-spore-forming bacterium, offers promise for the control of New Zealand grass grub, *Costelytra zealandica* (White), an important pest of pastures causing amber disease in target insect (Jackson 2007). Hurst et al. (2004) observed that 155-kb amber disease-associated plasmid (pADAP) carries the genes *sepA*, *sepB* and *sepC*, which are essential for the production of amber disease symptoms by *S. entomophila* in grass grub. Based on deletion analysis of pADAP and subsequent sequence data, a 47-kb clone was constructed, which when expressed in either an *E. coli* or a *Serratia* background exerted strong antifeeding activity and often led to rapid death of the infected grass grub larvae.

15.3.2 Baculoviruses

Baculoviruses are among the best known and most thoroughly studied insect pathogens and belong to family Baculoviridae. The baculovirus genome consists of a single circular double-stranded DNA molecule which is

covalently closed and supercoiled. Baculovirus replication is distinguished by the production of two different virion phenotypes: the occlusion-derived virions, which occur within proteinaceous viral occlusions and initiate infection of host, and the budded virions, which spread infection to other cells and tissues within the host. Baculoviruses have been isolated exclusively from insects and possess many of the attributes required in ideal biopesticides for use in insect pest management (Harrison and Hoover 2012). The major successes with baculoviruses have taken place in forestry, particularly for sawflies control in Europe and North America, and in agriculture for velvet bean caterpillar control on soybean in Brazil. However, narrow host range and slow action are the major obstacles in the widespread use of entomopathogenic viruses (Battu et al. 2002). Genetic engineering provides an effective tool for improving the activity of baculoviruses as biocontrol agents (Table 15.3). To improve the efficiency of viral insecticides, focused on increasing virulence, expanding host range and enhancing photostability, several strategies comprising both conventional (strain selection, serial passage and mutation) and DNA recombinant technology have been employed. These include expressing physiological effectors (hormones, enzymes or antisense), interrupting the normal metabolism of the insect, expressing insect-specific toxins such as neurotoxin and *Bt* crystal toxins or deleting baculoviral genes that may interact with the reaction of the viruses to the insect (Bonning et al. 2003; Hu et al. 2003; Narayanan 2003).

15.3.2.1 Expanding Host Range

Baculovirus infections have been described in over 700 species of insects belonging to orders Lepidoptera, Hymenoptera, Diptera, Coleoptera, Trichoptera, Thysanura and Neuroptera. But most baculoviruses have limited host rages, usually restricted to one or a few closely related species within the same genus (Battu et al. 2002). An old-fashioned methodology of cross infectivity testing of baculoviruses that includes screening of long recognised strains for possible encroachments on generic, tribal, subfamilial, familial or even ordeal taxonomic frontiers in their host relationships may prove to be highly rewarding. Variants of baculoviruses may arise when virus infects alternate hosts. An isolate of spruce budworm, *Choristoneura fumiferana* NPV, was fed to the neonate cabbage looper larvae and the wax moth larvae. After passage, virus was able to infect new hosts. Baculovirus, hitherto restricted to one genus in Noctuidae, could also provoke lethal virus disease in various locusts and grasshoppers. An ELISA test showed that the baculovirus (from a moth) that spread systematically in the locust and killed it, sometimes within 4 days, was homologous to the virus extracted from the infected caterpillar. A reciprocal transmission of the virus from the locust back to the caterpillar, produced the same syndrome and the same causal agent (Harpaz 1987). Thus, it is very important that cross infectivity testing of baculoviruses should be undertaken to identify baculoviruses that improved from their original parent stock. After passage through the alternate host, the viruses can be further evaluated for enhanced virulence to their original (in back-feeding trials) as well as alternate insect hosts (Arora et al. 2000).

The recombinant DNA technology has its current applications in inserting foreign genes into insect baculoviruses and achieving their rapid and efficient expression in the recipient host systems. There are many avenues to explore the further development of baculovirus-based viral insecticides with extended host range, and detailed methods for genetic manipulations are available. The effective host range of baculoviruses might be expanded by inserting insect-specific neurotoxin and/or behaviour-modifying gene into the baculovirus genome so that the gene is under the control of promoter that expresses early on virus entry into cells. This would not actually alter the host range for virus replication but could alter the ability of virus to influence host behaviour and thereby increase the effective host range of virus as pesticide. The necessity for application of a variety of viruses to protect those crops affected by a multitude of pests could be avoided by specifically broadening the host range of one virus. It is envisaged that this may be achieved by engineering specific genes from

