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BIOTECHNOLOGICAL TECHNIQUES-DNA BARCODING, RNAI, SIT, AND BIOPESTICIDES IN INSECT PEST MANAGEMENT FOR SUSTAINABLE AGRICULTURE DURING 21ST CENTURY

REKHA[≡] [a*], SHIKHA RANA[a] AND ANU [b]

[a] W. R. S. Govt. College Dehri, HPU Shimla, India.
[b] Department of Zoology, Punjabi University Patiala, India.

AUTHOR'S CONTRIBUTION

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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Review Article

ABSTRACT

Biotechnology is based on the understanding of DNA and thus helps in the manipulation of genes through genetic engineering with molecular markers and tissue culture. Recently, Biotechnology not only provides new varieties of crops (agriculture biotechnology) but has also played an important role in insect pest management with Integrated Pest Management (IPM). IPM is an environment-friendly approach that focuses on keeping the pest population at below economic threshold levels by employing all available alternate pest control methods and techniques such as cultural, mechanical, and biological(habitat manipulation) with emphasis on the use of bio-pesticides and pesticides from the plant (By Ministry of Agriculture and farmers welfare, India). Biotechnological techniques integrated with entomology include -DNA barcoding play important role in molecular taxonomy through recombinant DNA technology, RNAi gene regulatory process, Insect-resistant crop varieties, sterile insect technique, and biopesticides formulation which dynamically control the insect pest population.

Keywords: DNA barcoding; RNAi; SIT; Biopesticides.

1. INTRODUCTION

Recently biotechnology has provided additional tools to restraint the damages caused by insect pests integrated with solutions against the hazardous effect of traditional and chemical methods of insect pest control [1][2]. The benefits of biotechnology are especially meaningful at a time when our global

[≡] *Assistant Prof. of Zoology;*
**Corresponding author: Email: r.tapasvi3@gmail.com;*

population is growing and our demand for food is increasing these insect pests are the biggest challenge for our generation. Last year in 2020, India has faced one of the worst locust attacks in decades which caused large-scale destruction and threatened the food security of the country. So, the foremost step to control these insect pests is – timely identification. DNA barcoding provides a new method for the identification of species that is swifter and more accurate than traditional methods. Through this technique, insect pest species can be identified at any stage of life and also the biotypes which certainly not possible through traditional morphology identification methods [3]. Genetically RNA interference (RNAi) is a natural mechanism in which RNA molecules through translation or transcriptional repression are involved in sequence-specific gene silencing (suppression). RNAi can play a most important role in sustainable agricultural projects and integrated pest management that are required globally. By the biotechnological techniques, the genetically modified organism has been produced whose genome altered at molecular level thus help in controlling the vector-borne diseases in plants and animals.

2. DNA BARCODING

Agriculture plays a vital role in the boosting up of the economy of any country. But Pest management in agriculture is the biggest problem in the present scenario. Insects can become pests on the farm when they cause damage to the crop. Insects having chewing and piercing-sucking mouthparts cause a lot of damage to the crop. Most of the insect pest includes aphids, locusts, scales, spider mites, and whiteflies. These cause two types of damage one- by direct mode in which the plant is directly injured and the other by indirect mode in which these transmit viral pathogens[4][5]. To effectively control a pest, its accurate identification is the most challenging task because of its complex life cycles and cryptic species [6]. In general, a barcode is a set of lines of different widths and sizes representing data, that when read help identify the scanned object similar to this in the genome of living organism analogous to barcodes used for their identification. DNA barcoding can be an important technique for molecular identification of species in their distinct life stages, types [7], host-associated genetic variation [8], and the distinction between cryptic species [9]. In this technique, standardized DNA sequences are used as markers for the recognition of species [10] and a taxonomically unknown specimen compared with a reference library of barcodes of known species origin to establish a species-level identification [11].

For identification of the animal species, the mitochondrial gene cytochrome c oxidase subunit 1 (cox1 or COI; 648bp) of a short fragment of DNA sequence act as a standardized single universal molecular marker [12][13]. The mitochondrial genome of animals is a more reliable objective for analysis than the nuclear genome because of its unique features-

a) The mitochondrial genome lack introns, limited exposure to recombination because mitochondria are maternally inherited and have the haploid mode of inheritance [14].
b) In most animal phyla, the universal primers for the mitochondrial genes are very vigorous and enable recovery of its 5' end [15] [16]. The mitochondrial protein-coding gene contains more differences than the ribosomal gene. Therefore they are more likely to distinguish among closely related species.
c) Cytochrome c oxidase gene has a high dimension of phylogenetic signal than any other gene in mitochondrial genome[12] and has a high mutation rate as compared to the nuclear genome, which results in a high ratio of intra-specific divergence, significant in evolutionary studies[17][18].

