NANO ENCAPSULATION OF dsRNA 0F METAMORPHOSIS RELATED GENE AND ITS INSECTICIDAL POTENTIAL AGAINST DIAMOND BACK MOTH

THESIS

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Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola
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By
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DECLARATION OF STUDENT

I hereby declare that the experimental work and its interpretation in the thesis entitled **"NANO ENCAPSULATION OF dsRNA OF METAMORPHOSIS RELATED GENE AND ITS INSECTICIDAL POTENTIAL AGAINST DIAMOND BACK MOTH"** or part there of has neither been submitted for any other degree or diploma of any University, nor the data have been derived from any thesis / publication of any University or scientific organization. The source of materials used and all assistance received during the course of investigation have been duly acknowledged.

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CERTIFICATE

This is to certify that thesis entitled **"NANO ENCAPSULATION OF dsRNA OF METAMORPHOSIS RELATED GENE AND ITS INSECTICIDAL POTENTIAL AGAINST DIAMOND BACK MOTH"** submitted in partial fulfilment of the requirement for the degree of **"Master of Science in Agriculture (Agricultural Biotechnology)"** of Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola is a record of bonafide research work carried out by **Pimpalzare Pranali Subhashrao** under my guidance and supervision.

The subject of the thesis has been approved by the Student's Advisory Committee.

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(D) Abbreviations

%	:	Percent
˚C	:	Degree Celsius
µl	:	Micro liter
bp	:	Base pair
CD	:	Critical difference
cDNA	:	Complementary DNA
cm	:	Centimeter
CV	:	Coefficient of variation
DBM	:	Diamond back moth
DOPA	:	3,4 dihydroxyphenylalanine
dsRNA	:	Double stranded ribonucleic acid
Dr. PDKV	:	Dr. Panjabrao Deshmukh Krishi Vidyapeeth
EcR	:	Ecdysone receptor
et al.	:	et alia (and others)
etc.	:	Etcetera
FDA	:	Food and Drug Administration
Fig.	:	Figure
FTIR	:	Fourier Transform Infrared Spectroscopy
JHE	:	Juvenile hormone esterase
Gm	:	Gram
i.e.	:	id est. (that is)
ml	:	milliliter
mM	:	Milli molar
nm	:	Nanometer
PAGE	:	Polyacrylamide gel electrophoresis
PCR	:	Polymerase chain reaction
PLGA	:	Poly (lactic-co-glycolic acid)
PVA	:	Poly vinyl alcohol
RISC	:	RNA induced silencing complex
RNA	:	Ribonucleic acid
rpm	:	Revolution per minute
S.E.	:	Standard Error
ζP	:	Zeta potential
UV	:	Ultra Violet
W/V	:	Weight by volume
ζP	:	Zeta potential

(E) Thesis Abstract

a)	**Title of the thesis**	**:**	**NANO ENCAPSULATION OF dsRNA OF METAMORPHOSIS RELATED GENE AND ITS INSECTICIDAL POTENTIAL AGAINST DIAMOND BACK MOTH**
b)	**Full name of student**	**:**	**Pimpalzare Pranali Subhashrao**
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ABSTRACT

The present investigation entitled “Nano encapsulation of dsRNA of metamorphosis related gene and its insecticidal potential against diamond back moth” was carried out at Biotechnology Centre, Department of Agricultural Botany, Post Graduate Institute, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola during the academic year 2019-2021.

RNA interference (RNAi) technology post transcriptional mechanism triggerd by dsRNA to silence specific genes by down regulating their expression. It has become a potential strategy for functional and regulatory studies of insect genes and has potential to control insect pest. Though it has been challenging to produce effective RNAi in lepidopteran Diamond back moth (*P. xylostella*). Some important factors significantly influencing the silencing potential effects are concentration and length of dsRNA, nucleotide sequence, and determination of the silencing effect and life stage of the target organism. The *P.xylostella* shows RNAi effects after feeding of nanoencapsulated dsRNA. Considering the potential impact on the normal physiological functions of healthy adults, Juvenile Hormone Esterase gene select as targets for dsRNA mediated gene silencing. Juvenile Hormone Esterase gene sequence obtained from NCBI database. Off targets were predicted to avoid non target effects. Possible RNAi sites were depicted to identify the region having maximum RNAi targets.

The aim of studies was to prepared PLGA nanoparticles, because of their possibility to target specific cells with high biodegradability and biocompatibility. It is approved for human use by the Food and Drug Administration. Here, we describes in great details about the formation and characterization of PLGA nanoparticles. Poly (lactic-co-glycolic acid) nanoparticles synthesized using double emulsification method. PVA (poly vinyl alcohol) used as stabilizer during the synthesis of PLGA nanoparticles. This protocol can be readily adapted to use alternative emulsifiers PVA (e.g. Poly vinyl alcohol) *or* solvent (e.g. methanol).

A typical peak at 380nm having 0.56 absorbance on UV- visible spectroscopy indicates synthesis of PLGA nanoparticles. Particle size and zeta potential are determined with Nanoparticle size analyzer and Zeta potential analyzer. The PLGA nanoparticles characterized during present study showed 244.9 nm particle sizes and -4.59 mV zeta potential. FTIR analysis, carried out in wave range 1000- 4000 $cm^{-1,}$ showed different functional groups like 3884 cm^{-1} and 3410 cm^{-1} (Alcohol), 1780 cm^{-1} (Carbonyl group), 1494 cm^{-1} and 1666 cm^{-1} (Glycolic

acid) 1382 cm-1, and 1187 cm^{-1} (Esters), 1472 cm^{-1} and 1096 cm^{-1}(Vinyl) in PLGA NPs synthesis.

The process of encapsulation of dsRNA with PLGA NPs done to increase the insecticidal potential of dsRNA-JHE(A) and dsRNA-JHE(B). The encapsulated nanoparticles were also characterized by UV spectrophotometry, particle size was determined with Nanoparticle size analyzer and Zeta potential analyzer. We provide representative images for nanoparticles produced.

Effect of dsRNA and encapsulated dsRNA with PLGA NPs was checked out by performing insect bioassay studies on *P. xylostella* larvae. The dsRNAs and encapsulated dsRNA with PLGANPs were individually spread on to the cabbage leaf discs and twelve larvae were released into 3 replication. We detected that encapsulated dsRNA-JHE(A) and encapsulated dsRNA-JHE(B) which had the highest mortality 60% and 72% as compared to control 0% (only water) and dsRNA 36% JHE(A) and 48% JHE(B).

These findings largely broaden the target selection for RNAi-based technology and deliver dsRNA into the insect and potential against insect damage and insect control management.

CHAPTER I

INTRODUCTION

1.1 Background Information

1.1.1 Need of new promising tools for insect pest management

Insect pests infestation is the major limiting factors for commercial cultivation of crucifers worldwide and it is prior to achieve maximum production of food and other products with reduced loss in agricultural crops incurred by DBM plus the management expenses cost about $ 4-5 billion annually worldwide (Furlong *et al.,* 2013) Also, diamond back moth causes threat of great loss of 90% and above to crucifer production.

Currently, different strategies such as cultural, mechanical, biological, chemical and transgenic approaches are utilized for effective control of insect pests. Amongst these most abundantly used treatment is chemical. Although the use of chemical pesticides is effective, the cost, toxicity to humans, the environment has motivated over increasing resistance in insect against these insecticide and the search for alternative pest control strategies (Whangbo and Hunter, 2008). However, DBM becomes resistant to any insecticides within a short period. Formerly, DBM has been found to be significantly resistant to organophosphates, carbamates, pyrethroids, spinosyns, avermectins, neonicotinoids, pyrazoles, and oxadiazines (Liu *et al.,* 2012) Production of transgenic plants as strategy to control insect pests has become globly used. Transgenic crops have revolutionized insect pest control, but their effectiveness has been reduced by evolution of resistance in pests. They analyzed global monitoring data reported during the first two decades of transgenic crops, with each case representing the responses of one pest species in one country to one insecticidal protein from *Bacillus thuringiensis* (Tabashnik, 2017).

Despite the tremendous utility of RNAi as a promising strategy for studying fundamental biological questions and for control of insect pests, there is still a need to analyze several aspects of RNAi before establishing it as a longterm effective pest control method in the field. RNAi application and efficacy remains variable among different genes, tissues, organisms and life stage of insect. For example, RNAi effect has been found to be more in hemocytes of *D. melanogaster* and *Manduca sexta* as compared to other tissues when injected with target gene dsRNAs (Mao and Zeng, 2014; Miller *et al.,* 2008).

1.1.2 RNA interference

RNA interference (RNAi) is a sequence-specific mRNA degradation mechanism triggered by small interfering RNAs (siRNAs). This phenomenon of gene silencing has now been considered as a potential strategy for the control of insect pests. The process is mediated by RNA sequences which are introduced into the cell as exogenous RNA, usually as double stranded RNA (dsRNA), and it occurs after export of mRNA from the nucleus. Studies of the nematode *Caenorhabditis elegans* have shown that the injection of both sense (the strand which has an identical sequence to mRNA) and antisense RNA strands (the complementary sequence of mRNA) separately causes gene silencing (Guo and Kemphues, 1995). Later, Fire *et al.* (1998) conducted an experiment by injecting a mixture of sense and anti-sense strands as dsRNAs into *C. elegans* and found that the dsRNA was ten times more effective in decreasing expression of the homologous endogenous mRNA than using either the sense or the antisense strand alone. Since then, the silencing of a certain genes using dsRNA has been called RNA interference.

However, the efficacy of RNAi depends on the vitality of target gene, site of dsRNA accumulation, delivery method, insect species, developmental stage of target insect, dsRNA/siRNA concentration, presence or absence of RNA-dependent RNA Polymerase (RdRP), which is involved in amplifying silencing signal, etc. (Lim *et al.,* 2016; Zhu and

Palli, 2020). This technology is deliberately used in entomology to study the gene function, regulation and expression particularly in *Drosophila melanogaster* (Miller *et al.,* 2008), Tribolium castaneum (Tomoyasu and Denell, 2004) and *Bombyx mori* (Hossain *et al.,* 2008). Most of these experiments have been conducted by injecting dsRNA directly to the organism through microinjection or droplet feeding, which is inadequate to control insect pests in the field condition. The organism should be able to separately take up the dsRNA, for example through feeding and digestion in the gut.

1.1.3 dsRNA Uptake and Intracellular RNAi Mechanism

RNAi pathway involves formation of interfering molecules through activity of dicer enzyme. Theys interfering molecules can be small interfering RNA (siRNA), the microRNA (miRNA) and piwiRNA (piRNA). Although siRNA and miRNA differ in the way they enter in the RNAi pathway, both of them are generated from larger more complex dsRNA by a ribonuclease III enzyme, called Dicer-2, into small interfering RNAs (siRNAs). These 21–24 nucleotide duplexes are subsequently incorporated in the so-called RNA induced silencing complex (RISC) where the duplex is unwound. Subsequently, an Argonaute2 (AGO2) protein cleaves the passenger (sense) strand and the guide (antisense) strand remains connected with the RISC. Afterwards, the guide strand of the siRNA guides the RISC and allows Watson-Crick base pairing of the complex to complementary target mRNA for cleavage of target mRNA by AGO2 protein. By this degradation of the target mRNA, specific post-transcriptional gene silencing occurs (Agrawal *et al.,* 2003). In 2007, Baum *et al.,* demonstrated the fact that gene silencing in insects can be achieved through dsRNA strategy via consumption of plant material expressing hairpin dsRNA construct against target gene. The most effective dsRNA, directed against a gene encoding V-type ATPase A, demonstrated rapid knockdown of endogenous mRNA within 24 h of ingestion and triggered a specific RNAi response with low concentrations of dsRNA. There are two types of RNAi: cell-autonomous RNAi and non-

cell-autonomous RNAi to determine the systemic RNAi signal inside the body of an organism (Whangbo and Hunter, 2008; Huvenne and Smagghe, 2010). Cell-autonomous RNAi refers to RNAi that happens inside the cell while non-cell-autonomous RNAi entails uptake into the cell and/or transport of the silencing signal from one cell to another and from one tissue to another. Non-cell-autonomous involves the phenomenon of environmental RNAi, which triggers the RNAi by environmental exposure either by soaking or feeding (Baum and Roberts, 2014).

