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Molecular approaches for the improvement of natural enemies

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Abstract
Integrated Pest Management (IPM) tactics employing living entities and chemical control measures need to be adopted in such a manner that the biological control agents are least affected by chemical pesticides, environmental vagaries and other factors hindering their progress. Conventional and molecular methods can be adopted to enhance their level of tolerance. The former utilizes the techniques of artificial selection and hybridization, while mutagenesis and recombinant DNA technology constitutes the latter. Suitable interventions adopted at genetic level can make entomopathogens, predators and parasitoids more tolerant at field scale. Hence, there is ample scope for the adoption of genetic improvement projects of natural enemies. Meanwhile, we should also take into consideration various underlying risk factors concerned with the development of Genetically Modified Organisms.

Keywords: Molecular, recombinant DNA technology, genetic improvement

Introduction
Insect pests affecting crops often pose a great menace to the farming community. According to a report published by the Food and Agricultural Organisation (FAO, 2018) [4], the total loss in food grains caused by pests, including insects is around 1.3 billion tonnes per year. Here emerged the vigour and vitality of the concept 'Integrated Pest Management' (IPM). For the successful implementation of this very concept of IPM, there should be a vivid synchronization between biological control measures employing living entities, environmental conditions prevailing in the region and chemical control measures. Natural enemies can be integrated into the system of IPM after making desirable changes at the genome level. Hence interventions are needed at the molecular level for the effective manipulation of natural enemies.

Factors affecting natural enemies
Climatic conditions
Parasitoids and predators are affected by the climatic conditions in the region where they are being introduced. Entomopathogens often fail to tolerate temperature and humidity beyond a threshold limit. Nematodes are highly vulnerable to dessication which reduces their shelf life.
The optimum temperature required for the survival and greater predatory potential of natural enemies depends on the ambient conditions prevailing in their native. The investigations by Singh and Jalali (1994) [27] have shown that temperature higher than 35 ^{0}C prevents the emergence of trichogrammatids. According to Lord (2005) [12], preincubation of *B. bassiana* conidia at 30 ^{0}C and 75 per cent humidity for five days resulted in reduced mortality in adults of lesser grain borer, *Rhizopertha dominica* Fabricius.

Host range
Due to high specificity in the mode of entry of entomopathogens, they have restricted host range.

Chemical pesticides
According to Stark *et al*. (2007) [30], sub lethal application of insecticides may disrupt predatory behaviour and reduces their efficacy in locating prey due to its repellent and antifeedant effects.
The acaricides belonging to the organophosphate and organostanic chemical groups completely inhibited conidial germination, vegetative growth and sporulation of *B. bassiana*

(de Oliviera and Neves, 2004) [2]. The detrimental effect of pesticides on the parasitic wasp *Cotesia marginiventris* (Cresson) was studied by Ruberson and Roberts (2005) [23] who have found out that the development of both males and females of the wasp was delayed by lambda-cyhalothrin, dicrotophos and emamectin benzoate by a day. Survival of the parasitoid was also marginally affected by insecticides, with the lowest survival occurring in emamectin benzoate (76.7 per cent) and lambda-cyhalothrin (80 per cent) treated populations. Laznik and Trdan (2013) [11] reported that the nematode *Heterorhabditis bacteriophora* Poinar is sensitive to the insecticides abamectin and lufenuron.

Time taken to induce mortality

It takes two to three weeks for entomopathogens to kill the insects whereas chemical insecticides may need only two to three hours. This may be due to the fact that as the natural enemies are living entities, they produce toxin only after completing certain physiological processes within the insect body, resulting in more time of kill.

Methods employed for the improvement of natural enemies

1. Conventional approaches

Conventional methods employed for the improvement of natural enemies include artificial selection and hybridization.

a. Artificial selection

Artificial selection or selective breeding refers to a process whereby the breeder chooses to perpetuate only those forms with certain desirable inheritable characteristics. The procedure of artificial selection as detailed by Hoy (1985) [8] is as follows:

i. Determination of traits that need improvement in a potentially effective natural enemy
ii. Population sampling of natural enemy
iii. Rearing and maintenance of natural enemy
iv. Selection of natural enemy
v. Assessment of fitness of natural enemy through lab, green house or field cage studies and genetic analysis.
vi. Determination of field release strategy, followed by release of natural enemies and evaluation of their efficacy. If found effective, the technology is implemented.