Procedure for the DNA barcoding (Fig-1): DNA barcoding has four main steps-DNA.

(I)**DNA Extraction**: First of all collect the sample/tissue from the unidentified specimen (take a small amount and rest of specimen preserved). The DNA extraction involved- lysis of cell and nucleus, precipitation- separates the freed DNA from cellular debris and purification of DNA. This is done by using various DNA extraction methods – Phenol-chloroform method, Proteinase K enzyme, Silica column-based extraction method, and DNA extraction by magnetic beads.
(II) **PCR amplification**: Universal primers like LCO1490 [15], LepF1, and LepR1 [19] are used to amplify a known region of the cytochrome oxidase I (COI) gene.
(III) **DNA Sequencing**: It is a process of determining nucleotides sequence in DNA by using the Sanger Sequencing method.
(IV) **Analysis:** Various programs can be used to analyze the DNA sequence- Barcode of Life Data Systems (BOLD) and National Centre for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST).

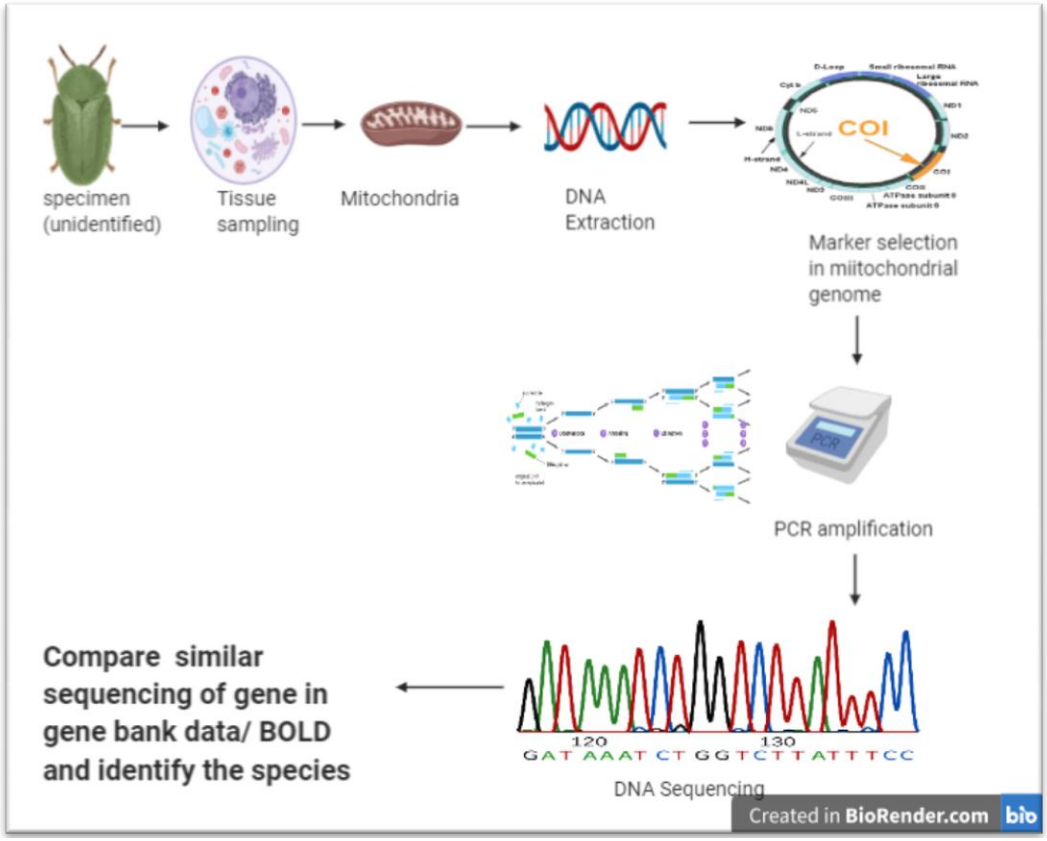

Fig. 1. Main steps of DNA barcoding – unidentified insect pest collected and DNA extracted, selected appropriate marker, amplify the DNA sequence with PCR amplification, DNA sequencing (nucleotide-influenced the amino acid sequences of proteins), and finally analyzed the data

The Barcode of Life Data Systems (BOLD) is an online - data storage and analysis program for DNA barcode records [20]. It consists of four main components, a data portal, an educational portal, a registry of Barcode Index Numbers (BINs) (putative species), and a data collection and analysis workbench.