Table 15.3 Genetically engineered baculoviruses for insect pest management

Insect species	Baculovirus	Gene/toxin	Impact on biological activity	References
Autographa californica	AcNPV	Scorpion, *Androctonus australis* toxin gene (*AaIT*)	Falling off infected *H. virescens* larvae 5–11 h before death and unable to climb plant to continue feeding, less foliage consumption	Hoover et al. (1995)
	AcMNPV	*AaIT*	Reduces time to kill (25–40 %) of *S. eridania*, *T. ni* and *H. virescens*	Stewart et al. (1991), McCutchen et al. (1991), Carbonell et al. (1988)
	AcNPV	Enhancin gene from *Trichoplusia ni* granulovirus	Increase in infection by 21-fold in *S. exigua*	Hayakawa et al. (2000)
	AcMNPV	*Agelenopsis aperta* (*Mu-Aga-IV*)	Increase in effectiveness to kill of *S. frugiperda* and *T. ni*	Prikhodko et al. 1996
		Anemonia sulcata (*AsII*)		
		Stydactyla (*sh1*)		
	AcMNPV	Juvenile esterase from *Heliothis virescens*	Increase in speed of kill by 20 %	Hammock et al. (1990), Bonning et al. (1999)
	AcMNPV	Viral gene (*egt*) – deletion	Increase in speed of kill, reduced feeding	O'Reilly and Miller (1991)
	AcNPV	Mite toxin	Increase in speed of kill	Tomalski and Miller (1991)
	AcNPV	Scorpion *Leiurus quinquestriatus hebraeus* toxin gene (*LqhIT1*, *LqhiT2*)	Improvement in speed of kill (32 %), decrease in median survival time (34 %) in *H. virescens*	Gershburg et al. (1998), Harrison and Bonning (2000)
	AcNPV	Spider *Diguetia canities* toxin gene (*DTX9.2*)	Induced paralysis and improved speed of kill	Hughes et al. (1997)
Rachiplusia ou	RoMNPV	Scorpion *L. quinquestriatus hebraeus* toxin gene (*LqhiT2*)	Improvement in speed of kill (40 %)in *O. nubilalis* and *H. zea*	Harrison and Bonning (2000)
Bombyx mori	BmNPV	*AaIT*	Increase in biological activity	Maeda et al. (1991)
	BmNPV	Diuretic hormone	Haemolymph of the infected larvae was decreased by 30 % with an increased mortality	Maeda (1989)
Heliothis zea	HzNPV	*AaIT*	Mortality at faster rate	Treacy et al. (2000)

one NPV into the genome of other. A future approach would be to synthesise biologically active and authenticated insect pheromones and other behaviour-modifying proteins that are used in IPM by way of expressing the genes responsible for pheromones in insect cells through baculovirus vector instead of depending on in vitro production technique, which is cost prohibitive and time consuming. Engineering viruses by insertion of insecticidal proteins in many cases should also result in expanded host range. This is because, in many lepidopteran host species that do not develop disease upon inoculation with conventional baculoviruses, there can be limited viral replication, and these less susceptible hosts infected by an engineered virus that expresses a potent insecticidal protein will likely succumb as the virus need not replicate

extensively to paralyse or kill the larva (Narayanan 2003). Some of the baculoviruses genes identified to date that influence the host range are *p143* (putative DNA helicase), apoptotic suppressor genes (*p35* and *iap*) and some line transcriptional factors like *lef-7*, *hrf-1*, *hcf-1*, etc. These represent future DNA segments for genetic engineering of baculoviruses.

Helicase Gene *(p143)*: In case of AcMNPV, the putative helicase gene p143 is essential for DNA replication as reported by Lu and Carstens (1991). BmNPV and AcMNPV infect *B. mori* and *S. frugiperda* cells, respectively, but cannot replicate in heterologous cell line, but the recombinant AcMNPV carrying helicase gene from BmNPV was capable of replicating both in *B. mori* and in *S. frugiperda*.

Apoptotic and Anti-apoptotic Gene *(p35)*: Apoptosis may be defined as 'a process where the cell dies in a controlled manner in response to specific stimuli, apparently following an intrinsic programme'. A specific gene *p35* which is required for AcMNPV late gene expression and virus DNA replication in Sf-21 cells was identified as being responsible for blocking the apoptotic response (Clem and Miller 1994).

Host Range Factor Gene *(hrf 1)*: The broad spectrum AcMNPV does not infect either gypsy moth larvae or its cell line IPLB-Id 6527, but the recombinant AcMNPV carrying *hrf-1* gene from LdMNPV was able to replicate both in gypsy moth larvae and in its cell line.

15.3.2.2 Improvement in Speed of Kill/Virulence

It has been known for sometime that different isolates of same viral species from different geographical locations can vary significantly in efficacy against the same target pests. Moreover, a virulent NPV sprayed at an appropriate rate will kill the first and second and in some cases the third instar within 2–4 days, but the later instars may live for a week or more, causing further damage to the crop. Current approaches improving the efficacy of viral insecticides, therefore, are aimed at developing broad spectrum viruses that will cause cessation of feeding within 24–48 h. NPVs isolated from different populations of fall armyworm, *S. frugiperda*, in Louisiana have been shown to vary by 16-fold in their LC_{50} against these pests (Fuxa et al. 1988). Rabindra (1992) reported that among the isolates of NPV of *H. armigera* collected from three agroclimatic regions of India, there existed tremendous variation in virulence. Arora et al. (1997) reported that PAU isolate of HaNPV was more virulent than isolates from other parts of India. Similarly, Padmanaban et al. (2002) observed significant variation in virulence of different isolates of HaNPV collected from different areas of Punjab. These findings suggest that natural variations in pathogenic populations and virulence of viruses isolated from different geographical areas should receive more attention towards the selection of isolates to be used as viral insecticides.