Aim of the present study is to silence the candidate genes viz. Juvenile Hormone Esterase (JHE) from DBM. It plays a crucial role in preventing precocious metamorphosis and stimulating reproduction. Juvenile hormone esterase (JHE) performs a rapid breakdown of residual JH in the hemolymph during last instar to induce a larval to pupal metamorphosis. Juvenile hormones (JHs) are a family of sesquiterpenes that regulate a wide spectrum of critical biological events in insects including development, metamorphosis, reproduction, polyphenism, and alteration in behaviour. A whole genome of the diamondback moth (DBM), *Plutella xylostella*, has been annotated and proposed 11 JHE candidates. Sequence analysis using conserved motifs commonly found in other JHEs proposed a putative JHE (Px004817). Px004817 (64.61 kDa, pI = 5.28) exhibited a characteristic JHE expression pattern by showing high peak at the early last instar, at which JHE enzyme activity was also at a maximal level. RNA interference of Px004817 reduced JHE activity and interrupted pupal development with a significant increase of larval period. (Xiaojun, 2015) JH homologs have been identified that posses an α, β-unsaturated methyl ester at one end of the molecule and an epoxide at the other. Therefore, we envisioned that silencing the gene encoding JHE might impact individual *P. xylostella* larvae and result in reduced populations.

1.2 Nanotechnology

Nanotechnology is an emerging and rapidly growing field with its application in science and technology for purpose of manufacturing new material at the nanoscale level (Albercht, 2006).

Nanotechnology encompasses the production and application of physical, chemical, and biological systems at scales ranging from individual atoms or molecules to submicron dimensions, as well as the integration of the resulting nanostructures into larger systems. (Bhushan, 2010). The prefix “Nano” developed from the Greek word meaning “dwarf” used to indicate one billionth of a meter or 10^{-9}. The term “nanotechnology” was given by Norio Taniguchi in 1974 to describe materials whose dimensions are less than a micrometer. The concept of Nanotechnology was given by physicist noble laureate Professor Richard P. Feynman (Feynman, 1959) in his speech “There’s plenty of room at the bottom” at California Institute of Technology. Science and technology research in nanotechnology promises breakthroughs in areas such as materials and manufacturing, nanoelectonics, medicine and healthcare, energy, biotechnology, information technology and national security. It is widely felt that nanotechnology will bring the next industrial revolution (Bhushan, 2010).

1.2.1 PLGA nanoparticles

The biodegradable and biocompatible polymer poly (D,L-Lactide-co-Glycolide acid) (PLGA) is a copolymer of glycolic acid and lactic acid. It is approved for human use by the US Food and Drug Administration (Woodrow *et al.,* 2009). The polymer also caught interest for the delivery of plasmid DNA and siRNA in recent years. The advantages of PLGA-based siRNA delivery include high stability, facile cellular uptake by endocytosis, ability to target specific tissues or organs by adsorption or ligand binding, biodegradability, low toxicity, and sustained release characteristics (Singha *et al.,* 2011). However, PLGA could not be applied efficiently in siRNA delivery due to the lower electrostatic interaction between PLGA and siRNA and less efficient endosomal escape and release of siRNA. To overcome these problems, the surface of PLGA can be decorated with various cationic nanoparticles such as DOTAP, PEI, or polyamine (Wu *et al.,* 2012).

The polymers derived from lactic and glycolic acids have received a great deal of attention in research on alternative biodegradable polymers (Elnashar, 2010). Various studies demonstrating their low toxicity, including their approval by the Food and Drug Administration (FDA) for use as drug delivery systems (DDS) can be found in the literature. PLGA copolymers have desirable properties, such as a constant biodegradation rate, mechanical resistance, and regular individual chain geometry (Jain, 2000).

The first copolymer made up of units from lactic and glycolic acid, known as PLGA, was commercialized in the 1970's. The great interest in this copolymer stems from the fact that it's mechanical and degradation time properties can be controlled through monomer ratios (Stevanovic *et al*., 2008).

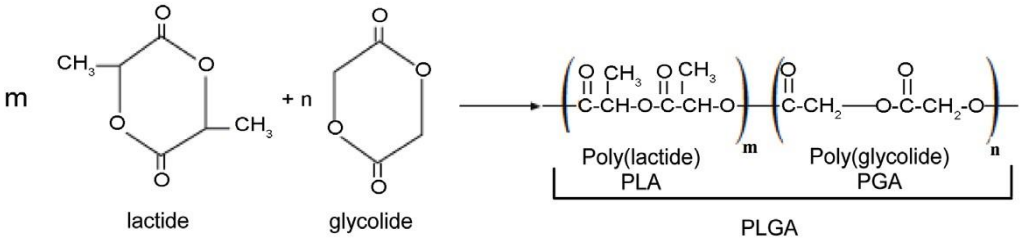


Fig 1. Chemical structure of the dimers, polymers, and copolymerization reaction

The copolymerization of PLGA can basically be done in two ways: 1) Through a direct polycondensation reaction of lactic acid and glycolic acid, resulting in copolymers of low molecular weight (Lunt, 1998) 2) Through an opening polymerization of cyclic dimers of lactic acid (lactide) and glycolic acid (glycolide), resulting in co- polymers with high molecular weight and therefore with better mechanical properties (Gildingand, 1979). The bulk polymerization generally takes from two to six hours for temperatures around 175°C and in the presence of initiator. Lauryl alcohol can be added during the process in order to control the molecular weight (Chasin *et al.,* 1990).

Oil-water (single) or water-oil-water (double) emulsion is one method by which PLGA can be used to encapsulate hydrophobic and hydrophilic drugs in micro- or nanoscale form. Briefly, PLGA is dissolved into an organic phase (oil) that is emulsified with a surfactant or stabilizer (water). Hydrophobic drugs are added directly to the oil phase, whereas hydrophilic drugs (water) may be first emulsified with the polymer solution prior to formation of particles. High intensity sonication bursts facilitate the formation of small polymer droplets. The resulting emulsion is added to a larger aqueous phase and stirred for several hours, which allows the solvent to evaporate. Hardened nanoparticles are collected and washed by centrifugation (McCall and Sirianni, 2013). Copolymer poly (D, L-lactideco-glycolide) is used for the controlled delivery of several classes of medicaments like anticancer agents, anti-hypertensive agents, immunomodulatory drugs, hormones, and macromolecules like nucleic acid, proteins, peptides, antibodies, and DLPLG nanospheres are very efficient mean of transdermal transport of medicaments in the body, for example, ascorbic acid (Yokoyama and Huang, 2005).

Gene silencing method providing an effective amount of a nanoparticle comprising a polymer matrix and dsRNA and placing the nanoparticle in a location where insects may come into direct contact therewith. The nanoparticles are orally ingested by the target insect, and the ingested nanoparticles trigger gene silencing of the target gene in the target insect. In one or more embodiments, the dsRNA of the ingested nanoparticles is cleaved into siRNAs in the target insect, which triggers the gene silencing (Zhang, 2006).

1.3 *Plutella xylostella* L, a destructive pest

The diamondback moth (DBM), *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), is one of the most destructive insect pests of crucifers worldwide. Larvae of *P. xylostella*, feed on the foliage of the cruciferous plants from the seedling stage to harvest, and greatly reduce the yield and quality of produce. *P. xylostella* has only become a significant pest, with major problems caused by the evolution of

insecticide resistance (Talekar and Shelton, 2003) It is an oligophagous species feeds on plants of Brassicaceae, which include economically important crops such as cabbage, cauliflower, broccoli, canola and Brussels sprouts family. In India the estimated annual crop losses due to this pest amount to 16 million USD (Mojanand, 2003). It was first observed in North America in 1854. The most important insect pest of cabbage crop is DBM (Plutellidae: Lepidoptera), recorded from more than 128 countries of the world (Tsunoda, 1980). Cabbage (*Brassica oleracea* L. var. capitata) is being grown in an area of 3088 hectares with production of 8.75 million tonnes. Major cabbage growing states in the country are Uttar Pradesh, Orissa, Bihar, West Bengal, Assam, Karnataka, Maharashtra, Madhya Pradesh and Tamil Nadu (Meghana *et al.,* 2018) recorded this pest for the first time in India on cruciferous vegetables and perusal of literature revealed that the pest is distributed all over India. In India, diamondback moth has national importance on cabbage as it causes 50-80% annual loss in the marketable yield (Devjani and Singh, 1999). The most devastating pest that causes severe damage in cabbage production is the DBM (Kwarteng and Towler, 1994). It has shown significant resistance to almost every insecticide applied in field including biopesticides such as crystal toxins from *B. thuringiensis* and spinosyns from *Saccharo polyspora spinosa* under field conditions (Tabashnik *et al*., 2003).

1.3.1 Need of study

Over the past 50 years, protection of crops from insect pests has relied heavily on the use of broad-spectrum chemical insecticides. However, overuse of some of these compounds has led to resistance, with over 500 insect species estimated to be resistant to one or more insecticides (Andrade and Hunter, 2016). While dsRNA holds great promise as a pest control strategy, employing it in the field will require careful consideration of its advantages, drawbacks, and risks, Many insecticides are toxic, and direct exposure can cause pesticide

poisoning of farm workers and animals, as well as loss of beneficial insects (Trivedi *et al.,* 2010). In addition, some pesticides persist in the environment for decades, with their effects accumulating along the food chain. Given these issues, RNAi-based strategies could provide an environmentally friendlier method to control insect pests while also reducing the use of chemical pesticides. Moreover, since RNAi can be highly specific (Yu *et al.,* 2016; Cooper *et al*., 2019), farmers should be able to control infestations of crop pests without impacting beneficial insects or other animals present in the field. Another advantage is that RNAi is a natural process that occurs in almost all eukaryotes. All plants and animals produce siRNAs to regulate the expression of endogenous genes and transposable elements, as well as to fight viral infection. Since humans are exposed to sRNAs in their diet every day, RNAi-based pest control is expected to be non-toxic (Trivedi *et al.,* 2010).

In addition, topical application of dsRNA may not pose an environmental hazard because dsRNAs are rapidly degraded in soil and water (half-life under 30 h or 72 h, respectively; Fischer *et al.,* 2017). Studies in the laboratory and greenhouse have provided proof-of-concept that RNAi can be used to reduce plant predation and the viability or fertility of a variety of insects. Together, these findings suggest that RNAi-based pest control strategies could have broad agronomic significance. Besides enhancing dsRNA uptake, nanoparticles can increase dsRNA stability. In comparison to naked dsRNA, dsRNA loaded on layered double hydroxide (LDH) clay nano-sheets exhibited less degradation following an RNase treatment and greater stability when sprayed on *N. tabacum* leaves (Mitter *et al.,* 2017). dsRNA-LDH also had better adherence to leaves after rinsing and provided prolonged protection against viral infection. In another study, analyses of three different types of dsRNA-nanoparticle complexes subjected to acidic, neutral or basic conditions revealed that dsRNA complexed with carbon quantum dot (CQD) nanoparticles was substantially more stable than that complexed with chitosan or silica, and this correlated with greater RNAi efficiency in

Aedes aegypti larvae (Das *et al*., 2015). Since instability due to dsRNases and inappropriate pH is a major obstacle for oral dsRNA delivery in insects (Cooper *et al.,* 2019), nanoparticles may provide an effective way to circumvent these issues. However, the ability of nanoparticles to improve RNAi efficacy in insects may be associated with increased toxicity in animals and elevated environmental risks. Thus present investigation is planned with following objectives.

1.4 Objectives of study

1. Identification of suitable RNAi targets in metamorphosis related genes of *Plutella xylostella* and synthesis of dsRNA.
2. Nano encapsulation studies of dsRNA and its insect bioassay against Diamond Back Moth.