3. RNAi EFFICACY

In recent few years, Genetically modified crops have been very successful in controlling insect pests and have also reduced the use of chemical pesticides[1][21] but the insect pests have evolved field resistance against insecticidal toxin by the swift evolutionary process [22], so not so successful to completely control the insect pest. Although,RNA interference (RNAi) provides a great assurance for effective control of agricultural insect pests [23] through sequence-specific gene silencing mechanism. RNA interference (RNAi) is activated by double-stranded RNA (dsRNA) and is likely to be the novel approach for the next generation of insect pest-resistant transgenic plants [24]. RNAi pathway is found naturally in many animals; performs the function in defense mechanism against pathogens like viruses and transposable elements. dsRNA is processed into short RNA duplexes which perform the following activities- used to guide the recognition of their target, cleave a complementary mRNA, repress their protein synthesis at a posttranscriptional silencing level, and alter their chromatin structure at the transcriptional level[25].

RNAi Pathways- There are three RNAi pathways in insects [26][27]:

i) siRNA(small interfering RNAs- 20–25 nucleotides) mediated pathway.
ii) miRNA(micro RNAs- 21–24 nucleotides) mediated pathway.
iii) piRNA (Piwi-interacting RNAs- 24–30 nucleotides) mediated pathway.

Mechanism: At the cellular level, these RNAi pathways are interconnected and protect the organism from pathogens by gene regulation [28] [29]. In the RNAi pathway, siRNAs are found to be excised from long, fully complementary double-stranded RNAs (dsRNAs) [30], and these dsRNAs can derive directly

from RNA virus replication (exogenous in origin) naturally. The biogenesis of siRNA requires the RNase-III Dicer enzyme [31] also known as endoribonuclease dicer. miRNAs are generated from endogenous transcripts that form stem-loop structures that guide RNA silencing. Inside the cell, in the nucleus, the biogenesis of miRNAs requires- RNase-III-family enzyme Drosha that recognized these hairpin structures and cleaved them into precursor pre-miRNAs then pre-miRNAs are transported to the cytoplasm by Exportin-5[32][33][34]. In the cytoplasm, the pre-miRNA is further processed by the RNase III endonuclease Dicer into a mature miRNA duplex [35]. Finally, RNA-mediated gene silencing complexes are formed by both siRNA and miRNA[36]. Finally, miRNA duplex is loaded into the Argonaute (AGO) family of proteins to form a miRNA-induced silencing complex (miRISC) or miRNA containing effector complex of RiboNucleo Protein particles(RNP)[37][38] and siRNA also form RISC (RNA Induced Silencing Complex) or RITS (RNA-Induced Transcriptional Silencing) complexes. Argonaute proteins are the highly specialized components for silencing complexes (RISC, RITS,RNP)[39] and perform a function- guide RNA to associate with target RNAs and slicing of these targets, blocking the translation in miRNP and with rasiRNA (repeat-associated short interfering RNA) - a specialized nuclear Argonaute-containing complex- guides the condensation of heterochromatin by the RITS complex[40].

piRNAs (PIWI-interacting RNAs) are found in clusters form in the genome and protect germ cells from transposable elements expressed in invertebrates and vertebrates e.g. in the genome of *Drosophila melanogaster* have three Piwi proteins - Piwi, Aub (Aubergine), and Ago3 (Argonaute). The biogenesis of piRNAs is initiated from long single-stranded precursor transcripts and this mechanism requires endoribonucleases- zucchini (ping-pong mechanism) [41] instead of Dicer. After the recognition of mRNA of a transposable element, piRNAs are formed in the cytoplasm through the slicer activity which is done by endonuclease- Piwi proteins, Aub, Ago3 and cleaved by Zucchini and trimmed by the exonuclease Nibbler [42]. Finally, piRNAs silencing is processed by promoting-methylation of DNA and heterochromatinization [43].