In 2003, decreased susceptibility in several *Cydia pomonella* populations to *C. pomonella* granulovirus (CpGV) products in apple and pear orchards has been reported from Germany and from France (Fritsch et al. 2005; Sauphanor et al. 2006; Asser-Kaiser et al. 2007). The resistance was observed to be 60,000-fold in resistant colonies as against susceptible ones (Berling et al. 2009). The resistance to CpGV was found to be based on a single, dominant gene that was located on the Z-chromosome (Asser-Kaiser et al. 2010). Resistance to Mexican isolate (CpGV-M) was also observed when budded virus of CpGV was directly injected into the haemocoel of resistant individuals, thereby circum-passing the midgut which clearly indicated that not a change in the midgut but a cellular factor must be responsible for the CpGV not being able to replicate in resistant *C. pomonella* individuals (Jehle et al. 2010). Intensive search for alternatives to the conventionally used CpGV isolate resulted in the finding of new isolates for overcoming CpGV resistance. Several of these isolates have been tested in the field and are being registered for codling moth control in different European countries. Among them, two isolates (112 and NPP-R1) presented an increased virulence to resistant codling moth larvae. CpGV-I12 identified from Iran was found to be as effective against resistant codling moth larvae in Germany and was observed to

partially overcome the resistance (Jehle et al. 2006; Eberle et al. 2008). However, isolate NPP-R1 showed an even higher pathogenicity on resistant CpGV than other isolates. In addition, CpGV virus (Madex Plus) was also selected by subsequent passage of CpGV-M through resistant codling moth larvae. The 2016-r4 isolate obtained from four successive passages of NPP-R1 in RGV larvae had a sharply reduced proportion of the CpGV-M-like genotype and an increased virulence to against insects from the resistant colony (Berling et al. 2009).

Serial passage of viruses leads to rapidly accumulating mutants also known as 'the passage effect' which results in changing of virus efficacy. Increase in virulence of *Helicoverpa zea* SNPV against *H. zea* after repeated passage through the host has been reported (Shapiro et al. 1997; Ignoffo et al. 1995). Similarly, 15-fold increase in virulence of *Autographa californica* (Speyer) MNPV has been observed after repeated passages through *P. xylostella* (Klondy-Hirsch and Beck 1997). The direct application of conventional genetics through chemical mutagens so as to create variants of viruses has also been documented. Reichelderfer and Benton (1973) reported ninefold increase in virulence of *S. frugiperda* NPV after 4 treatments of virus with a chemical mutagen 3-methylcholanthrene. Similarly, Wood et al. (1981) obtained variants of *A. California* NPV with enhanced virulence after treating virus with 2-aminopurine.

The strategies used for genetically modifying the baculoviruses include (a) use of very late gene promoters like the polyhedron and p10 promoters to drive gene expression, (b) use of alternative promoters to produce relatively an early expression of the foreign gene, (c) use of multiple expression vectors to produce polyhedron-positive recombinant viruses and (d) deletion of gene ecdysteroid UDP-glucosyltransferase (*egt*) which the virus has acquired to protect the host from premature death. Candidate genes for hyperexpression in the baculovirus insecticide system include those encoding insect-specific enzyme gene (juvenile hormone esterase gene), hormone genes (eclosion hormone gene, diuretic hormone gene) and insect-specific foreign toxic genes (scorpion venom toxin genes, predatory mite toxic gene, predatory spider toxin gene, parasitic wasp venom gene and *Bt* δ-endotoxin genes). Recombinant baculoviruses expressing such genes have been constructed and their insecticidal activity assessed in vivo (Sharma 2009).

Expression of Juvenile Hormone Esterase Gene: The metamorphic changes of insect caterpillars into pupae and ultimately into adults are regulated by the juvenile hormone (JH). A reduction in the titre of JH is associated with drastic increase in the levels of juvenile hormone esterase (JHE) (Bonning and Hammock 1994). The speed of kill of AcMNPV was improved by 20 % by the expression of JHE (Hammock et al. 1990). First instar larvae of *H. virescens* or *T. ni* were killed 20 % faster when these were infected with AcJHE-KK (AcMNPV expressing mutant JHE-KK) as compared to control larvae infected with recombinant AcMNPV expressing authentic JHE (AcHE) (Bonning et al. 1999); however, survival time was only marginally reduced in older AcJHE-KK-infected instars.

Expression of Hormone Genes: Neurohormones are the master regulatory hormones of insects and affect critical physiological processes that include moulting, metamorphosis, reproduction and general homeostasis and kill or debilitate the treated insects. Further, neurohormones are proteins which are unstable and unsuited for application. So the use of insect viruses as highly efficient cloning-expression vectors for neurohormone gene comes to the rescue for their efficient utilisation in the management of insect pests.

Eclosion Hormone Gene: The eclosion hormone triggers the shedding of the old cuticle. Eldrigde et al. (1991) expressed eclosion hormone in *Manduca sexta* through AcMNPV baculovirus expression system with high level of biological activity.

Diuretic Hormone Gene: Diuretic and antidiuretic hormones are considered to play important roles in maintaining the water balance in insects. This balance might be disrupted if a recombinant virus produced elevated levels of either hormone in the infected larva. Maeda

(1989) expressed a synthetic diuretic hormone gene of tobacco hornworm in a recombinant baculovirus, viz. *Bombyx mori* NPV. The silkworm larvae infected with recombinant baculovirus showed alteration in the larval fluid metabolism; the haemolymph of the infected larvae was decreased by 30 % with an increased mortality in comparison with wild-type-infected larvae.