1.5 Scope and importance

Brassica vegetables, like cabbage (*Brassica oleracea* var. capitata) and cauliflower (*B. oleracea* var. botrytis), and open leaf kales, like rape (*Brassica napus*) and covo (*Brassica carinata*), are the popular staple relish and most widely grown leafy vegetables in the tropical and subtropical regions of india. These vegetables are grown throughout the year and form the fastest growing agricultural subsector that contributes significantly to national and regional incomes. This has resulted in higher rates of pest infestation, especially by the DBM and higher pesticide use. The 98% of sprayed insecticides and 95% of herbicides reach a destination other than their target species, because they are sprayed or spread across entire agricultural fields (Sparks and Riley, 2018). Insect infectivity has the unique problem of being difficult to control with insecticides because killing the insect often does nothing to diminish the infectivity. This in turn, has contributed to insecticide resistance, environmental degradation, and human health impacts, which have triggered a growing interest in alternative management techniques. A survey was conducted in the states of Gujarat, West Bengal, and Karnataka from October 2006 through January 2007 (Weinberger, 2009).

Three hundred farmers were interviewed to obtain information on pesticide use in cabbage and cauliflower production, the cost of pesticide use, and socioeconomic characters that influence cabbage and cauliflower production. Farmers relied on pesticides as the major and often exclusive crop protection strategy. Ten of the active ingredients (16.4% of all pesticides reported by all farmers in this survey) were listed as extremely or highly hazardous by the World Health Organization.

RNAi-based strategies could provide an environmentally friendlier method to control insect pests while also reducing the use of chemical pesticides. Moreover, since RNAi can be highly specific (Yu *et al.,* 2016; Cooper *et al.*, 2019), farmers should be able to control infestations of crop pests without impacting beneficial insects or other animals present in the field. Another advantage is that RNAi is a natural process that occurs in almost all eukaryotes. The sequence-specific gene silencing via RNA interference (RNAi) holds a great assure for effective management of agricultural pests. RNAi is naturally occurring conserved process responsible for gene regulation and defence against pathogens. A successful RNAi response can be achieved by using efficient delivery method (Mamta and Rajam, 2017). In nanoparticle based delivery method, dsRNAs are made to entrap into chitosan polymer via electrostatic forces to form a dsRNA nanoparticles (Howard *et al*., 2006). However, dsRNA-based nanoparticles delivery methods for RNAi have not been fully utilized for insect pest control.

1.6 Hypothesis

Gene silencing encoding JHE might impact individual *P. xylostella* larvae and result in reduced populations. The gene silencing by RNAi technology, various strove have been made by different scientists using transgenic methods of gene delivery. Many people believe those transgenic organisms are bad and have adverse environmental effect.

Another efficient delivery system by design nanoparticles, nanoparticle will be help in protecting and delivering dsRNA. The present

study attempts for Nano encapsulation of dsRNA of metamorphosis related gene and its insecticidal potential against DBM through encapsulating PLGA nanoparticles with dsRNA delivery which could be environmentally safe, low toxic, trans-membrane abilities, and possibilities to target nanoparticles to the specific organs or cells and have broad prospects of immense application.

1.7 Limitations

RNAi can be successfully employed as a control strategy against insect pests. In most of functional genomic studies, injection or feeding of bacteria expressing the dsRNA was used as a delivery method to silence different target genes. Silencing of some genes had produced devastating effects on the insect growth, development, and survival (Xu *et al.*, 2016). These studies suggested the possibility of utilization of feeding bioassay approach for control of insect pests through RNAi However, there are many limitations using RNAi-based technology for pest control, with the effectiveness target gene selection and reliable double stranded RNA (dsRNA) delivery being two of the major challenges. With respect to target gene selection, at present, the use of homologous genes and genome-scale high-throughput screening are the main strategies adopted by researchers. Hence, RNAi-mediated crop protection has been considered as a potential new-generation technology for pest control, or as a complementary method of existing pest control strategies; however, further development to improve the efficacy of protection and range of species affected is necessary. Current progress has proven that RNAi technology has the potential to be a tool for designing a new generation of insect control measures. To accelerate its practical application in crop protection, further study on dsRNA uptake mechanisms based on the knowledge of insect physiology and biochemistry is needed. Despite the tremendous utility of RNAi as a promising strategy for studying fundamental biological questions and for control of insect pests, there is still a need to analyze several aspects of RNAi before establishing it as a long-term effective pest control method in the field.

CHAPTER II

REVIEW OF LITERATURE

Insects are our most serious competitors for food and fiber and are vectors of some of our most serious diseases. Chemical pesticides are routinely used to protect crops and to reduce the spread of insect-borne diseases In addition, there is increasing public concern over the risk that many of these chemicals pose to the environment and to human and livestock health. Together, these issues provide compelling reasons to find safer, more pest-specific alternatives to control pest insects. The potential of RNAi induced by dsRNAs is emerging new tool for developing plant resistance to coleopteran and lepidopteran pests (Gordon and Waterhouse, 2007). Hence in this review potential of RNAi in the management of the DBM is reviewed under the following points:

2.1 History

RNAi-like gene silencing phenomenon originated from studies of chalcone synthase (CHS), an enzymatic component of the pathway responsible for violet colouration in petunia flowers (petunias are cultivated hybrid plants, Petunia X hybrida, Family Solanaceae). When researchers over-expressed CHS (by introducing a chimeric CHS gene) with the aim of generating violet petunia flowers, they surprise after seeing the resulting petunias were white (Napoli *et al.,* 1990). Analysis of transcript levels revealed that CHS messenger RNA (mRNA) levels in the transgenic petunias were fifty times lower than in the wild-type plants. The group hypothesized that this effect was caused by the exogenous transgene suppressing the endogenous CHS gene and they coined this process “co-suppression”.

Waterhouse *et al.* (1998) assumed that dsRNA was important either as a silencing trigger or as an intermediate in all the RNAi related silencing pathways; it was not known whether other stimuli (besides dsRNA) could trigger silencing. For example, silencing in

response to a DNA transgene could still involve a dsRNA trigger: the transgene might integrate itself into the genome in such a way that a nearby promoter, or an inverted copy of the transgene itself, leads to the production of dsRNA, which could in turn enter directly into the RNAi pathway.

Montgomery (2004) gene expression is manipulated by researchers working in three different fields resulted in unanticipated gene silencing. Rather than ignoring such results, they further investigate the nature of such silencing, which was named “co-suppression” in plants, “quelling” in fungi, and “RNA interference” (RNAi) in nematodes. By the late 1990s, it was discovered that silencing could be initiated in this diverse set of organisms by exposing cells to double-stranded RNA (dsRNA), which directed the destruction of mRNAs containing similar sequences.

RNAi commonly referred as double-stranded RNA (dsRNA)-mediated gene silencing, this mechanism is becoming a widely used as functional genomics tool in insects to ascertain the function of the many newly identified genes accumulating from genome sequencing projects reported (Hannon, 2002). The basic components of the RNAi process, namely the endonuclease Dicer, which first chops long dsRNAs into short interfering RNAs (siRNAs), and the RNA-induced silencing complex (RISC), which facilitates the targeting and endonucleolytic attack on mRNAs with sequence identity to the dsRNA, are evolutionarily conserved across virtually all eukaryotic taxa, and consequently, RNAi could be readily applied to any insect species.

2.2 RNAi in insect pest management

Tomari *et al*. (2007) explained the core machinery in somatic tissues in fruitflies (*Drosophila melanogaster*), and presumably in insects in general, two RNA silencing pathways exit, characterized by siRNAs and miRNAs that seem to be at least partially separated with respect to biogenesis and function. The siRNA pathway is thought to

primarily function as a defense response against exogenous dsRNAs, while the miRNA pathway primarily uses endogenous products transcribed from the cell's genome with dsRNA structure to regulate developmental processes, as for instance generated from viruses, *Drosophila* mutants in the core siRNA machinery (Dicer-2, the RNA binding protein R2D2 and Argonaute-2) indeed show increased sensitivity to infection by RNA viruses.

It is interesting to note that while RNAi components and pathways have been studied intensively in the insect model organism *Drosophila melanogaster*, and RNAi pathway genes have been identified in all insect orders, the efficiency of environmental RNAi in insects is highly variable (Terenius *et al.,* 2011; Gu and Knipple, 2013; Cooper *et al.*, 2019). Efforts to elucidate the cause(s) of this variability have revealed multiple contributing factors, including instability of the dsRNA, as well differing ability of insects to uptake the dsRNA and/or upregulate or express RNAi machinery genes.

The siRNA designed to target *P. xylostella* AChE1 and AChE2 genes. Mortality caused by siRNA was monitored and also checked the level of down-regulation of the transcripts as well as the AChE activity. Effects of the siRNA on treated *Brassica oleracea* and *B. alboglabra* plants were also investigated with the different dosages Gong, *et al.,* (2013). Based on our data we discuss creating novel bio-pesticides based on the RNAi technology by chemically synthesizing siRNAs.

Ellango *et al*. (2014) take attempt to assess the potential of *tyrosine Hydroxylase* (TH) as a target gene, which is a key regulator in the biosynthesis of 3, 4 dihydroxyphenylalanine (DOPA). DOPA is a precursor for agents that function in neurotransmission, melanization, sclerotization of the cuticle and immune responses in insects. Here, we provided to DBM larvae treated with 3 different concentrations (1.04, 2.08, and 3.12 μg/cm^2) of cognate dsRNA coated on discs of cabbage leaf, the natural diet for the larvae. We recorded the influence of the dietary dsRNA on TH transcript levels, larval growth, and larval survival rate. The dietary dsRNA

led to reduced target gene transcript level and larval feeding level and caused larval mortality in a concentration-dependent manner. These results demonstrate that the TH gene has potential as a target gene for RNAi-mediated management of *P. xylostella*.

The excess use of chemical insecticides such as organophosphates, pyrethroids, and endosulfan in controling notorious sap-sucking insect pests such as the *melon aphid, Aphis gossypii Glover*, resulted in insecticide resistance. RNAi is a sequence-specific gene silencing mechanism triggered by dsRNA Chaitanya, *et al.*, (2015). The efficiency of RNAi is dependent on concentration of dsRNA, mode of delivery, gene targets, insect species, etc. The objective of this study to assess the extent of down-regulation by using two different concentrations of dsRNA specific to sodium channel (AgSCN) and ultraspiracle genes (AgUSP) (0.0625 and 0.125 mg/ml) with two-time intervals (48 and 96 hours) through oral delivery. The extent of declined gene expression and percentage mortality chronicled for sodium channel and USP is proportional to the dsRNA concentration. Thus, this study affirms the outlook in the development of RNAi which can form a potent species-specific tool in the management of insect pests such as *A. gossypii.*

Xiaojun, *et al.* (2015) suggested juvenile hormone esterase (JHE) performs a rapid breakdown of residual JH in the hemolymph during last instar to induce a larval-to-pupal metamorphosis. A whole genome of the diamondback moth (DBM), *Plutella xylostella*, has been annotated and proposed 11 JHE candidates. Sequence analysis using conserved motifs commonly found in other JHEs proposed a putative JHE (Px004817). Px004817 (64.61 kDa, pI = 5.28) exhibited a characteristic JHE expression pattern by showing high peak at the early last instar, at which JHE enzyme activity was also at a maximal level. RNA interference of Px004817 reduced JHE activity and interrupted pupal development with a significant increase of larval period. This study identifies Px004817 as a JHE-like gene of *P. xylostella*.

RNAi is a reverse genetic tool with high sequence specificity, elicited by double-stranded RNA (dsRNA) for silencing the target genes, Chaitanya *et al.* (2017) The objective of this study was to silence two candidate genes from DBM viz., Juvenile Hormone Epoxide hydrolase (PxJHEH) and Ecdysteroid receptor (PxEcR) involved in regulating metamorphosis and molting respectively. In this regard, we have custom designed the dsRNAs (500 bp) by cloning and sequencing the candidate genes and delivered it orally (non-invasive mode). The extent of gene silencing and mortality recorded for both genes were proportional to the dsRNA concentration. Our study projects that PxJHEH and PxEcR genes would be good target for dsRNA mediated gene silencing in management of insect pests such as DBM in near future.