Insects can acquire dsRNA from the outer environment thus spreading it to different cells of the body [23]. The uptake of dsRNA in insects is explained by two types of mechanisms- transmembrane channel-mediated uptake mechanism based on transmembrane proteins [44] and an endocytosis-mediated uptake mechanism based on receptor-mediated endocytosis [45]. The strategy of RNAi in insect pests control – Identification of target gene, designing of dsRna molecule, Protein stability, and phenotypic analysis, delivery of dsRNA and effect on insect pests as shown in Fig.2-

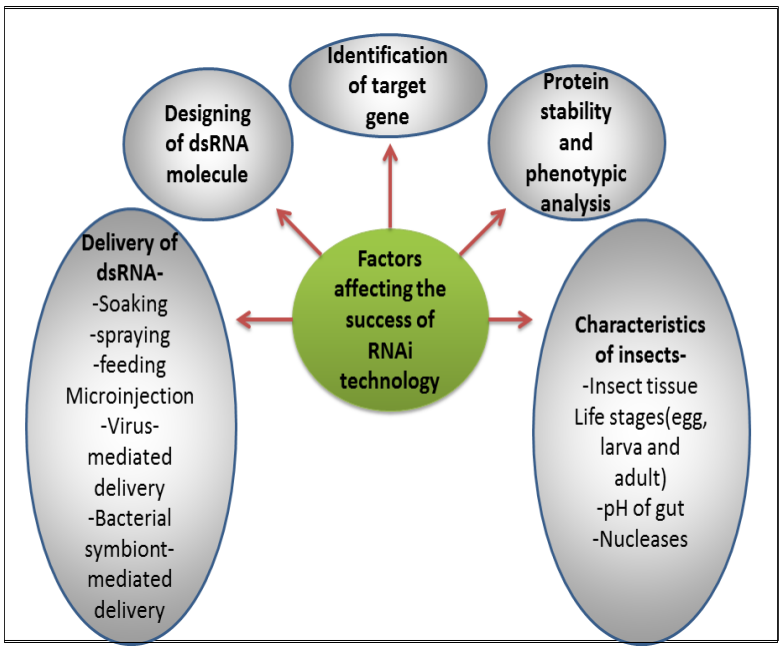

Fig.2. RNAi strategy in insect pests management

3.1Use of RNAi in genetically modified (GM) plant against insect-pest

The most successfully commercialized genetically modified (GM) crops for insect-pest management have are based on Bacillus thuringiensis (Bt) toxins [46], this toxin kills the insect pest by attacking on the gut epithelium [86]. For plant-mediated RNAi pathway- dsRNA derived from hairpin RNA (hpRNA) which engineered in plant crop to target the genes in insect pests[47][23]. There are some examples of some insect pests-

Common name (with order)	Scientific name	Target Gene	Effect on insect pest	Reference
Western corn rootworm (Coleoptera)	*Diabrotica virgifera virgifera*	vATPaseA	larval stunting and mortality	[48]
Cotton bollworm (Lepidoptera)	*Helicoverpa armigera*	CYP6AE14	Larval growth retardation	[47]
Colorado potato beetle (Coleoptera)	*Leptinotarsa decemlineata*	LdJHAMT	Reduced pupation rate	[49]
Cotton bollworm (Lepidoptera)	*Helicoverpa armigera*	HaHR3	larval mortality	[50]
Green peach aphid (Hemiptera)	*Myzus persicae*	MpC001, Rack1	Progeny reduced	[51]

There are several challenges in the application of RNAi-based technology used for insect pest control-off-target and non-target effects of dsRNA, the evolution of RNAi resistant insects, and efficient dsRNA delivery, antibiotic-resistant marker genes, and most important point highly efficient dsRNA with a low-cost price. Nanoparticles are emerging as a new tool for dsRNA delivery for insect pest management.

4. STERILE INSECT TECHNIQUE (SIT)

In this technique, large numbers of sterile individuals are released in the wild population which compete for mating and causes blocking of population growth mechanisms for inducing sterility including [52]-.

(I)**Irradiation** – Mostly used for sterilization the insect exposed through X-rays and γ rays affect germline-cells. It causes chromosomal disintegration and the death of progeny in the early stages of development.

(II)**Chemosterilization**- chemical compounds are used to control insect pests mainly vectors of diseases by causing temporary or permanent sterility. This chemical includes- Aziridine-phosphoryl, Non-alkylating Dimethylamines, Non-aziridine Alkylating Agents, and Juvenile Hormone Analogs[53].