15.3.2.3 Expression of Insect-Specific Foreign Toxic Genes

Arthropod venom offers a rich source of insect-selective toxins. Carbonell et al. (1988) were the first to attempt to improve the insecticidal activity of baculovirus by expressing biologically active scorpion toxin, insectotoxin-1 of *Buthus eupeus* Koch. Unfortunately, biological activity was not detected in insect bioassays using larvae of *Trichoplusia ni* (Hübner), *G. mellonella* and *Sarcophaga* sp. with any of the constructs. However, the first genes that were inserted into baculoviruses which successfully altered their biology were insect-selective toxin genes derived from other arthropods, viz. scorpion *Androctonus australis* (Linnaeus) and the predatory mite, *Pyemotes tritici* (LaGreze-Fossat & Montagne). The scorpion neurotoxins are an ideal choice to improve the efficacy of baculoviruses owing to their high selectivity as toxin proteins bind to sodium channel proteins and affect neuronal membranes (Lester et al. 1982; Zlotkin 1988). Genes that code for scorpion and mite venom have been engineered into the AcNPV from *A. californica* (Speyer) to increase the effectiveness of this virus (Tomalski and Miller 1991). It has been reported that recombinant AcMNPV expressing gene-encoding insect-specific toxin (*AaIT*) from scorpion significantly reduces time of kill by 25–40 % in southern armyworm, *Spodoptera eridania* (Cramer); cabbage looper, *T. ni*, and tobacco budworm, *Heliothis virescens* (Fab.) (Carbonell et al. 1988) and also acted more quickly and significantly reduced the crop damage by cabbage looper *T. ni* on cabbage under field trials. The *AaIT* gene expressed under the control of various promoters has also been inserted into NPV of mint looper, *Rachiplusia ou* (Guenee) (RoMNPV) (Harrison and Bonning 2000), cotton bollworms, *H. zea* (HzNPV) (Treacy et al. 2000) and *H. armigera* (HaSnPV) (Chen et al. 2000; Sun et al. 2004). Expression of *AaIT* under late p6.9 promoter of AcMNPV by recombinant RoMNPV resulted in 34, 37 and 19 % improvements in speed of kill in comparison to control larvae infected with RoMNPV when tested on neonates of *O. nubilalis*, *H. zea* and *H. Virescens*, respectively (Harrison and Bonning 2000). Treacy et al. (2000) evaluated insecticidal properties of corn earworm *H. zea* (Boddie) NPV (Hz*AaIT*) genetically altered with toxin from *A. australis* against Heliothine species and found that Hz*AaIT* killed the larvae of *H. virescens* and *H. zea* at faster rate than non-transformed HzNPV. In addition to *A. australis*, venom of yellow Israeli scorpion, *Leiurus quinquestriatus hebraeus* (Birula), also contains insect-selective toxins (*LqqIT1*, *LqhIT1*, *LqhIT5*, *LqhIT2* and *LqqIT2*). Gershburg et al. (1998) showed that recombinant AcMNPV expressing the excitatory LqhIT1 toxin from scorpion resulted in improvement in speed of kill by 32 % as against wild AcMNPV. Harrison and Bonning (2000) observed 34 % decrease in median survival time though expression of LqhIT2 as compared to control larvae in *H. virescens*. Similarly, recombinant RoMNPV expressing LqhIT2 gene construct showed 40 % improvement in speed of kill as against wild-type virus on larvae of European corn borer, *O. nubilalis*, and *H. zea*. Maeda et al. (1991) achieved a significant increase in activity by expression cDNA of *AaIT* in NPV from silkworm, *Bombyx mori* L.

Helicoverpa zea (Boddie) NPV has been improved by way of inserting mite toxin gene and expressed toxin during infection resulting in 50 % mortality within 40 h after virus treatment. The toxins NPS-901 (Krapecho et al. 1995) and *DTX9.2* (Hughes et al. 1997) discovered from spider *Diguetia canities* (McCook), when injected, induced paralysis that was specific to insects, and cloning it into a baculovirus enhanced the virus activity. Likewise, spider toxins *μ-Aga-IV* from *Agelenopsis aperta* (Gertsch) and *TalTX-1* from *Tegenaria agrestis* (Walckenaer) also showed an improved speed of kill (Prikhodko

et al. 1996; Hughes et al. 1997). The toxins from sea anemones, *Anemonia sulcata* (Pennant) (*AsII*) and *Stichodactyla helianthus* (Ellis) (*ShI*), have also shown 38 and 36 % improvements in speed of kill in neonates of *T. ni* and *S. frugiperda*, respectively. Quistad et al. (1994) purified and characterised insecticidal toxin from the venom of the parasitic wasp, *Bracon hebetor* Say, and had shown that the toxin paralyses insects. It has shown 400-fold higher biocidal activity against *Spodoptera* when compared to scorpion toxin and 100-fold higher activity against *Galleria* when compared to mite toxin.

15.3.2.3.1 Expression of Proteases

Expression of basement membrane degrading proteases is one of the most remarkable improvements in speed of kill of recombinant baculoviruses. The baculovirus faces several obstacles within insect midgut, and the final midgut barrier is basement membrane. Expression of basement membrane degrading protease cathepsin L from flesh fly, *Sarcophaga peregrina*, in AcMNPV resulted in recombinant which showed 51 % faster killing speed in comparison to AcMNPV in neonates of *H. virescens* (Harrison and Bonning 2001).

15.3.2.3.2 Expression of *Bt* Toxin Genes

Bt toxin as the fused product, i.e. polyhedron-Cry 1Ac-green fluorescent protein (GFP), has been expressed in baculovirus AcMNPV. The recombinant AcMNPV (Color*Bt*rus) expressing this fused product produced polyhedra that occlude *Bt* toxin and GFP and released toxin and GFP proteins in the insect midgut. Bioassay using second and third instar larvae of *P. xylostella* showed 100-fold reduction in LD_{50}, and ST_{50} decreased by 60 % as compared to wild-type AcMNPV (Chang et al. 2003).