RNAi mechanism uses dsRNAs to silence specific targeted genes by inhibiting their expression. Though it has been challenging to generate effective RNAi in lepidopteran insects, in the current study this technology was applied to develop specific RNAi-based molecular tools that could be used to negatively impact the invasive lepidopteran forest pest, gypsy moth (GM). GM midgut-specific genes were selected for dsRNA design from larval transcriptome profiles, Gosh and Daw, (2017). Two different methods were used to produce specific dsRNAs, bacterial expression and in vitro synthesis, which were then fed to GM larvae. Depletion of uncharacterized gene targets known as locus 365 and locus 28365, or their stacked combination, depleted target transcripts in a sequence specific manner and resulted in 60% reduction in body mass. Treated GM females that were able to moult to the adult stage displayed an approximately two-fold reduction in egg masses. These have potential to be developed as molecular biopesticides for GM.

Chandra *et al.* (2019) reported that concatemerizing short double-stranded RNA corresponding to acetylcholinesterase (*AChE*) enhanced the RNAi efficiency in terms of higher target gene silencing and consequently resulted in lower larval weight gain and higher mortality of *P. xylostella*. Bautista *et al*., (2009) studied the influence of silencing the

cytochrome P450 gene CYP6BG1 that is over-expressed in a permethrin resistant DBM strain. When the gene was silenced after consumption of a droplet of dsRNA solution, the moths became significantly more sensitive to the pyrethroid insecticide.

We focused on studying a gene encoding a JHE enzyme because of its importance for insects' survival and development. Also, due to its specificity is a potential target for RNAi-based pest management. Usually, JHE is produced in the insect fat body, but the expression of JHE-encoding genes has been observed in other tissues, such as the gut (Huang *et al.,* 2019), as confirmed by our transcriptome. Furthermore, local activity of JH in the gut (Rahman *et al.,* 2017) and neofunctionalization of JH isomers in nervous tissues.

2.3 Off-target search

Jackson and Bartz (2003) described Off-target effects Using genome-wide microarray profiling as a method of detection, the authors identified modest, 1.5- to 3-fold changes in the expression of dozens of genes following transfection of individual siRNA. The defference of complementarily between the sense or antisense strand of the siRNA and suggesting a sequence specific component to the phenomena. The off-targeted genes varied considerably, and the overall off-target expression profile was unique for each siRNA.

Saxena and Jonsson (2003) established that single base pair mismatches between the siRNA and the target transcript dramatically alter siRNA functionality. From this result it was inferred that overall sequence identity played a role in siRNA specificity, thus leading to the adoption of local alignment algorithms, such as BLAST and Smith-Waterman to minimize off-target effects. To achieve this, users identified optimal target sequences using one or more selection criteria, and then filtered the resulting collection to eliminate sequences that shared significant levels of identity (such as > 15/19 bp) with other genes in the target genome.

Off-target effects are describes as the silencing of nontarget genes in the same organism or in non-target organisms. Off-target effect is one of the major limitation associated RNAi technology. Sequence homology of siRNAs to the non-target genes especially in its 3′ UTR region can cause off-target effects (Birmingham *et al.,* 2006, Jonathan and Jian, 2013). However, this limitation can be partially overcome by specific selection of the target region.

Naito *et al.* (2012) RNAi is commonly recognized as a powerful tool not only for functional genomics but also for therapeutic applications. Twenty-one nucleotide long siRNA suppresses the expression of the intended gene whose transcript possesses perfect complementarity to the siRNA guide strand. Hence, its silencing effect has been assumed to be extremely specific. However, accumulated evidence revealed that siRNA could down regulate unintended genes with partial complementarities mainly to the seven-nucleotide seed region of siRNA. This phenomenon is referred to as off-targete ffect. We have revealed that the capability to induce off-target effect is strongly correlated to the thermodynamic stability in siRNA seed-target duplex. For understanding accurate target gene function and successful therapeutic application, it may be critical to select a target gene-specific siRNA with minimized off-target effect.

2.4 Synthesis of PLGA nanoparticle

The homogenization of water and organic phases performed to encapsulate the ascorbic acid in the polymer matrix for controlled delivery (Stevanovic *et al*., 2007). Particles of the DLPLG with the different content of ascorbic acid have different morphological characteristics, that is, variable degree of uniformity, agglomeration, sizes, and spherical shaping. Mean sizes of nanoparticles, which contain DLPLG/ascorbic acid in the ratio 85/150%, were between 130 to 200 nm depending on which stereological parameters are considered (maximal diameters Dmax, feret X, or feret Y). By introducing up to 15% of ascorbic

acid, the spherical shape, size, and uniformity of DLPLG particles are preserved.

Tyrell *et al*. (2010), Yokoyama *et al*. (2004) studied the drug loading efficiency of camptothecin in poly (ethylene glycol) blockpoly (aspartic acid) (PEG-b-Asp) micelles according to three different preparation methods: dialysis, oil-in-water (O/W) emulsion and solution casting. The strong influence of the preparation method of polymeric micelles on the particle size, drug loading and release. The drug loadings were found to increase from 1% to 26% to 58% respectively. When sonication was applied in conjunction with the dialysis and emulsion methods, the drug loading efficiencies increased up to 45% and 37%, respectively. The solvent can also have an impact on the drug loading for a given polymer. By using a solvent evaporation process for the loading of amiodarone in poly (e-caprolactone)-block-poly (ethylene glycol) (PCL-b-PEG) micelles, it was shown that acetone gave a significantly higher drug loading, compared to chloroform (87% vs. 67%) Elhasi, *et al.*, (2007).

Dongmei *et al*. (2011) preparation of small interfering RNA (siRNA)-loaded PLGA nanoparticles by the double emulsion solvent evaporation method and characterize their properties. The volume ratio between the inner water phase and the oil phase, the PLGA concentration, the sonication time, the siRNA load, and the amount of acetylated bovine serum albumin (Ac-BSA) in the inner water phase added to stabilize the primary emulsion. The effects on the siRNA encapsulation efficiency and the particle size were investigated. The viscosity of the oil phase was increased at high PLGA concentration, which explains the improved encapsulation by stabilization of the primary emulsion and reduction of siRNA leakage to the outer water phase. Addition of Ac-BSA increased the encapsulation efficiency at low PLGA concentrations. These results enable careful understanding and definition of optimal process parameters for preparation of PLGA nanoparticles encapsulating high amounts of siRNA with immediate and long-term sustained release properties.

Srivastava *et al.* (2013) to achieve improved efficacy, polyphenolic constituents of black (theaflavin [TF]) and green (epigallocatechin-3-gallate [EGCG]) tea in poly (lactide-co-glycolide) nanoparticles (PLGA-NPs) were entrapped with entrapment efficacy of ~18% and 26%, respectively. Further, their preventive potential against 7, 12- dimethyl benzanthracene (DMBA)-induced DNA damage in mouse skin using DNA alkaline unwinding assay was evaluated. Additionally, TF- or EGCG loaded PLGA-NPs showed significant potential for induction of DNA repair genes (XRCC1, XRCC3, and ERCC3) and suppression of DNA damage responsive genes (p53, p21, MDM2, GADD45α, and COX2) as compared with respective bulk TF or EGCG doses. Taken together, TF- or EGCG-loaded PLGA-NPs showed a superior ability to prevent DMBA-induced DNA damage at much lower concentrations, thus opening a new dimension in chemoprevention research.

Aimed to synthesize the 82/18 PLGA to characterize and study the in vitro degradation in the form of rods in phosphate buffer solution (PBS). The PLGA is main bioreabsoable polymers used in the field of medicine. The copolymer was synthesized by opening the cyclic dimer rings of the monomers D, L-lactide and glycolide, in the presence of the tin octanoate initiator and of the Lauryl alcohol co-initiator (Silva *et al.,* 2015). This copolymer is widely applied in sutures, devices geared toward the controlled release of medication, and the guided regeneration of bone tissue as it presents a short degradation time.

2.5 Chracterization of nanoparticles

Vila *et al.* (2004) prepared the nanoparticles of PLGA & chitosan, they compared the size and entrapment, and the study suggested the potential utility of SEM and TEM for the identification of particle size and morphology of the nanoparticles.

This study observed the effects of some process variables on the size and size distribution of nanoparticles Rubiana and Raul (2005) developed PLGA based spherical nanoparticulate drug carriers with

controlled size. Praziquantel, a hydrophobic molecule, was encapsulated in that. Sonication time, PLGA concentration and PVA concentration was found be the process variables that can influence the design of nanoparticels when prepared by double emulsion solvent evaporation method.

Poly (lactide-co-glycolide) (PLGA) nanoparticles with different physical characteristics (size, size distribution, morphology, zeta potential) can be synthesized by controlling the parameters specific to the synthesis method employed. The aim of this review is to clearly, quantitatively and comprehensively describe the top–down synthesis techniques available for PLGA nanoparticle formation, as well as the techniques commonly used for nanoparticle characterization (Astete, 2006). Many examples are discussed in detail to provide the reader with an extensive knowledge base on the important parameters specific to the synthesis method described and ways in which these parameters can be manipulated to control the nanoparticle physical characteristics.

Ramalho and Pereira (2016) mention in this work, a laboratory experiment performed to prepared poly (lactic-co-glycolic acid) (PLGA) nanoparticles using two different synthesis procedures, a single and a double emulsion–solvent evaporation method. They also performed a physicochemical characterization of the prepared nanoparticles (NPs) by determining the hydrodynamic size and zeta potential values through Dynamic and Electrophoretic Light Scattering, respectively.

Several studies have shown that the encapsulation of pcDNA with PLGA nanoparticles (NPs) protects them against DNase enzyme action and increases the efficiency of gene delivery to the cells. The purpose of this study was to encapsulate pcDNA encoding IFN-λ1 (pIFN-λ1) with a simple and cost-effective method using PLGA NPs. The pIFN-λ1-loaded PLGA NPs were produced by a double-emulsion-solvent evaporation method and characterized in terms of size, size distribution, and zeta potential by DLS and morphologically by SEM and TEM. Parisa *et al.* (2019) The NPs were spherical in shape with a mean diameter of

380 ± 3 nm, a zeta potential of −3.3 ± 7.6 mV, an encapsulation efficiency of 75 ± 5%, and a loading capacity of 0.83 ± 0.06. The NPs were also bioactive and easily engulfed by RAW264.7 cells.

2.6 Nanotechnological inventions in insects

Benefits of nanotechnology, though limited, have driven efforts to develop advanced nanoparticles. Nanotechnology-based advanced materials are rapidly expanding development of better medicines. Long-standing efforts with lipid and polymer colloidal delivery systems, i.e. nanoparticles, have yielded better imaging and therapy. This is particularly the case for targeted nucleic acid (gene) therapeutics based on short interfering ribonucleic acid (siRNA), which is a new gene inhibitor that is highly potent and selective. Here, they evaluate the use of modular conjugates to construct targeted nanoparticle therapeutics for nucleic acids. These nanoparticles are beginning to emulate the sophistication of virus particles–nature's own nanoscale assemblies for nucleic acids (Woodle and Patrick, 2005).

Katas and Alpar (2006) explored chitosan as a siRNA vector due to its advantages such as low toxicity, biodegradability and biocompatibility. Chitosan nanoparticles were prepared by two methods of ionic cross-linking, simple complexation and ionic gelation using sodium tripolyphosphate (TPP). Both methods produced nanosize particles, less than 500 nm depending on type, molecular weight as well as concentration of chitosan. Chitosan–TPP nanoparticles with entrapped siRNA were shown to be better vectors as siRNA delivery vehicles compared to chitosan–siRNA complexes possibly due to their high binding capacity and loading efficiency.

This review made by Tamura and Nagasakai (2010) describes the progress and strategies of siRNA delivery systems based on polyion complexes, including synthetic polymers, biopolymers and nanoparticles, for the therapeutic application of siRNA. Numerous siRNA-containing polyion complex systems bound together through electrostatic

interactions between the negatively charged siRNA and positively charged components. Additionally, stimulus-sensitive smart siRNA carrier systems, including bioreducible polycations and hydrophilic polymer–siRNA conjugates, have been developed to enhance the gene silencing efficacy of siRNAs.