(III)**Transgenic technology (Genetically modified insect)** - In this technology - insertion of non-transposons elements are inserted into the genome (especially to produce males) through transgenic technology. This process reduces the reproductive capacity of the population because transgenic males when mate with the wild female population leads to embryonic lethality [54]. This Technique is helpful in controlling Anopheles populations. As referred by Harvey [55] Male selecting gene OX4319L (transgene) engineered in *Plutella xylostella*(crucifers pest) into wild-type populations led to rapid pest population decline, and then cause elimination.

RIDL (Release of Insects carrying a Dominant Lethal) is as similar to SIT in this technique sterile insects are engineered with having lethal gene in their genome so when released in wild population these insects cause decline population decline – progeny died before maturity.

5. BIOPESTICIDES AND BIOTECHNO LOGY

In the last few years many chemical pesticides have been banned in India, because of their hazardous effect on the environment(highly toxic to honeybees) as well as causing severe health issues [91] such as carcinogenic and neurotoxic [56]. So, Biopesticides are the best alternative to chemical pesticides because of their eco-friendly approach and fewer side effects on humans. These are categorized (Fig.3) as microbial biopesticides derived from viruses, bacteria, fungi, nematodes, botanical bio pesticides derived from plant secondary metabolites [57], and biochemical pesticides derived from pheromones. For making these biopesticides highly specific and highly targeted biotechnology play a very significant role by manipulating desirable trait.

Microbial biopesticides: ***Bacillus thuringiensis* (Bt)**, a soil-inhabiting bacterium produces Cry-toxin (Bt toxin) protein which is fatal to many insect orders including Lepidoptera, Diptera, and Coleoptera that causes major damage to crops. Cry-toxin mainly attacks the digestive system of insects when it comes in contact with proteases in the midgut resulting in its activation and causing rupturing of the midgut thus

ultimately leading to the death of insects [58]. Entomopathogenic fungi include *Beauveria bassiana, Metarhizium anisopliae, Lecanicillium lecanii, Nomuraea rileyi, and Hirsutella thompsonii* which are used widely to control insect pests [59] and with the biotechnological techniques, new strains have been developed on the commercial scale to get more accurate results. Their mode of infection possess when fungal conidia come in contact with insect pests, these fungal conidia develop hyphae which penetrate the insect's cuticle through enzymatic action and entered into the body of the insect pest. Inside the body, the fungal hyphae vegetatively propagate and thus lead to the death of the insect [60]. Baculoviruses are a large group of viruses that infect a broad range of insect pests mainly belonging to orders *Diptera*, *Hymenoptera*, and *Lepidoptera*. With the biotechnological techniques (Recombinant DNA technology) the efficiency of isolated baculoviruses can be increased by inserting foreign genes [61] mainly insect's toxin gene. There are two categories of baculoviruses- nucleopolyhedroviruses (NPVs) and granuloviruses (GVs) [57]. These may affect larval stages as well as adult stages when these are ingested by larvae; in the midgut leads to the breakdown of the protein and the release of the virions which destroys internal tissue [58] and ultimately causes death and in adults, these may paralyze the insect [62]. Entomopathogenic nematodes (EPNs) are the soil-inhabiting nematodes that have the tendency to kill the insects having soil-dwelling stages like larval and pupal stages, with the help of symbiotic bacteria [63]. There are two main genera- Steinernema and Heterorhabditis form where the EPNs are produced. For the action of EPNs- infective juveniles of the nematode play a major role, these invade the host through the oral, anal, spiracles openings [57] and finally release the symbiotic bacteria into the intestine that ultimately causes the death of the insect. In India, plant products have been used to control household insects from the ancient time but now in the last few years, these products are used as pesticides on large scale not only in India but also on the global platform. *Nicotiana tabacum*, *Azadirachta indica*, and *Chrysanthemum cinerariifolium* [64] are the main plants from which botanical biopesticides are derived. Biochemical pesticides include- Semiochemicals-pheromones, growth hormones [65], and enzymes. Pheromones are the chemical used by insects to attract mates (sex attractants), used against predators (release of volatile substances), and also to found food. Pheromones are highly species-specific and highly effective to control the insect pest population by attracting the insects, trapping, and finally leading to death [66].

In the formulation of biopesticides, the major challenge is the shelf life [67], storage, and efficacy consistency. Biopesticides consist primarily of living microbes so these are affected by physical environmental factors like temperature fluctuations, humidity, and exposure to ultraviolet radiations that reduce their efficacy.