15.3.2.3.3 Deletion of Ecdysteroid UDP-Glucosyltransferase (*egt*) Gene

Baculoviruses have acquired a gene known as *egt*, which encodes for enzyme ecdysteroid UDP-glucosyltransferase. The enzyme can transfer either glucose or galactose from UDP-sugar to ecdysteroids such as 20-OH-ecdysone, the hormone of insects which triggers moulting by governing gene expression. The deletion of the *egt* gene from viral genome accelerates the virus-induced mortality by allowing the infected larvae to begin moulting, resulting in feeding cessation during infection and premature degeneration of Malpighian tubules. Larvae infected with *egt*-deleted AcNPV displayed approximately 40 % reduction in larval feeding and early mortality with 20 % reduction in LT_{50} as compared to wild-type AcMNPV-infected larvae. An AcNPV lacking *egt* was the first genetically improved recombinant baculovirus to be approved by USEPA for field testing in the USA and was first field tested by American Cyanamid in 1993 (Black et al. 1997).

Some of the baculoviruses genes identified to date that influence the virulence include chitinase gene (*chi A*) and enhancin genes which can be potential DNA segments for genetic engineering of baculoviruses.

Chitinase Gene (*Chi A*): The chitinous cuticle of the insect covers virtually all external surface, even extending through the foregut, hindgut and tracheal tubes, constituting the first line of passive defence in insects. The median time for mortality of fourth instar larvae of *S. frugiperda* infected with a recombinant virus containing chitinase gene was approximately 20 h shorter than that for insects infected with a wild-type virus (Gopalakrishnan et al. 1995). Such engineered baculoviruses possessing the chitinolysis activity, in addition to their standard infectivity, should be more effective in the field against pests, rather than directly applying chitinase-based insecticidal spray formulations (Mazzone 1987).

Enhancin Genes: Wang and Granados (1997) have identified an invertebrate intestinal mucin (IIM) from a lepidopteran insect *T. ni* similar to the mucus layer found in mammals, protecting the digestive tract from microbial infections. It has been shown that *T. ni* granulosis virus (TnGV) can overcome this IIM barrier and disrupt the integrity of peritrophic membrane due to the presence of 'enhancin gene' or viral enhancing factors (VEF). Since baculovirus 'enhancing' protein genes are virus coded, they

can be better utilised to improve the efficacy of viral pesticides in the future. A virus enhancing factor from armyworm, *Pseudaletia unipuncta* (Walker) (=*Mythimna separata*), has been successfully expressed in *E. coli*, and the resulting lysates of transformed *E. coli* cells enhanced PsunMNPV infection when treated with either trypsin or thrombin. The VEF enhanced PsunMNPV infection in larvae of armyworm (Hukuhara et al. 2001).

15.3.2.3.4 Improvement in Photostability

One of the major obstacles to expansion in use of commercial baculovirus preparations for the control of crop pests is their sensitivity to photodegradation. The exact mechanism by which UV radiation inactivates the virus is not fully understood, although it has been opined that hydrogen peroxide produced by the near UV irradiation (300–380 nm region of solar spectrum) of one or more amino acids reduced both the vitality and pathogenicity of baculoviruses. A number of formulations containing sunlight protectants and application techniques have been used to improve photostability. However, genetic methods offer the greatest potential in this regard also.

Insect viruses have been selected for resistance to UV irradiation and for an increased rate of vertical transmission. One such strain of GV of codling moth (CpGV) was quite resistant to the effect of UV irradiation both under natural sunlight and in laboratory conditions (Brassel and Benz 1979). The modified strain was 5.6 times as resistant to UV light as the original isolate and remained infective for twice as long in the field. A UV-resistant strain of NPV of *Lymantria dispar* (Linnaeus) has also been obtained (Shapiro and Bell 1984). The genes that might be responsible for the effective DNA repair system, if isolated, inserted and expressed into the genomes of baculoviruses, can produce UV-resistant baculoviruses. Considering the available biodiversity in the tropics, such investigations in tropical agroecosystems will yield fruitful dividends. The biotechnological tools provide exciting opportunities for improving photostability, but thorough understanding of the mechanisms of photodegradation is a prerequisite for using these techniques (Arora et al. 2000). In an attempt to reduce UV inactivation of baculoviruses, Petrik et al. (2003) have developed a recombinant AcMNPV, vHSA50L, that expresses an algal virus pyrimidine dimer-specific glycosylase, cv-PDG, which is involved in the first steps of the repair of UV-damaged DNA. Although the polyhedra of vHSA50L showed no differences in UV inactivation in comparison to AcMNPV, the BV of vHSA50L was threefold more resistant.

15.3.3 Fungal Entomopathogens

Of the estimated 1.5–5.1 million species of fungi in the world, approximately 100,000 have been described (Blackwell 2011). Of these, approximately 750–1,000 species are entomopathogens placed in over 100 genera (St. Leger and Wang 2010). However, based on the number of cryptic species revealed by recent molecular phylogenic studies, it is evident that these estimates are low (Vega et al. 2012). The fungi infect individuals in most of the orders of insects including Hemiptera, Diptera, Coleoptera, Lepidoptera, Orthoptera and Hymenoptera. The majority of entomopathogenic fungi identified to date belong to fungal orders Entomophthorales (class, Zygomycetes; division, Zygomycota) and Hypocreales (class, Sordariomycetes; division, Ascomycota). Entomophthorales are chiefly parasitic on lower animals, and host death occurs by tissue colonisation with little or no use of toxins. Important entomopathogenic genera include *Entomophaga*, *Entomophthora*, *Erynia*, *Massospora*, *Pandora*, *Zoophthora* and *Neozygites* (Webster and Weber 2007). Although some of these genera cause frequent epizootics in insect pest populations, they are hard to mass produce. On the other hand, the members of Hypocreales are generally considered as opportunistic pathogens infecting many species of several insect orders, and host death is commonly associated with toxin production overwhelming host defence mechanisms. Important entomopathogenic genera include *Beauveria*, *Cordyceps*, *Isaria*, *Metarhizium*, *Nomuraea*, *Lecanicillium*, *Paecilomyces* and *Sorosporella*.