Moon *et al.* (2011) Suggested that loosely aggregated conjugated polymer nanoparticles (CPNs) were used as nontoxic and efficient small interfering RNA (siRNA) delivery vehicles with delivery visualization. A significant down regulation (94%) of a target gene was achieved by transfection of HeLa cells with the CPNs/siRNA complexes, supporting CPN as a promising siRNA delivery carrier. The interfering nanoparticles, or iNOPs, made from repetitively branched molecules of a small natural polymer called poly-Llysine, Rana (2013) there team synthesized.

Das *et al.* (2015) reported that in spite of devastating impact of mosquito borne pathogens on humans, widespread resistance to chemical insecticides and environmental concerns from residual toxicity limit mosquito control strategies. They tested three nanoparticles, chitosan, carbon quantum dot (CQD), and silica complexed with dsRNA, to target two mosquito genes (SNF7 and SRC) for controlling *Aedes aegypti* larvae. Relative mRNA levels were quantified using qRT-PCR to evaluate knockdown efficiency in nanoparticle-dsRNA treated larvae. The knockdown efficiency of target genes correlated with dsRNA mediated larval mortality. Among the three nanoparticles tested, CQD was the most efficient carrier for dsRNA retention, delivery, and thereby causing gene silencing and mortality in *A. aegypti*.

2.7 Method to delivery of nanoencapsulated dsRNA in insects

Microinjection was already used by Fire and Mello in the early days of RNAi (Fire *et al.,* 1998) as a method to introduce dsRNA into *C. elegans.* later, some easier and cheaper methods such as soaking and feeding have found their way into nematode and insect RNAi

experiments, but microinjection is still a widely used and very efficient research tool to introduce dsRNA into organisms in vivo, not only for nematodes but also for arthropods specifically at laboratory level. After the initial discovery in this nematode, dsRNA-mediated RNAi research by ingested dsRNA was applied in various insects such as *Spodoptera exigua, Diabrotica virgifera virgifer and Epiphyas postvittana* (Turner *et al.,* 2006; Baum *et al*., 2007; Tian *et al*., 2009; Surakasi *et al*., 2011). Two main strategies can be used in dsRNA-feeding experiments: dsRNAs can either be expressed in bacteria, or they can be synthesized *in vitro*, and then fed to insects either by mixing with food or by supplying as solution droplets.

Nanoparticles formulated from PLGA are being extensively investigated as non-viral gene delivery systems due to their controlled release characteristics and biocompatibility. However, encapsulation of plasmid DNA/protein has been beset by various problems, mainly low drug loading within the nanoparticles and the fact that the homogenization procedures employed in their manufacture usually compromise DNA integrity (Hsu *et al.* 1999). Efforts are being made to modify the procedure or the properties of PLGA for better loading, bioactivity etc. Hence encapsulation efficiency and release rates through nanoparticle-mediated drug delivery system can be optimized to improve their therapeutic efficacy. There has been much interest in using nanoparticles made from biodegradable polymers such as the most used poly (lactide-co-glycolide) because of its biocompatibility (Aminabhavi *et al.,* 2001).

Nanoparticles can be used to reduce dsRNA degradation and to increase the cellular uptake of intact dsRNA. Polymeric nanoparticles are produced using natural and synthetic polymers by wet synthetic routes. These are used because of their stability, ease for surface modification (Vauthier *et al.*, 2003; Herrero Vanrell *et al*., 2005) as well as their biodegradability and environmental safety.

Jaganathan *et al.* (2005) prepared a stable polymeric microsphere that can release TT over a period ranging from days to over months. They encapsulated TT in PLGA, chitosan and the proteins were stabilized by a protein stabilizer (trehalose) and the immune responses were compared. They also utilize $Mg(OH)_2$ to neutralize the acids liberated by the biodegradable PLGA polymer, they also added an antacid, which neutralizes the acidity during degradation of the polymer and prevented aggregation caused by PLGA, this study help us to understand the biggest drawback of PLGA polymer.

The dsRNAs of a species-specific E-subunit of the vATPase gene of *T. castaneum, A. pisum*, and *M. sexta* were synthesized in cell-free condition by Whyard *et al*. (2009). The dsRNAs can also be synthesized *in vitro* and the dsRNA solutions were dissolved in liquid artificial food or overlaid on the surface of solid foods for these insects. The ingestion of vATPase dsRNA led to 50%–75% mortality for all three insects. a similar dsRNA feeding experiment with *A. pisum* aphids using a microsachet feeding apparatus where dsRNA was mixed in 100µL of liquid artificial diet according to Sadeghi *et al.*, (2009).

2.8 Insect bioassay

Examined the effects of a feeding-based RNAi technique to target the gene trehalose phosphate synthase (TPS) in the *brown planthopper, Nilaparvata lugens,* Chen *et al.* (2010). The dsRNA feeding resulted in rapid and significant reduction in expression levels of TPS mRNA and enzymatic activity. Developmental abnormalities were observed in dsRNA fed *N. lugens* larvae which resulted in lethal effects. Insecticides play an integral role in the control of mosquito-borne diseases. With resistance to insecticides on the rise, surveillance of the target population for optimal choice of insecticides is a necessity. The Centers for Disease Control and Prevention (CDC) bottle assay and the World Health Organization (WHO) susceptibility test are the most frequently used methods in insecticide resistance monitoring Owusu, *et al.* (2015). However, the two bioassays differ in terms of insecticide

delivery and how insecticide susceptibility is measured. To evaluate how equivalent data from the two assays are, we compared the two methods side-by-side.

Dan *et al.* (2016) Owing to its increasing resistance to conventional pesticides, new strategies need to be developed for diamond back moth control. Investigated factors that modulate juvenile hormone esterase (JHE) activity and jhe (Px004817) transcription and determined the effects of these factors on subsequent growth and development in diamond back moth. Starvation inhibited JHE activity and jhe transcription, increased mortality, and decreased the rate of molting from the third- to the fourth-instar stages. Larvae kept at 32°C molted earlier and showed increased JHE activity and jhe transcription after 24-h treatment. Exposure to 1,325 mg/liter OTFP (3-octylthio-1,1,1-trifluoro-2-propanone) delayed molting and pupation, increased pupal weight, and decreased JHE activity and jhe transcription at both 24 and 48 h.The results demonstrated that JHE and jhe (Px004817) were involved in the responses of diamondback moth to external modulators and caused changes in growth and development. The combination of OTFP and pyriproxyfen increased the effectiveness of action against diamond back moth.

Paramasivam and Selvi (2017) performed Laboratory bioassays have become increasingly important because of their predictive value, generating comparative toxicity data on many chemicals in relatively short times at relatively low expense. investigated and provide a better understanding of insect-insecticide or insect-plant-insecticide interactions. It is a simple, versatile, easy, and sensitive technique for determining toxicity of wide range of chemicals, which greatly facilitates the determination of the LD_{50}, LC_{50} or any other lethal concentration/dose.

The leaf-dip bioassay is usually used to measure the toxicity to for systemic Insecticides. In this bioassay, insects are exposed to technical grade/ formulated insecticide and responses (mortality) recorded at a specific post-exposure interval. The mortality data are subjected to

log-dose probit analysis to generate estimates of a lethal concentration. These data collected over space and time could be used for monitoring insecticide resistance so as to develop appropriate insecticide resistance management strategies (Sabtharishiand Chandrain, 2017).

Christiaens *et al.* (2018) showed PAG87L polymer was selected as nanocarrier in the in vivo feeding bioassays. Chitine synthase B (ChSB) was chosen as a target gene for RNAi in *S. exigua*. Polymer dsRNA complexes were formed at a 2:1 N/P ratio. One hundred microliters of a 500ng dsRNA/µl solution were coated on a 2 × 2 cm Chinese cabbage leaf disc, for both naked and polyplex treatments. Mortality and the developmental stage of the larvae were recorded daily for 13 days and weight of the larvae was recorded every 2 days over the course of the experiment. statistical analysis for the weight data was performed using the SPSS software.

CHAPTER III

MATERIAL AND METHODS

The present research work on ‘Nano encapsulation of dsRNA of metamorphosis related gene and its insecticidal potential against Diamond Back Moth’ was carried out at the Biotechnology Centre, Dr. PDKV, Akola. All the chemicals were purchased from HiMedia unless otherwise stated.

This chapter elaborates the experimental material and methods adopted during the investigation. Various methodologies and activities that were undertaken during the course of investigation were standard research practices and protocols that are described in literature and were adopted either as such or with minor modifications, wherever required. The details of the material used, and the methods adopted during present investigation are as under,

3.1 Material

3.1.1 Insect culture

Larvae of *P. xylostella* collected from Department of Entomology, Dr. PDKV, Akola obtained by rearing the homogeneous culture.

3.1.2 Glassware

Glassware used in various experiments was of Rivera make. All the chemicals and reagent used in the present investigation were of bacteriological and molecular biology grade. For preparation of stock and other experimental work, Erlenmeyer flask, beakers, measuring cylinders, petridishes, falcon tubes and conical flask with screw cap were used.

3.1.3 Mass rearing of *P. xylostella*

Materials like Bell jars (oviposition chamber), plastic boxes, multicellular tray, forceps, fine camel hairbrush, absorbent cotton, muslin

cloth, tissue culture blades, test tubes, beakers, rearing rack, adult diet, electronic balance, refrigerator, measuring cylinder, fresh cabbage leaves, muslin cloth, 0.01% solution of sodium hypochlorite, camel hairbrush, cotton, rubber band and thread were used during experiment.

3.1.4 Chemical requirement

Various chemicals used for the synthesis, characterization studies of different metal nanoparticles are detailed in specific protocol; chemicals were purchased from Himedia and SRL.

3.1.5 Instruments used

Table 3.1. List of different instruments used in the present study

Sr.No.	Instrument	Source
1	Ultracentrifuge	Eppendorf
2.	Thermal cycler	Eppendorf
3	Water bath	WiseCircu
4	Double distillation unit	Borosil
5	Vacuum concentrator	Eppendorf
6	Filtration assembly	Sartorius
7	FTIR	Shimadzu
8	Particle size analyzer	Nanosight
9	Nano photometer	Implen
10	Weighing balance	Sartorius
11	Refrigerator (-20^{o}c)	Vestfrost
12	Autoclave	Hospharma
13	Magnetic stirrer	Remi
14	Mortar and Pestle	Fischer Scientific
15	UV-visible spectrophotometer	Systronics
16	Laminar Air Flow	Micro-Filt (India)
17	Orbital Shaker Incubator	Remi
18	Hot Air Oven	Swastik Scientific Co.
19	Sonicator	Bandelin

3.2 Bioinformatics work

A) Identification of *P. xylostella* specific genes: potential candidate for its control

i. *P. xylostella* specific genes were selected, based on its morphological importance and essentiality of that gene to *P. xylostella* for its development.

ii. Juvenile Hormone Esterase (JHE) gene of *P. xylostella was used* for further study.