Jalali *et al.* (2006) [9] developed strains of *T. chilonis* adapted to low temperature (18-24°C) by rearing it for 30 continuous generations. *T. chilonis* with tolerance to organophosphate insecticide, endosulfan was developed by Jalali *et al*. (2006) [10] and it was marketed under the trade name 'Endogram' (Navik and Varshney, 2018) [16]. Venkatesan *et al*. (2009) [35] reported that populations of common green lacewing, *Chrysoperla zastrowi arabica* (Esben-Petersen) developed resistance towards the organophosphorus insecticide, monocrotophos. Hexythiazox resistant populations of predatory mite, *Neoseiulus californicus* was developed by Salman and Ay (2013) [24]. Srivastava and Singh (2015) [29] developed high temperature tolerant strains of *T. chilonis* (HTTS) which can tolerate 27-31 ^{0}C temperature through selective breeding. Venkatesan (2016) [33] developed *T. chilonis* (strain HTTS) with resistance to high temperature (32-40^{0}C) for using in high temperature affected fields. Multiple Insecticide Tolerant Strain of *T. chilonis* (MITS-TC), with resistance to a wide array of insecticides with broad spectrum of action *viz*., profenophos, chlorpyriphos, chlorantraniliprole and fenvalerate was developed by Nayak *et al*. (2018) [17].

B. bassiana can tolerate upto 32 times the field dose of imidacloprid, 16 times the dose of carbendazim and carbosulfan and eight times the dose of chlorpyriphos, lambda cyhalothrin, malathion and mancozeb after 10 passages through respective poisoned media (Joseph, 2014) [10]. Nilamudeen (2015) [18] reported that the isolates Bb5 and Bb21 of *B. bassiana* and Ma4 of *M. anisopliae* were able to thrive four, eight and eight times the field doses of acephate, chlorantraniliprole and thiamethoxam, respectively after passing 10 times through respective poisoned media.

b. Hybridization

Hybridization refers to the process of crossing two genetically different individuals of different species (interspecific hybridization) or same species (intraspecific hybridization).

Interspecific hybrids of *Chrysoperla* with increased fertility were developed by Naka *et al*. (2005) [15] by crossing between green lacewings of indigenous origin, *C. carnea* and that introduced from Germany, *Chrysoperla nipponensis* (Okamato). Mukuka *et al*. (2010) [14] developed intraspecific hybrid strains of *H. bacteriophora* which were heat-tolerant (40-42^{0}C) and desiccation tolerant (water activity – 0.65 a_w). Venkatesan and Jalali (2015) [34] developed *T. chilonis* (strain-MITS) which can tolerate high temperature (32-38^{o}C) and chemical insecticides *viz*., endosulfan, monocrotophos and fenvalerate.

There are certain limitations for the adoption of conventional breeding techniques as pointed out by Simmonds (1963)[26]. Due to inherent complexity of the natural environment, the exact factor to which a beneficial organism is susceptible is difficult to determine *ex situ*, and therefore cannot be dealt with in the laboratory. Furthermore, lack of knowledge concerning the genetic basis for inheritance of desirable characteristics in beneficial organisms makes selective breeding a difficult and lengthy proposition. It is also necessary to obtain a sufficiently broad genetic diversity to assure the availability of suitable characters on which selection can be adopted.

2. Molecular approaches

a. Mutagenesis

Mutagenesis is a process by which the genetic information of an organism is changed, resulting in mutation. It may occur spontaneously in nature or as a result of exposure to mutagens. Mutagens can be classified into three types based on their origin. They are physical mutagens, chemical mutagens and biological mutagens. Physical mutagens include ionizing radiations such as X-rays, gamma rays, alpha particles and ultraviolet radiations. Chemical mutagens include arsenic, nickel, chromium and benzene and biological mutagens include transposons and viruses.

Mutagenesis of vegetative cells of *B. thuringiensis* using Ultra Violet radiation (UV) and nitrous acid resulted in enhanced production of delta-endotoxin (Ghribi *et al*., 2004). Avanti *et al*. (2014) [1] developed thermotolerant mutants of *B. bassiana* and *Lecanicillium lecanii* Zare & Gams using physical mutagens *viz*., moist heat stress (35^{0}C) and UV. It was observed that the mutants yielded more conidia and higher biomass. Shinohara *et al*. (2013) [25] developed mutants of *Isaria fumosorosea* Wize using ion beams and gamma rays to provide enhanced resistance against the broad spectrum

fungicide, benomyl. Fungicide tolerant strains of *Beauveria* were developed by Fitriana *et al.* (2015) [5]. The strains BB22 and BB24 with tolerance to the broad spectrum fungicide benomyl were developed by exposing these to ion beams and gamma rays. The study revealed that the strains were 500 and 800 times more tolerant to benomyl than wild strains in exhibiting vegetative growth. Hmani *et al.* (2018) [7] conducted mutagenesis of vegetative cells of *B. thuringiensis* by using UV and nitrous acid and found that the mutagens enhanced the production of Vip3Aa16 toxin. Vegetative insecticidal proteins (Vip) are insecticidal proteins produced during the vegetative growing phase of fungi. These include Vip1, Vip2 and Vip3, out of which Vip3 is infective to lepidopterans. These proteins bind with specific receptors, different from that of Cry protein, resulting in pore formation in midgut which finally leads to paralysis and death. Sumaya *et al.* (2018) [31] created mutants of *H. bacteriophora* with high longevity (5.8 days longer than susceptible line), using Ethyl Methyl Sulphonate (EMS). *H. bacteriophora* is an entomopathogen associated with the enteric bacterium, *Photorhabdus spp.* and it is used to control all pests including moths, beetles, flies etc.