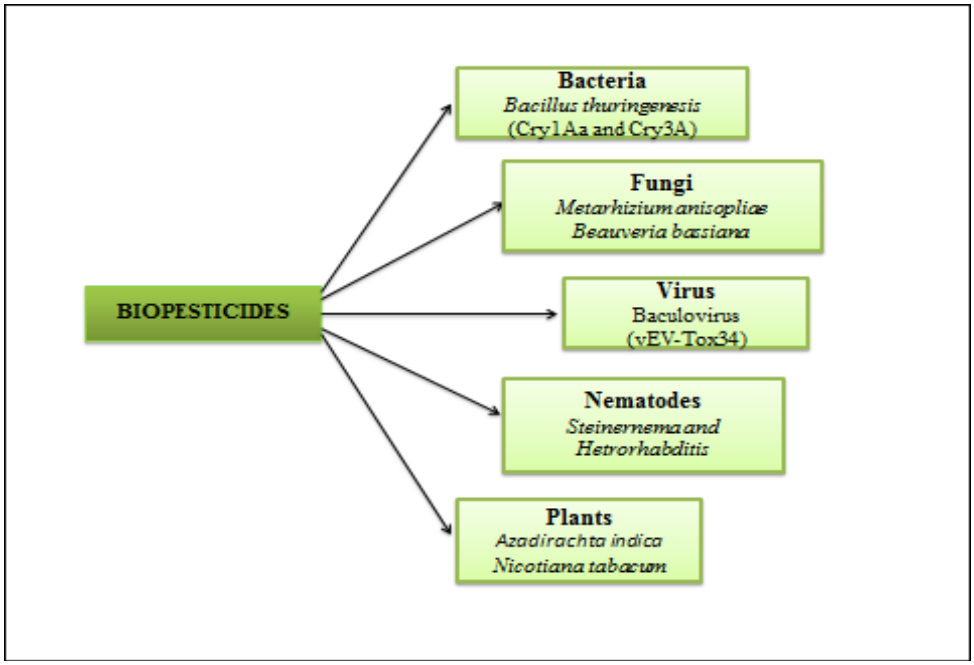

Fig. 3. Biopesticides from the different living sources are used to control the insects in natural ways

6. DISCUSSION

DNA barcoding makes the identification of insect pests more reliable and accurate by discriminating the intraspecific and interspecific diversity of species. Recently, millions of records of DNA barcode data are available in the BOLD system for identification [68]. RNA interference is an effective tool in insect genomic projects in entomology, also can protect the crops from insect pests, and can become next-generation biopesticide[69][71][72]. For the perfect result of RNAi technology in insect pest management-the target gene should be selected precisely [23] [70]. RNAi mediated technology can be applied on most of the insect pests that cause maximum damage[73] belongs from order coleoptera, Lepidoptera, and hemiptera including- red flour beetle (*Tribolium castaneum*)[74], western corn rootworm(*Diabrotica virgifera*)[75][76], light brown apple moth(*Epiphyas postvittana*)[77], cotton bollworm (*Helicoverpa armigera*)[47], European corn borer(*Ostrinia nubilalis*) [78] sugarcane borer, (*Diatraea saccharalis*) [79], whitefly (*Bemisia tabaci*)[80]and Rice weevil (*Sitophilus oryzae*)[81]. SIT depends on mass rearing, which causes ripple effects on insect pest population [82]. SIT integrated with various methods helps to eradicate the different types of insect pests mainly a pest of order Lepidoptera [83] [84] [85]. Nosemosis is a widespread disease of adult honeybees that can be treated with RNAi-based strategy by specific gene silencing [87] [88] thus also helping in the beneficial insect's diseases[89][90]. In biopesticides, different types of enzymes (chitinases, cellulases, and proteases) are used which eliminate the insect pests and do not cause damage to crops [92]. The biopesticides market growing in a very swift way and in the coming future, these will equalize synthetic pesticides [93] [94].

7. CONCLUSION

Biotechnology integrated with entomology has huge potential to protect the crops from insect pests and with agriculture, the production of crops also increased with transgenic crop varieties. India has diversified geographical regions which support many different types of insects due to this reason, in recent few years many invasive insect species attacked India which cause a lot of damage to crops and horticultural crops but their timely identification through DNA barcoding make it possible to get rid of this type of attack. RNAi transgenic crops provide a new approach to controlling insects and also provide protection to the beneficial insects. New varieties of silkworm and honeybee are possible through genetic engineering which increases the prosperity of farmers. For the more successful application of biotechnology – new genes should be identified which have multiple functions and more work should be done to make nanoparticles more effective in insect pest management which causes zero damage to the environment.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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