Besides these groups, microsporidia have also been included among fungi based on recent phylogenetic studies (Hibbett et al. 2007). Most of the more than 1,300 described species in approximately 180 genera are pathogens of invertebrate animals, with insects being type hosts of nearly half of the described genera (Becnel and Andreadis 1999). The major obstacles in the successful utilisation of entomopathogenic fungi as biocontrol agents include relative instability, requirement of moist conditions for spore germination, invasion and growth and also slow rates of kill. The optimisation of entomopathogenic fungi by genetic engineering is in its infancy because of a limited knowledge of the molecular and biochemical basis of fungi. Moreover, due to large size of the fungal genome and most fungal toxins being complex molecules determined by several genes, their genetic manipulation still remains a knotty task. The major objectives in genetic improvement of entomopathogenic fungi are to increase virulence and improve photostability. In addition, they have also been improved for resistance to fungicides. Breeding techniques like mutagenesis, transformation using plasmid vectors and protoplasts fusion are used for field stability and effectiveness.

15.3.3.1 Enhancing Virulence

Protoplast fusion technique has been utilised to develop hybrid strains that possess more pathogenicity than that of the parents. Protoplast fusion of diauxotrophic mutants of *B. bassiana* (entomopathogenic strain) and *B. sulfurescens* (toxicogenic strain) resulted in hybrids that were more pathogenic than parents, and some possessed very high virulence and killed the insects more rapidly (Viaud et al. 1998). Entomopathogenic fungi such as *B. bassiana* invade insects by direct penetration of host cuticles via the action of diverse hydrolases including proteases and chitinases coupled to mechanical pressure. In order to better target cuticle protein-chitin structures and accelerate penetration speed, a hybrid protease (CDEP-BmChBD) was constructed by fusion of a chitin-binding domain BmChBD from *Bombyx mori* chitinase to the C-terminal of CDEP-1, a subtilisin-like protease from *B. bassiana*. Compared to the wild type, the hybrid protease was able to bind chitin and released greater amounts of peptides/proteins from insect cuticles. The insecticidal activity of *B. bassiana* was enhanced by including proteases, CDEP-1 or CDEP-BmChBD produced in *Pichia pastoris*, as an additive; however, the augment effect of CDEP-BmChBD was significantly higher than that of CDEP-1. Expression of the hybrid protease in *B. bassiana* also significantly increased fungal virulence compared to wild type and strains overexpressing the native protease (Fan et al. 2010).

Apart from cuticle-degrading proteinases, a strain of *M. anisopliae* was identified that produced an acute protein toxin active at 0.7 μg/100 mg, and other toxins from *M. anisopliae* and *B. bassiana* are being isolated (Quesada-Moraga et al. 2006). A toxic protein from *B. bassiana* (bassiacridin) had an LT_{50} of 3 μg per insect when injected into fourth instar locust nymphs *Locusta migratoria* (Linnaeus) (Quesada-Moraga and Vey 2004). The various toxins from *M. anisopliae* and *B. bassiana* affect different aspects of insect biology and, therefore, could be used synergistically to increase the magnitude of hypervirulence and to reduce the probability of resistance evolving to a single transgene product (St. Leger and Wang 2010). The molecular and biochemical bases of pathogenicity of *M. anisopliae*, which causes green muscardine disease, have been particularly well studied. Various genes relating to formation of the appressorium, virulence and nutritional stress have been cloned from *M. anisopliae*. *Pr1*gene, which encodes a subtilisin-like protease, is involved in insect host cuticle penetration and has been found to activate trypsin, which in turn activates the phenoloxidase system involved in the initiation of the melanisation process and tyrosine metabolism. Additional copies of this gene were introduced into the genome of *M. anisopliae* with the objective to enhance the efficacy as *M. anisopliae*, which in general takes 5–10 days to kill a host. Infection with recombinant strain resulted in partial hydrolysis of haemolymph proteins and extensive melanisation of insect (St. Leger et al. 1996). As a result, the larvae infected with the recombinant strains were killed

at 25 % faster rate in comparison to larvae infected with wild-type *M. anisopliae*, and feeding damage was reduced by 40 %. Moreover, the melanised cadavers were poor substrates for fungal sporulation, which would restrict dissemination of the recombinant fungus, thereby limiting the environmental impact and necessitating repeat sales of the control agents. Although it would be possible to incorporate other insecticidal genes into *M. anisopliae*, the use of a homologous gene such as *Prl* may be more acceptable from a regulatory viewpoint (St. Leger 2007). *M. anisopliae* has also been genetically improved for increased pathogenicity and virulence through expression of insect-specific neurotoxin from scorpion *A. australis* under the control of promoter that is active only in the presence of insect haemolymph. The modified fungus exhibited same mortality rate in tobacco hornworm at 22-fold lower doses and, at certain concentrations, reduces survival of infected mosquitoes by > 40 % as compared to wild-type fungus (Wang and St. Leger 2007).