Recombinant DNA Technology

Recombinant DNA (rDNA) technology or genetic engineering refers to the artificial synthesis or isolation of specific genes or Deoxyribo Nucleic Acid (DNA) fragments and introducing these into the genome of host organism.

The steps in recombinant DNA technology involve isolation of DNA, fragmentation, amplification of gene of interest and insertion of recombinant DNA into the host. Recombinant DNA can be introduced into the host by means of vector-mediated or vectorless methods.

In vector-mediated method, vectors like plasmids and bacteriophages help in carrying and integrating the desired gene into the genome of the host. St. Leger *et al.* (1996) [33] developed the first recombinant fungi gene encoding insect cuticle degrading protease PR1A into *M. anisopliae*, resulting in 25 per cent reduction in time of kill in *M. sexta.* Lu *et al.* (2008) [13] developed genetically engineered *B. bassiana* by incorporating insect-specific scorpion neurotoxin gene AaIT from *Androctonus australis* Hector and PR1A gene from *M. anisopliae*. It was observed that Bb13T and Bb13TPR1A were able to reduce the Median Lethal Times by 40 per cent and 36.7 per cent, respectively against Masson pine caterpillar, *Dendrolimus punctatus* Germar and 24.4 per cent and 20.9 per cent, respectively against greater wax moth, *Galleria mellonella* Fabricius. Incorporation of tyrosinase gene in *B. bassiana* using *Agrobacterium tumefaciens* Smith & Townsend resulted in development of UV tolerant strains of *B. bassiana*. (Shang *et al*., 2012) [26]. As per the findings by (Zhang *et al*., 2014) [39], strains of *L. lecanii* transformed with carbendazim tolerant gene (mrt) from *Botrytis cinerea* exhibited 380-fold resistance to the fungicide. A recombinant *H. armigera* NucleoPolyhedroVirus (HearNPV), Ar1b-HearNPV, was constructed and identified as an improved bio-control agent of *H. armigera* larvae by Yu *et al.* (2015). The plasmid pFastBac HTb was used to express the insect-specific neurotoxin gene, *ar1b*, which was originally isolated from the Sydney funnel-web spider (*Atrax robustus*). This recombinant virus also showed a 32.87 per cent decrease in LT_{50} assays compared with the wild type virus. Deng *et al.* (2019) [3] developed a transgenic strain of *B. bassiana* expressing Cyt2Ba toxin from *B. thuringiensis* using the plasmid pBARGPE1 *via* electroporation. They have observed that the LT_{50}s of the female *A. aegypti* treated with Bb-Cyt2Ba were reduced by 47 per cent compared with the treatment with wild strains @ 10^6 conidia mL^{-1}. Cytolytic δ-endotoxin (Cyt2Ba) is toxic to the larvae of *Culex*, *Aedes* and *Anopheles* and the adults of these mosquitoes act as vectors of several viral diseases.

Vector-less methods include protoplast fusion, electroporation, microinjection and microprojectile method.

Protoplast fusion

Protoplast fusion refers to adherence of protoplasts with one another either simultaneously or in the presence of fusion inducing chemicals. Sirisha *et al.* (2010) [28] successfully accomplished Poly Ethylene Glycol (PEG) mediated protoplast fusion between strains of *M. anisopliae* and *B. bassiana* with fast mycelia growth and abundant sporulation. Moreover, the activities of genes coding for Pr1 and Pr2 genes which code for protease enzyme were found to be doubled.

Electroporation

Electroporation is the introduction of foreign DNA into cell by a brief exposure to very high voltage electric pulses or more exposure to low voltage pulses ranging from 4000-8000 Vcm^{-1}. This induces transient pores in cell membrane which facilitates the uptake of DNA. The advantages of this method is that it is applicable to almost all the cell types, most effective method as majority of cells take up the target DNA, only small quantity of DNA is needed to adopt this technique. But, irregular pulse intensity can result in irreversible damage in cell wall. Development of herbicide tolerant strains of *L. lecanii*, with tolerance to phosphinothricin herbicide with broad spectrum of action (Timofeev *et al*., 2019) [32].