Table 3.2. Identification of *P. xylostella* specific genes, *Plutella xylostella* juvenile hormone esterase (LOC105387861), mRNA NCBI Reference Sequence: XM_038121711.1

GenBank
>XM_038121711.1 PREDICTED: Plutella xylostella juvenile hormone esterase (LOC105387861), transcript variant X4, mRNA
ATTTTAAGTCGCACGTTGGCAACACTGCAGTTACTTTACCGAGGTCACGAGTGCGGTAGCCGGGTTGCGGTTTTA
AAACAAATACAACTCAGTGCAATATAGTAAATTAAGGATTTATCAACGATATTACAAACGGGGAGGCAGGATGTCA
GACGCTCCGGTGGTGTCAGTACAGCAGGGCTCCCTGCAGGGCCGCCTGGTGTCAGCTGCTGACGGGAAGGCGT
ACTACAGCTTCCAGGGCATCCCGTATGCCAAGCCGCCGCTGGGCAGCTTGAGGTTTAAGGCACCGCAACCCGTG
GAGCCATGGGAGGGCGTCCGCGAGGCCACGGCCGAGGGCAACGTCAGCGCACAGATAGACCCCCTTTTCCACA
AAGAATACATCGGGGACGAGAACTGTCTGTTCCTCAACGTATACACCCCCAATCTGGACGGAGAGTTCCTTCCCG
TCATGGTCTTCATACATGGAGGAGGGTTCAAATGGGGCTCAGGGAATACCTCGCTTTATGGACCAGATTATTTGG
TAGACAGGGATGTGGTGGTTGTGACGTTAAACTACAGATGCGGACCGCTGGGCTTCCTCTGTTTGAACACGCCAA
AGTGCCAGGAAACGCCGGTCTAAAAGACATAGTGCAGGCGGTCAAATGGGTCAAGGATAACATTCAAAACTTCGG
TGGGAATCCTGGAAATGTGACCGTTTTCGGGGAGAGCGCCGGTGGGGTTGCAGTCAGTCTGTTAACTGCTAGTC
CGCTGACCAAAAATCTTATTAGCAAAGCGATTATACAGTCTGGTACCGGATTGAATGGATGGGCGTTCCAAAAGAT
CCTTTGGAGAACGCTAAAGCTTTGGCTAAAACCCTTGGATGCGAGTCGGAAGACTACGAAGACATATTGGAGTTT
CTGTCAACAACGCCGGTGAAGGACCTGGTTGTAGCCAACAGCAAATTGGCTCCACCAGAACTGTTCTACGAAAAA
GCGTCCTCAATCTTCGCTCCGGTAGTCGAGAAAGAATTCCCAGGAGTCGAAGCGGCTTTGACCGAACCTTTCATA
GACCTGCTAACGTCCGGCCGAACAGCCGAAATCCCGATCATGATTGGTTCGACCACTCTCGAATTCATGCTCGGC
CATACGCCAGAAGACTTCCAAATCTTCATTCCTGACAACTTGAGCTTGAAGCGAAACTCCGAAGAGTCTTTAGCTG
TCGCTGACAAAATCAAGAGCCTCTACTTCAAAGACGGCCACACGGGGGTGGAGCATTTAACTGAGTATTACCAGC
TACTGTCAGACAAACTGATCAACATCGACACCCATCGCTATGTGAAATACCTGATCAATGTATCCAAAAAGCCGAT
CTATTACTACAAGTTCGACTACGTAGGGGAGTTAAACGTCCATGGGAAGCTGTTGACAAGTCTGGGGTTGAAGCA
CGCAAGCCACATGGACGAGTTGGGGTATTTGTTCAAGAATGTGCTTCAGAATGATGTGGAGCCGACGGCGGCTG
ATGTGAAGATGAGGGAACGCATGCTGAGGCTTTGGACCAACTTTGCTAAGGGAGGAAACCCTACCCCCGAAGAG
AACCACTTCATAACCGTCAACTGGCAGCCAGTGACCAAAGACAACCTCTACTACTTGAAACTAGGTTCTGAATATC
ACTGGACACGAACCCTGACAAAGAAAAGATGGAATTCTGGGAGGAGCTCTACAGCAAATACTTCAAAATCTGGGA
AGAACCCATCAAAAACGACGTAGCAGTAGAACAAGTGACACCAGTAGATGAAACACAAGAGACAGTAGATGAAAA
ACAAGAGACAGTAGATGAAAAACAAGAGACAGTAGATGTAAAAGAAGAAAATGTTGAGACAGAAACAGTTGTAGAT
GATTCTAAGTCAGAACCTAATGCTAACAATGTGGATGAACAAGAATTTGTTACCGACACAAATGGAATTGTGACTG
AAACTGAAGAAGTAGTACAAATAGTTACCAAATCTGAAGAAATTATTGAAACAGTCACTGAATCAGAAAATCACGAA
GAAGTAAAACCAGACATTGTTGAATCAGTCACTGCATCTGAAACATTAGTTAAAGAAGTGAAACCAGAAGTTGTTG
AAGAACCGGAACCAATTGTAGAAGTATCCCCTCTGAAGGAAGAAGTCAAAATAGTAGCAGAAGAAATACAAGGGC
CGAAAGTGACAGAAATAGCGAAGGAATACCAGAATGGGAACGCGAAGATTCACCAAAATGGCAACGGATATTTGG
ACAAAGCTCCATCAAGAACGTCCAACGAAATTAAAATGGCTGTCAAAGCTAATGGCGCTCCTAAAGACGTGATCA
GAGCAAACGACCCACCAGAAGATGATTTGCCTAAGAATATTGGTGTCAACAAATTCGTCAACTTTTTCGAATCACT
CGGTGGCAAGAAGTAAATATCCTTGAAGTAGGTACAGTTTAGTGCTAATTTCTCCATTTTCGGTTAGAGCATAACC
AGGTAGTAGTGGTTGACTTTTGACACATCTGTCAAGAATTTTTTATGGAGATGACGTTTAATTTATAAATCAATCCC
TGTCGGTTAGATAACCGACTATGGAGAAATTGGGGCTTAATCTATTAAGTTCTTGAGTATAGTGATAGTGACACTA
ACTTATCTGATCCACCCTAACAAATTAATTTATGGATTAGAAGAATTTATTTATTTTATTCACCAAGCTTGGATTAGG
TAGGTATAATAAAATTATGCTAAAAGCAATTACGAAAATGCCATCAGTAGGCCAGATAAGTTAGTTTTACTATACTC
TCTTTTTATTTTAACGTATGACTGGTCATAGATACTACTGGTTAAGTTATTTCTATTA

B) Off target prediction

i. Homologous sequences were searched in the target insect / parasitoid / predators and mammals. Off target homologies was rejected.

ii. 200bp to 500bp gene sequence was selected based on its uniqueness in *P. xylostella* NCBI reference sequence XM_011558648.1 and XM_038121711.1

C) Primers designing for the potential target genes

i. Primers for the *P. xylostella* specific gene were design using pick-primer software, available on NCBI web server. Suitable primer pairs were selected, amplifying 200-500bp gene unique to *P. xylostella*

ii. The complete gene sequence of Diamondback moth downloaded from NCBI Gene bank and used for dsRNA designing. Amongst these possible targets, promising target(s) was identified by using some stringent selection criteria's. (Fig 2).

a. Selected dsRNA should not possess any homology with other genes of *P. xylostella*. DBM genome will be used as a reference genome to rule out the homology with existing genes by performing BLAST (Basic Local Alignment Search Tool).

b. Similarly, homology with other nonredundant genes were ruled out by performing BLAST by taking 21 nucleotide (nt) long dsRNA sequence as a query sequence and redundant genome database as a refere

3.2.1 Rearing of *Plutella xylostella* (DBM)

The larvae and pupae of homogenous *P. xylostella* were obtained from Department of Entomology, Dr. PDKV, Akola. They were reared in the laboratory on the mustard seedlings till further use. The rearing procedure described by Lu and Sun (1984) was followed to maintain the test culture of *P. xylostella*. Mustard seeds were used for raising seedlings. The treated seeds were sown in plastic cups containing soil rite. The seedlings were placed in the mating chamber as a substrate for oviposition of *P. xylostella*. The moths (approximately 50 numbers) of *P. xylostella* were released in the mating chamber. Once in two days seedlings were replaced with another fresh set. The adults were provided with liquid adult diet (Moharil *et al.,* 2008). The eggs were hatched within

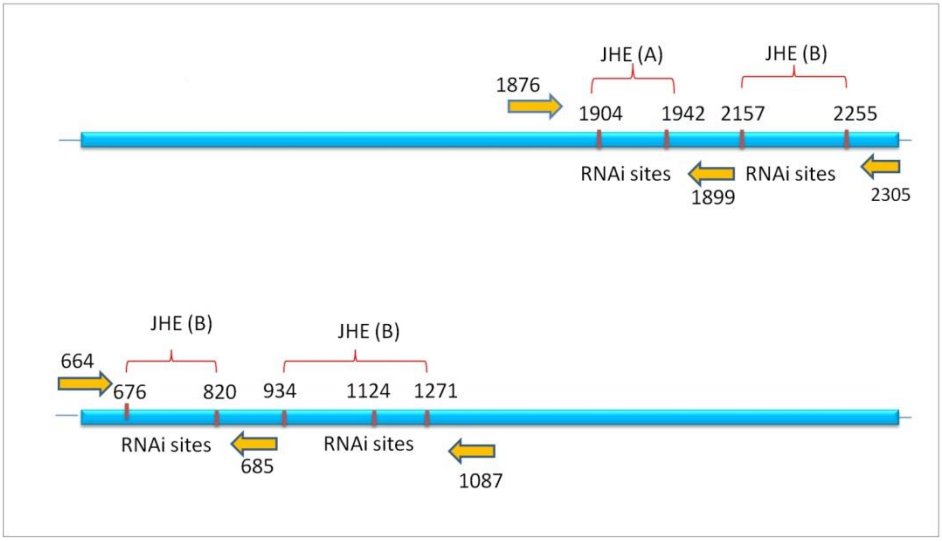


Fig. 2. Primers Location after predicting RNAi targets

12 days after oviposition and the neonates could feed on the same seedlings. After the consumption of the seedling's larvae were transferred to the fresh mustard seedlings. The rearing was done in rearing chamber at temperature 27^0C ± 1^0C, relative humidity 75 ± 1 percent and photoperiod approximately 13:11 light: dark hours. Aseptic conditions were maintained to avoid contamination with fungus and other pathogen by daily cleaning and sterilization. Pupae were sorted out carefully and transferred to adult emergence chamber.

3.2.2 DNA isolation

The DNA isolation was done using a manufacture's protocol with minor modifications. stepwise protocol for DNA isolation is as given below:

1. Collected the 5 larvae from maintained rearing culture and keep this into liquid nitrogen to keep them freeze.
2. Homogenized the frozen larvae in mortar and pestle and add 500 µl Lysis buffer (100 mM Tris HCl. (pH -8.0) 50 mM EDTA. Na_2. (pH-8.0) 1.5 M NaCl, 2% CTAB and 0.1% BME) and vertex for 30 second at full speed.
3. Homogenate was incubated for 30 to 60 minutes at 65°C with occasional mixing at 10 minutes intervals.
4. Phenol, Chloroform, Isoamyl Alcohol (25:24:1) added and mix for 5 to 10 minutes also add proteinase K (20mg/ml) and again mix thoroughly.
5. Centrifugation of sample was done at 15000 rpm for 10 to 15 min. Transfer supernatant in new tube.
6. Equal volume of pre chilled absolute isopropanol was added and mix for 5 minutes and centrifuge at 10000 rpm for 10 minutes.
7. Supernatant discarded by saving pellets.
8. Equal volume of 70% ethanol added and mix for 5 minutes and centrifuge at 10000 rpm for 10 minutes.

9. Supernatant was discarded and dry the tube at room temperature for 30 minutes.
10. Pellet was dissolved in Tris EDTA (TE buffer) and freeze for downstream processing.

Total DNA concentration was determined using nanophotometer (IMPLN, Germany) and was electrophoresed in 1.2% agarose gel to check for DNA quality and integrity.

3.2.3 Agarose gel for DNA electrophoresis

10X TBE recipe and preparation

Table 3.3. Required components

Component	Mass (100ml)	Molarity
Tris free base (mw: 121.14 g/mol)	12.11 g	1 M
Boric acid (mw: 61.83 g/mol)	6.18 g	1 M
EDTA (mw: 372.24 g/mol)	4 ml	0.5 M

Procedure

1. Take 10 ml of 10X TBE and add 90 ml of distilled water to make 100 ml 1X TBE.
2. 1.2 g of agarose was added to the 100ml of 1X TBE.
3. Boil the sample to dissolve the agarose
4. Once the temperature came down, add 6 µl of EtBr to the solution.
5. Fill the tank with 1X TBE buffer to run the DNA.
6. Total RNA was run on 1.2% agarose gel.

3.2.4 Amplification of DNA using primers

Extracted DNA should be used as a template for further amplification studies. Amplification was carried out by using a thermal cycler. Gene specific PCR probability was carried out by using *Taq*

polymerase (HiMedia). However, promising samples were used for dsRNA synthesis by using MEGAscript T_7 RNAi Kit.