Epiphytic fungus, *Erwinia herbicola* (Lohnis) Dye, has been successfully transformed with a *Bt* toxin gene *cry1Aa1* present in plasmid pUN4. The pUN4 was expressed into three isolates of *E. herbicola*. The transformed *E. herbicola* strains Eh4, Eh5 and Eh6 expressed the toxin protein and conferred insecticidal activity. One of the transformed *E. herbicola* strains, Eh4, when sprayed on cabbage leaves for colonisation to test its insecticidal efficacy and persistency against the diamondback moth, *P. xylostella*, showed 36.7–60 and 40–83.3 % mortality after 48 and 72 h, respectively (Lin et al. 2002).

15.3.3.2 Resistance to Fungicides

Benomyl- or carbendazim-resistant isolates of entomopathogenic fungi have been obtained by mutagenesis as well as by DNA-mediated transformations so that they can be used along with these pesticides in insect control programme. The susceptible strains of *M. anisopliae* have been treated with ultraviolet light and mutagenic agents to obtain benomyl- or carbendazim-resistant isolates (Tsai et al. 1993). A *β-tubulin* gene from the fungus *Neurospora crassa* (Draft) encoding resistance to benomyl was transferred to *M. anisopliae* via cosmid p^{50}. The transformed fungi showed normal cell division when cultured on nonselective agar and retained the ability to infect and kill the larvae of tobacco hornworm, *Manduca sexta* (Bernier et al. 1989). Another gene *benlate* A3 from *Aspergillus nidulans* has also been used to transform *M. anisopliae* via cosmid p^{50} as vector (Goettel et al. 1990). The transformants were pathogenic to *M. sexta* in the presence of 50 μg/ml of benomyl, producing infection structures (appressoria) and enzyme chymoelastase which dissolve insect cuticle. The gene encoding the cuticle-degrading protease (Pr1) has also been inserted into genome of the same fungus. *B. bassiana* has also been transformed using electroporation for methyl 1, 2-benzimidazole carbamate (MBC) resistance with *N. crassa* '*β-tubulin*' gene. The transformants were stable and able to grow in the presence of 5 μg MBC/ml. Similarly, *B. bassiana* transformed with conventional protoplasting and electroporation and polyethylene glycol treatment via pSV50 harbouring 'β-*tubulin*' gene from *N. crassa* grew well on benomyl concentrations at 10 μg/ml. The transformants were found to be mitotically stable on wither-selective or non-selective medium (Sandhu et al. 2001). These studies demonstrated that the genetically engineered strains of entomopathogenic fungi having tolerance to fungicides can be used along with other components of pest control, thereby promoting their utility in integrated management programmes.

15.3.3.3 Improving Photostability

The efficacy of mycoinsecticides is significantly influenced by environmental abiotic factors including solar UV radiation, temperature and humidity (Rangel et al. 2008). Various UV protectants have been employed to protect fungal conidia against UV radiation, especially UV-B (280–320 nm) (Jackson et al. 2010). To improve resistance to UV damage, Shang et al. (2012) used agrobacterium-mediated transformation to engineer *B. bassiana* with an exogenous tyrosinase gene. Tyrosinases are type-3 copper-containing

monooxygenases involved in the conversion of L-tyrosine or L-Dopa to form melanin (Ito et al. 2000). Melanin absorbs light at all wavelengths, especially the UV range. The mitotically stable transformants produced larger amounts of yellowish pigments than the wild-type strain, and these imparted significantly increased UV resistance. Moreover, the virulence of the transgenic isolate was also significantly increased against the silkworm *B. mori* and the mealworm *Tenebrio molitor* (Linnaeus).

15.3.4 Entomopathogenic Nematode

Nematodes comprise a tremendously diverse phylum whose members exploit habitats more varied than any other groups of animals except arthropods. The nematodes associated with insects are diverse phylogenetically, belonging to 13 different suborders of the Nematoda (Lewis and Clarke 2012). Nematodes in the families Steinernematidae and Heterorhabditidae have the ability to quickly infect their insect hosts. Their unique association with symbiotic bacteria (*Xenorhabdus* for Steinernematidae and *Photorhabdus* for Heterorhabditidae) makes them effective biocontrol agents. Entomopathogenic nematodes (EPNs) are persistent and recycle inside host causing long-term and sustainable effects on pest populations. They can be cultured easily in vitro and have a high reproductive potential. However, susceptibility to environment stress and diverse host-finding behaviour limits their efficiency. Genetic improvement in entomopathogenic nematodes may increase their searching capacity, virulence and resistance to environmental factors. Techniques such as selective breeding, hybridisation, mutagenesis and gene transformation have been employed for genetic manipulation in nematodes.

15.3.4.1 Improvement in Host-Finding Ability and Infectivity

A successful breeding programme must have for a desired trait either a moderately high heritability value or a very large genetically diverse base population from which to select. Artificial selection for enhancement of entomopathogenic nematode host finding and infectivity has been demonstrated in several selection programmes. A 72-fold increase in host-finding ability of a strain of *Steinernema carpocapsae* Weiser against grubs of Japanese beetle, *Popillia japonica* (Newman), was obtained, but when assayed in field and laboratory, no difference in infectivity was detected when compared to wild-type strains (Gaugler and Campbell 1991). Similarly, an increase of 20–27-fold for host-finding ability has been demonstrated in *S. carpocapsae* towards wax moth, *Galleria mellonella* (Linnaeus), after 13 rounds of selection (Gaugler et al. 1989; Gaugler and Campbell 1991).