Microinjection

Microinjection is the technique of delivering foreign DNA directly into the cytoplasm or nucleus of the cells using micromanipulators, *viz*., micropipette and microinjection needle. Through the technique of microinjection/maternal injection, Presnail and Hoy (1992) [20] were able to transform predatory mite, *Metaseiulus occidentalis* (Nesbitt), with a heat shock protein (hsp 70) from Drosophila. Gravid females of *M. occidentalis* with eggs that are nearly, but not fully mature were selected for the research. The plasmid used for transformation contained *Escherichia coli* β-galactosidase gene (*lacZ*) regulated by the Drosophila *hsp70* heat-shock promoter. Plasmid suspended in an injection buffer was injected with the help of microinjector and Zeiss inverted compound microscope. It was injected between third and fourth coxae such that the needle penetrates the egg. Progeny emerged from these eggs (G_1) was assayed for transformation using β-galactosidase assay. For assessing the efficacy, G_1 larvae were given heat shock of 37 ^{0}C for 30 minutes. Followed by this, the transformed strains were tested for β-galactosidase activity. It was observed that transformed larvae with *lacZ* gene were visualised in blue colour and out of the 49 established lines, only seven were able to express lac-z gene. Maternal microinjection was also attempted with a braconid parasitoid wasp *Cardiochiles diaphaniae* by the same authors in the year 1996.

M. occidentalis is Western predatory mite, which belongs to the family, Phytoseiidae. They are used as predators of mites, which act as insect pests in North American orchards.

Drosophila is usually taken as the model organism for genetic investigations due to its short life cycle, ease of culture and maintenance and presence of a low number of chromosomes. High temperature often acts as one of the barrier for the application of natural enemies. Heat Shock Proteins (HSPs) or stress proteins refer to a family of proteins that are produced by cells in response to exposure to stressful conditions. They were first described in relation to heat shock, but are now known to be expressed during other stresses including exposure to cold, UV light and during wound healing. HSPs are named according to their molecular weight, out of which hsp70 is the most common. According to them, this technology can be extended to other mites of the family Phytoseiidae too.

Hashmi *et al*. (1995) [6] introduced foreign genes from *Caenorhabditis elegans* (Maupas), a free-living nematode that encodes for the roller phenotype and 16 kDa HSP (hsp16) into *H. bacteriophora* HP88 by microinjection using vectors carrying the gene.

Microprojectile method

In microprojectile or biolistic or particle bombardment method, foreign DNA is delivered into cells when heavy microparticles of tungsten or gold-coated DNA are pushed into plant cells at a very high speed using high voltage electric discharge. The advantages of this technique are that DNA can directly penetrate the cell, genome of sub-cellular organelles can be manipulated and use of harmful viruses as gene carrier can be avoided. Disadvantages include lower transformation efficiency, need of special equipments and chances of cell damage. Inglis *et al*. (2000) transferred bar gene from *Streptomyces hygroscopicus* (Jensen) Yuntsen offering resistance to the herbicides, bialaphos and Glufosinate Ammonium (GA) into *M. anisopliae* var. *acridum*.

Risks concerned with genetically modified organisms

Prakash *et al*. (2011) [19] outlined various risk factors linked with Genetically Modified Organisms (GMOs). Genetic contamination or interbreeding occurs when Genetically Modified Organisms (GMOs) interbreed with the wild-type, resulting in disappearance of novel traits in wild types. Faster growth of GMOs can enable them to have a competitive advantage over the native organisms. This may allow them to become invasive, to spread into new habitats and cause ecological and economic damage. Selection pressure may increase on target and nontarget species to adapt to the introduced changes and genetic change can destruct the ecosystem. Once the GMOs have been introduced into the environment and some problems arise, it is impossible to eliminate them. In genetically modified microorganisms, there can be horizontal transfer of recombinant genes to other microorganisms, which can further lead to adverse effects on the health of people or the environment, unpredictable and unintended effects, long term effects and ethical concerns.

Genetic improvement projects of natural enemies

According to Routray *et al*. (2016) [22], the following are the factors to be considered while undertaking genetic improvement projects of natural enemies. Initiation of genetic improvement projects demands the need to identify the factors limiting the efficacy of natural enemy. A great deal must be known about the biology, ecology and behaviour of the natural enemy. Moreover, genetic variability must be available upon which one can select using artificial selection. If such variability does not occur in natural populations, it must be provided for through mutagenesis or through recombinant DNA methods. The improved natural enemy must be documented to be effective in the field and the cost of the project should be justified by the benefits achieved.

Conclusion

We should have a definite and distinct knowledge on various factors affecting the sustenance of natural enemies, which act in the ecosystem. Various molecular methods can be used for the manipulation of natural enemies with recombinant DNA technology standing in the forefront. Development of 'lab creatures' with improved traits through molecular methods can enhance their efficiency thereby providing assured rates of pest control. Hence, we should utilize the emerging techniques in the field of molecular biology so that 'farmers' friends' can be successfully manipulated.

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