Table 3.4. Gene specific primers used in present investigation

Sr. No.	Gene name	Sequence	GC%	Product Size	Tm
1.	**Juvenile Hormone Esterase (JHE) A**				
	Left primer	CAGAACCTAATGCTAACAATGTGG	41.67	430	59.30°C
	Right primer	GGAGCGCCATTAGCTTTGAC	55.00		59.35°C
2.	**Juvenile Hormone Esterase (JHE) B**				
	Left primer	AAACTTCGGTGGGAATCCTGG	52.38	424	59.82°C
	Right primer	GATCGGGATTTCGGCTGTTC	55.00		59.35°C

The reaction was performed by adding following components to sterile thin-walled PCR tubes for each PCR amplification:

Table 3.5. PCR reaction mixture components

Sr. No.	Component	Volume
1	10X PCR Buffer	2µl
2	Taq Polymerase	0.3 µl
3	dNTP mix (10 mM)	0.4 µl
4	Primer	1µl
5	Template (250ng/µl)	2µl
6	ddH_2O	14.3 µl
	Total	**20 µl**

3.2.6 Resolution of amplified product

After completion of PCR reaction, separation of amplified fragments was carried out using Bio-rad gel electrophoresis assembly. PCR amplification products were analyzed by agarose gel electrophoresis on 2% agarose gel stained with ethidium bromide solution (0.5 µl/ml). and products were visualized using a gel documentation system (Alpha Innotech).

3.2.8 Primers for dsRNA with T_7 promoters

For the Juvenile Hormone Esterase gene, primers were designed both manually and with the aid of the online software (Primer3) for off-target minimized 500 bp dsRNA. It used to check the suitability of primers in respect to annealing temperature and G/C % etc. T_7 RNA polymerase promoter sequences were added to each primer and synthesized.

Table 3.6. Primers for dsRNA with T_7 promoters

Sr. No.	Oligo	Start	T_7 promoter + Primer séquence (5'⟶3')
1.	Left Primer	1876	GAATTAATACGACTCACTATAGGGAGACAGAACCTA ATGCTAACAATGTGG
	Right Primer	2005	GAATTAATACGACTCACTATAGGGAGAGGAGCGCC ATTAGCTTTGAC
2.	Left Primer	664	GAATTAATACGACTCACTATAGGGAGAAAACTTCGG TGGGAATCCTGG
	Right Primer	1087	GAATTAATACGACTCACTATAGGGAGAGATCGGGAT TTCGGCTGTTC

3.2.9. Preparation of dsRNA from PCR products (single tube synthesis)

A. PCR

PCR primers were designed to amplify 200 – 600 bp amplicons of the genes of interest. The targeted region was not located in high homology regions, and preferably located as close to the 3' prime end of the gene as possible. Both PCR primers were tailed with the T_7 promoter sequence GAATTAATACGACTCACTATAGGGAGA at their 5' end. Amplified the fragment by PCR using the following concentrations:

Table 3.7. Components for preparation of dsRNA synthesis

Sr. No.	Components	Volume
1.	10X PCR Buffer	5 µl
2.	Taq Polymerase	0.75µl
3.	dNTP mix (2.5 mM)	1µl
4.	Genomic DNA template (250ng/µl)	2.5µl
5.	Modified T7 Promotor Primer Mix (10 pmol/µl)	2.5µl
6.	ddH_2O	38.25µl
	Total	**50 µl**

3.2.10 Gel electrophoretic analysis

Separation of amplified fragments was carried out using Bio-rad gel electrophoresis assembly. PCR amplification products were analyzed by agarose gel electrophoresis on 2% agarose gel stained with ethidium bromide solution (0.5 µl/ml). The gel was run in 1X TBE buffer at 70-80 Volts for 45 minutes with standard ladders of 100 bp size used according to concentrations given on instruction manual. Gel Doc system (Alpha innotech) was used for further analysis.

B. Purification of PCR product

3.2.11 Gel elution

Gel elution of amplicons of intrest was performed using GenElute^{TM} gel extraction kit as per the protocol prescribed in technical bulletin. The GenElute Gel Extraction Kit is used to isolate DNA from polyacrylamide gels when using the following “crush and soak” method.

Table 3.8. Required components for gel elution

Sr.No.	Components	Volume
1.	Column Preparation Solution	60 ml
2.	Gel Solubilization Solution	140ml
3.	Elution Solution (10mM Tris-HCl, pH 9.0)	6ml
4.	GenElute binding Column G	60 each
5.	Collection tubes, 2ml	2 X 70 each
6.	Wash Solution Concentrate G	12 ml

3.2.11.1 Gel Diffusion Buffer

The gel diffusion buffer consists of 0.1% SDS, 1 mM EDTA, 10 mM magnesium acetate, and 500 mM ammonium acetate, pH 8.0. 50ml of nuclease free water were added to make 100ml solution to an appropriately size beaker. Components were added in the order that they are listed. While stirring mixed all components and nuclease free water

was added upto 100 ml. The ammonium acetate was added last to avoid precipitation of the SDS.

Table 3.9. Components for preparation of diffusion buffer

Sr.No.	Component	Amount for 100 ml
1	Water, Molecular Biology grade	100 ml
2	10% SDS Solution	1ml
3	0.5 M EDTA	0.2 ml
4	Magnesium acetate	0.215 g
5	Ammonium acetate	3.854 g

Procedure

1. The gel slice was excised containing the DNA band of intrest with a clean, sharp scalpel. The size of band was minimized by removing excess of gel.
2. The gel slice was weighed. 1-2 volumes of diffusion buffer to the 1 volume of gel were added. The gel slice was further incubated at 50°C for 30 min.
3. The sample was centrifuged for 1 min.
4. Removed residual polyacrylamide by measuring the volume of recovered supernatant and 3 volumes of the gel Solubilization solution was added. For every 100 ml of supernatant, add 300 ml of gel Solubilization solution.
5. 500 ml of the Column Preparation Solution was added to each binding column. Centrifuged 1 minute. Discarded flow through liquid. Solution was turned yellowish in color.
6. 100% isopropanol was added and mixed until homogenize properly.
7. Solublized gel solution mixture was loaded into the binding column that was assembled in a 2 ml collection tube. The color was change from yellow to red after sample was applied to the binding column.

8. Centrifuged the solution 1 minute after loading to column each time. Discarded the flow through liquid.
9. 700 ml of Wash Solution was added to the binding column. Centrifuged 1 minute. Removed the binding column from the collection tube and discarded the flow-through liquid.
10. Placed the binding column back into the collection tube and centrifuged again for 1 minute without any additional wash solution to removed excess ethanol.
11. Binding column was transferred to a fresh collection tube. 50 ml of Elution Solution was added to the center of the membrane and incubate for 1 minute.
12. Centrifuged 1 minute. The elution solution was preheated to 65°C before used.

C. Production of dsRNA

The purified PCR amplicons was used to synthesize dsRNA with the MEGAscript T_7 Kit or MEGAscript RNAi Kit (Ambion). RNase free tubes were used during the procedure in this order:

Table 3.10. Components for reaction mixture of dsRNA synthesis

Sr.No.	Components	Volume
1	ATP	2 µl
2	CTP	2 µl
3	GTP	2 µl
4	UTP	2 µl
5	Buffer (warm to 37˚C)	2 µl
6	Cleaned PCR product	8 µl
7	Enzyme mix	2 µl
	Total	**20 µl**

- Components were mixed gently and spin down.
- The samples were incubated at 37˚C for 6-12 hours (With constant shaking).

- Again, the sample was incubated at 75˚C for 5 min (Anneal), then cool down to room temperature (~25˚C) for about 3~4 hr or longer. Freeze dsRNA at -20˚C or -80˚C.

D. Digestion of dsDNA and ssRNA using MEGAscript RNAi Kit (Ambion)

Table 3.11. Required components for digestion of dsRNA

Sr.No.	Components	Volume
1	Nuclease free water	21 µl
2	Digestion buffer	5 µl
3	dsRNA	20 µl
4	DNase I	2 µl
5	RNase A	2 µl
	Total	**50 µl**

E. Purification of dsRNA

Table 3.12. Required components for gel elution

Sr.No.	Components	Volume
1	Nuclease-free water	150 µl
2	dsRNA	50 µl
3	Phenol	100 µl
4	Chloroform	100 µl
	Total	**400 µl**

dsRNA was air dried and add 15 µl nuclease free water. Then it was diluted 1 µl of dsRNA ten times. dsRNA concentrations were measure using nanodrop spectrophotometer and it was checked on an 2% agarose gel.

3.3. PLGA nanoparticle synthesis

3.3.1 Materials and methods

Poly (D, L-lactide-co-glycolide) (DLPLG) was obtained from Sigma-Aldrich and had a lactide to glycolide ratio of 50:50. Molecular

weight of polymer was 40000–50000 g/mol. Time of complete resorption of this polymer is 4–8 weeks. Methanol and Polyvinyl alcohol (5% PVA) was used. All other chemicals and solvents were of reagent grade (Stevanović *et a*l., 2007)

Procedure

1. Copolymer DLPLG was obtained by means of physical methods from commercial granules using solvent/nonsolvent systems.
2. Commercial granules poly (D, L-lactide-co-glycolide) (0.05 g) were dissolved in 1.5 ml of acetone and, after approximately two hours, 2 ml of methanol was added into solvent mixture.
3. DLPLG precipitated by the addition of methanol and the solution became whitish.
4. Organic phase was added dropwise into the aqueous phase containing 20 ml of PVA solution (0.02% w/w) while continuous stirring at 2000 rpm by a stirrer for 15 min (Sahin *et al.,* 2017)
5. Then sonicate the samples for 60 sec in an ice bath.
6. This emulsion was again diluted with 20ml of PVA solution. After that, the solution was centrifuged at 4000 rpm for 2 hours.
7. Discard the supernatant being careful not to disturb the nanoparticle pellet.
8. The remaining pellets collected as nanoparticle for further characterization method (Plate 1).

3.3.2 Characterization of PLGA nanoparticle

The synthesized polymer nanoparticles were characterized using UV-Visible spectroscopy, Zeta potential, particle size analysis and FTIR.

1. UV-Visible spectrometry

It is the primary characterization technique to monitor synthesis, stability, and molar absorptive of polymer nanoparticles. PLGA