15.3.4.2 Adaptability to Environment Stress

Selection has also been successful in enhancing infectivity at low and high temperatures. Screening natural entomopathogenic nematode populations for desirable biological traits, while very laborious, is feasible and has shown some success in isolating heat- and cold-tolerant strains (Glazer et al. 1996; Mracek et al. 1998). Grewal et al. (1996) showed that selecting nematodes for cold tolerance enhanced their virulence by 5.3 and 6.6 times after six and twelve passages at 8 °C. A high variability in tolerance among strains and comparatively high heritability for the adapted heat tolerance were observed in 36 natural populations and 18 hybrid or inbred strains of *Heterorhabditis bacteriophora* Poinar tested for response to high temperature (Mukuka et al. 2010a). Similarly, variability in desiccation tolerance has also been documented in 43 strains of *Heterorhabditis* and 18 hybrid/inbred line strains of *H. bacteriophora* (Mukuka et al. 2010b). Desiccation-tolerant mutants of *Heterorhabditis megidis* Poinar have been isolated after exposing to ethyl methanesulphonate (EMS). Mutagenesis induced by exposing young *H. bacteriophora* hermaphrodites to EMS has been also documented (Koltai et al. 1994). Hybridisation can also offer powerful tool for genetic

improvement of entomopathogenic nematodes to produce superior strains. A trait for heat tolerance was transferred from *H. bacteriophora* strain designated IS5 discovered in the Negev desert in Israel to the wild-type *H. bacteriophora* HP88 strain with no loss of fitness in hybrid progeny as compared to wild strain (Glazer and Segal 2003). Hybridisation of heterogeneous population of *S. feltiae* for desiccation tolerance and host-seeking ability resulted in a survival rate of 80–90 % for tolerance to rapid desiccation, more than 85 % for tolerance to slow desiccation and more than 75 % host-seeking ability after 10, 20 and 25 selection cycles, respectively (Salame et al. 2010).

Genetic engineering provides significant advantage over conventional methods in improving the efficacy of EPNs. This approach involves microinjection of foreign DNA into the gonads of a young adult female or a hermaphrodite or through biolistic DNA bombardment. For the first time, successful transformation of *H. bacteriophora* was made by microinjection of plasmid vectors carrying hsp-16 genes coding for 16-kDa heat-shock protein as well as *rol-6* gene coding for roller phenotype (Hashmi et al. 1995). *H. bacteriophora* has also been genetically improved for thermotolerance by transfer of gene from *Caenorhabditis elegans*, *hsp70A*, encoding heat-shock protein which enables cells to eliminate or renature proteins damaged by high temperature. The transgenic nematodes exhibited 18-fold tolerance to heat shock as against wild ones (Hashmi et al. 1998). Transformation of *tps-1* (trehalose-6-phosphate synthetase) gene and heat-inducible promoter derived from *C. elegans* into *Steinernema feltiae* resulted in transformed nematodes showing tolerance to desiccation and osmotic loss (Vellai et al. 1999). Currently, genome of TTO1 strain of *H. bacteriophora* is being sequenced at the Washington University Genome Sequencing Center in St. Louis. The completion of the genome sequence will establish a solid foundation for the much needed functional genomic studies on the genes that are involved in critical biological processes of nematodes, viz. dauer formation, stress resistance, sex determination, etc., and functions of these genes will be elucidated using RNA interference technology (Jindal et al. 2010).

15.3.4.3 Tolerance to Nematicides

Entomopathogenic nematodes have also been selected for enhanced resistance to nematicides and other insecticides. Genetic selection of *H. bacteriophora* strain HP88 to nematicides resulted in 70-fold increase in resistance to oxamyl and eight- to ninefold increase in resistance to phenamiphos and avermection (Brey and Hashmi 2003).

15.4 Conclusions

The exploitation of biocontrol agents (parasitoids, predators and pathogens) could provide an excellent alternative to chemicals for pest management as they are economically viable and ecologically safe. However, limited success has been realised with augmentative biological control due to limited research efforts. The major obstacles in their widespread use include sensitivity to adverse environment conditions, narrow spectrum of activity and slow speed of kill. Therefore, increasing efforts need to be devoted to overcome these limitations so that these can be utilised for the efficient biological control programmes. This may be achieved by conventional as well as genetic engineering or recombinant DNA techniques. The conventional methods include strain selection, serial passage, mutation, conjugation, transduction, natural selection, selective breeding, hybridisation, etc., whereas the genetic engineering approaches comprise indirect gene transfer methods (vector mediated) by using *Agrobacterium tumefaciens* as vector and direct gene transfer (vectorless methods) such as microprojectile bombardment with DNA or biolistics, direct DNA transfer into isolated protoplasts, electroporation, microinjection, etc. It is worthwhile to mention that the tools of molecular biology have led to the development of more effective, faster acting baculoviruses, bacteria, fungi and nematodes that could

provide a valuable tool for sustainable pest management. This has led to the improvement in their genetic makeup providing broad range insect control with desired pathogenicity, virulence, host range and persistence. Among these entomopathogens, genetically modified bacteria have reached the commercial sector, and recombinant baculoviruses are expected to mark their appearance in the near future. The engineering of entomopathogenic nematodes and fungi is in its infancy, slowed in part by the greater genetic complexity of these organisms. However, genetic improvement of arthropod natural enemies has received little attention and is only restricted to selection process for improved strains with tolerance to pesticides and high temperature. The thoughtful study of genetics and physiology of reproduction through biotechnological interventions could play an important role in improving mass production of arthropod natural enemies. The prospects for genetic improvement of biocontrol agents are exciting; however, as with all other initiatives that involve release of genetically modified bioagents, there is significant concern. The potential environment risks associated with the release of these genetically manipulated bioagents need to be addressed at all four trophic levels if we are to really exploit these biotechnological interventions in improving the biocontrol agents for sustainable pest management.

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