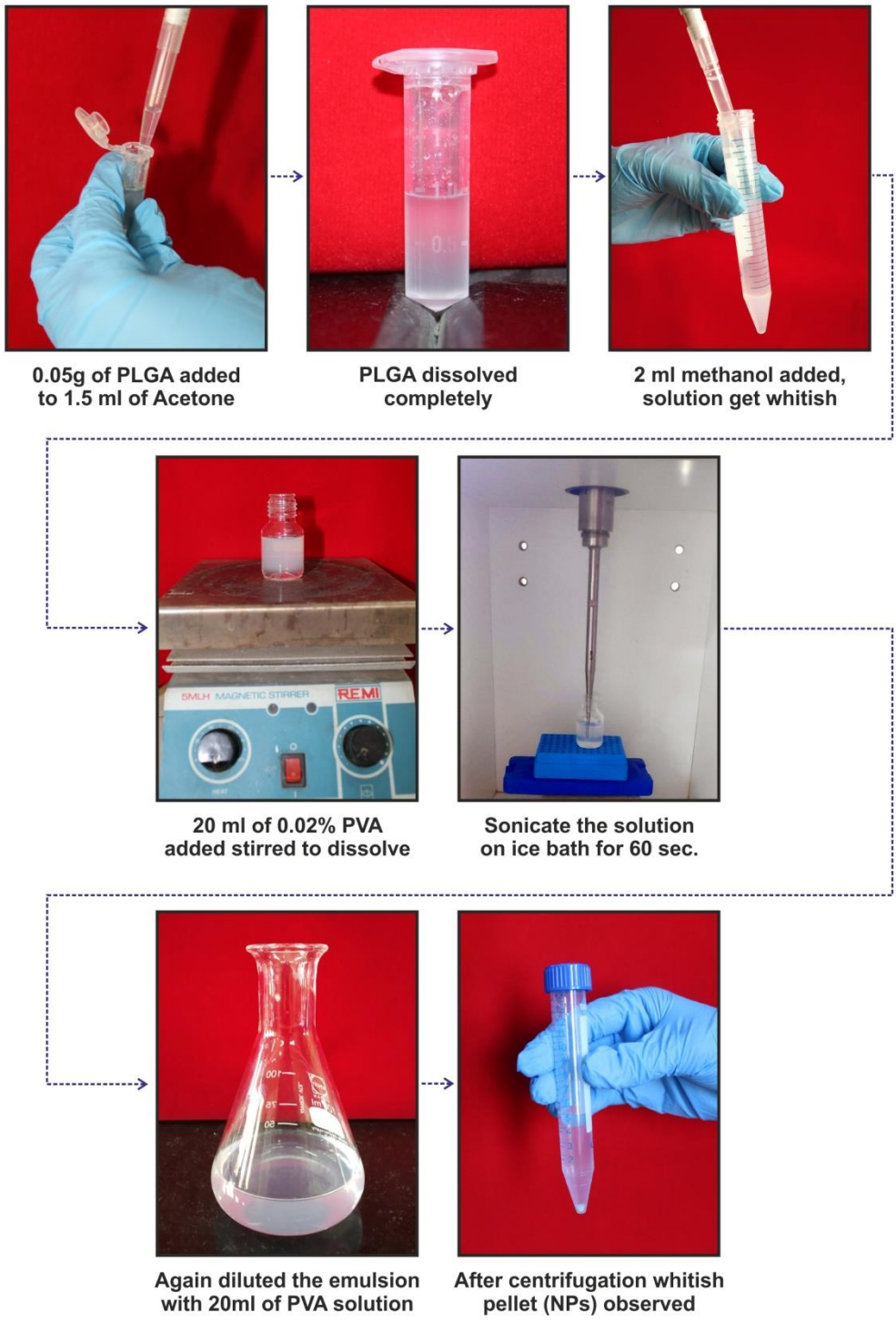


Plate 1. Synthesis of PLGA Nanoparticles using double emulsification method

particles in vitro in physiological solution (Acetone) were studied with UV spectroscopy. The UV measurements were performed on Perkin-Elmer Lambda 35 UV is spectrophotometer in the frequency interval of 200–600 nm.

2. Particle size analysis

Particle size and size distribution are the most important characteristics of nanoparticles systems. They determine the in vivo distribution, biological fate, and toxicity and targeting ability of nanoparticles in system. In addition, they can also influence the pesticide loading, drug release and stability of nanoparticles. Currently, the faster and most routine method of determining particle size is by photoncorrelation spectroscopy or dynamic light scattering.

Procedure Particle size analysis of PLGA nanoparticles

1. For dynamic light scattering measurements 12 mm cell was used.
2. One milliliter of distilled water (or ethylene glycol) was added to the cell and then 50 µl from stock dispersions were added.
3. Samples were again sonicated for 5 minutes, the supernatant was collected size distributions of the metal nanoparticles were determined with a computer controlled particle size analyzer.
4. The suitable parameters viscosity, absorption and refractive index were recorded for water and ethylene glycol.
5. The pH value of each suspension was adjusted by adding either NaOH or HCl.
6. Histograms with the distributions of sizes were recorded.

3. Zeta potential (ζP)

Zeta Potential analysis is a technique for determining the surface charge of nanoparticles in solution (colloids). Also known as electrokinetic potential, it is measured in mill volts (mV). Nanoparticles

have a surface charge that attracts a thin layer of ions of opposite charge to the nanoparticles surface.

This double layer of ions travels with the nanoparticles as it diffuses throughout the solution. The electric potential at the boundary of the double layer is known as the Zeta potential of the particles and has values that typically range from +100 mV to -100 mV. The magnitude of the zeta potential is predictive of the colloidal stability. Nanoparticles with Zeta Potential values greater than +25 mV or less than - 25 mV typically have high degrees of stability less negative value than -15 mV represents beginning of agglomeration of particles. Higher is the zeta ion potential more stable is the colloid of nanoparticles. When zeta potential becomes zero, the colloid will precipitate into solid. It cannot be measured directly and is calculated on basis of electrophoretic mobility based on rate at which a charged particle moves in response to electric field, nanoparticles with zeta potential will migrate towards opposite charged electrode and its rate of migration is equal to zeta potential.

Procedure for Calculations for zeta potential

1. Zeta cells were rinsed thoroughly with water, followed by ethanol, and finally water again. The zeta cells were dried using a gentle stream of nitrogen attached to a filter to remove any remaining solvent.
2. The zeta cells were capped until use to prevent any dust contamination. Samples were prepared in a low ionic strength medium; 10 mM NaCl Suspending medium was filtered prior to sample preparation using a 0.1 μm or smaller pore size membrane.
3. Zeta potential is dependent on pH and therefore the sample pH was measured before and after the zeta potential readings. Samples were loaded into Zeta Cells without any air bubbles.
4. Zeta cell were filled with sample by gently depressing the syringe plunger in a drop-wise manner. Before loading the zeta cell into the instrument, the measuring windows were carefully wipe with lens paper.

5. Then electrodes on the zeta cell were kept in contact with the leads in the instrument. The temperature was controlled and measured with an accuracy and precision of 0.3°C.
6. A minimum of three runs per sample were taken to establish measurement repeatability. The applied voltage was set to automatic mode.

4. FTIR

FTIR Fourier Transform Infrared Spectroscopy is the analytical technique that identifies organic, polymeric and inorganic materials. It used infrared light to scan samples. It detected the functional groups present in nanoparticles. FTIR relies on the fact that the most molecules absorb light in the infra-red region of the electromagnetic spectrum. This absorption corresponds specifically to the bonds present in the molecule. The frequency range is measured as wave numbers typically over the range 4000 – 600 cm^{-1}.

3.3.3 Encapsulation of dsRNA in PLGA nanoparticles

3.3.3.1 Materials

RNA samples and necessary chemicals used for encapsulation procedure. Use RNase-free pipette tips, plastic ware, water, buffers, and treated glassware.

Procedure

Synthesis of dsRNA encapsulated with PLGA nanoparticles using a double-emulsion solvent evaporation (w 1 /o/w 2) technique is summarized below,

1. RNAs was resuspended in RNase-free water. Briefly centrifuged the tube to bring down all droplets from the wall and lid of the tube (Pantazis *et al.,* 2012)
2. 200 µl of dsRNA was to 400 µl of 5 % PEG (Modified Yokoyama and Huang, 2005). Incubation was done at room temperature for 30 min

3. Dispense 1 ml of $CHCl_3$ into a glass vial containing a magnetic stir bar. 30 mg of PLGA was added. Stirred to dissolve.
4. Sonicate on ice for 30 sec.
5. 6 ml of 2 % PVA was added to a glass vial with a magnetic stir bar. The emulsion was added from step 4 to the PVA solution. Sonicate on ice for 1 min.
6. Evaporated the solution ($CHCl_3$) overnight (18 h) under continuous stirring. Recovered the PLGA NPs by centrifuged at 20,000 × g for 20 min at 4°C. Removed the supernatant and saved it for later evaluation.
7. The pellet wash three times with 10 ml of RNase-free water to remove any residual PVA and free (i.e., non-encapsulated) dsRNA. Saved the supernatant from each wash separately for later evaluation of un-encapsulated RNA remaining in the wash solutions.
8. Resuspend the NPs in 5 ml of 5% sucrose solution to stabilize the dsRNA inside the NPs.
9. Sonicate the NPs for 30 s over an ice bath. Freezed dry the PLGA NPs. The lyophilized NPs were stable up to 6 months at −20°C.

3.3.3.2 Insect bioassay with dsRNA

To examine the effect of the encapsulated dsRNA, the following bioassays were conducted. Feeding was selected as a simple method to deliver the synthetic dsRNA, which was more feasible with small insects such as aphids than injection or other methods; the experiment was run as described below.

Fresh cabbage leaf discs (5 cm diameter) were placed in a Petri dish. These dilutions were applied individually on the surface of the leaf disc with a clean soft paint brush and allowed to dry. We used the highest concentration, 20 µl were spread on individual leaf disc, of both dsRNA and encapsulated dsRNA. This method was implemented from studies by Furlong and Endersby (Furlong *et al.,* 1994; Endersby *et al.,*

2008) with slight modification. A treated control was also maintained with nuclease free water alone. *P. xylostella* larvae were transferred to each leaf disc and maintained until the end of the experiment. A total number of 5 treatments (20 µg) with 3 replications having 10 numbers of larvae in each replication (Chaitanya *et al*., 2017). The plates were kept at 27°C for 10 days and 60% relative humidity. Observations on the larval mortality rates were recorded on subsequent days up to 5 days (Ellango *et al.,* 2014).

CHAPTER IV

RESULTS AND DISCUSSION

The present investigation entitled “Nano encapsulation of dsRNA of metamorphosis related gene and its insecticidal potential against Diamond back moth” was carried out at the Biotechnolgy Centre, Department of Agricultural Botany, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola during the year 2019-2021.

The current investigation carried out with the aim to deliver Nanoencapsulated dsRNA to the Diamondback moth and to syudy its insecticidal potential against the DBM.

The expression of the Juvenile Hormone Esterase (JHE) essential in controlling the metamorphosis along with growth and development of insects hence were chosen as effective targets in the integrated pest management.

The study conducted into two phases, Phase I dealt with the Identification of suitable RNAi targets in metamorphosis related genes of *Plutella xylostella* and development of dsRNA, Phase II dealt with the Nano encapsulation studies of dsRNA and its insect bioassay against Diamondback Moth.

The findings of the investigation are presented in this chapter along with the pertinent discussion under the appropriate subheadings.

4.1 Identifying suitable RNAi targets in *P. xylostella*

Primers for the Juvenile Hormone Esterase (JHE) gene were designed by employing Primer-3 software using the sequence information for the available accessions in NCBI for *P. xylostella* (Table 4.1). The gene-specific sequences were subjected to synthesize the dsRNA. A 200 to 600bp off-target minimized region was selected by employing siDirect 2.0 (http://sidirect2.rnai.jp/doc/) software for single

gene. These regions were compared for other populations of the study and variations were recorded. Further, the selected sequences were subjected to Invitrogen Block-iT RNAi designer software, web-based online software.

Table 4.1. Gene sequences of *P.xylostella* available accessions in NCBI

1. PREDICTED: Plutella xylostella juvenile hormone esterase (LOC105387861), mRNA

NCBI Reference Sequence: XM_038121708.1

GenBank Graphics

>XM_038121708.1:1876-2305 PREDICTED: *Plutella xylostella* juvenile hormone esterase (LOC105387861), transcript variant X1, mRNA

CTGACAAAGAAAAGATGGAATTCTGGGAGGAGCTCTACAGCAAATACTTCAAAATCTGGGAAGAACCCATC
AAAAACGACGTAGCAGTAGAACAAGTGACACCAGTAGATGAAACACAAGAGACAGTAGATGAAAAACAAG
AGACAGTAGATGAAAAACAAGAGACAGTAGATGTAAAAGAAGAAAATGTTGAGACAGAAACAGTTGTAGAT
GATTCTAAGTCAGAACCTAATGCTAACAATGTGGATGAACAAGAATTTGTTACCGACACAAATGGAATTGTA
CTGAAACTGAAGAAGTAGTACAAATAGTTACCAAATCTGAAGAAATTATTGAAACAGTCACTGAATCAGAAA
ATCACGAAGAAGTAAAACCAGACATTGTTGAATCAGTCACTGCATCTGAAACATTAGTTAAAGAAGTGAAA
CC

2. PREDICTED: *Plutella xylostella* juvenile hormone esterase (LOC105387861), mRNA

NCBI Reference Sequence: XM_038121711.1

GenBank Graphics

>XM_038121711.1:664-1087 PREDICTED: *Plutella xylostella* juvenile hormone esterase (LOC105387861), mRNA

AAACTTCGGTGGGAATCCTGGAAATGTGACCGTTTTCGGGGAGAGCGCCGGTGGGGTTGCAGTCAGTCT
GTTAACTGCTAGTCCGCTGACCAAAAATCTTATTAGCAAAGCGATTATACAGTCTGGTACCGGATTGAATG
GATGGGCGTTCCAAAAGACTCCTTTGGAGAACGCTAAAGCTTTGGCTAAAACCCTTGGATGCGAGTCGGA
AGACTACGAAGACATATTGGAGTTTCTGTCAACAACGCCGGTGAAGGACCTGGTTGTAGCCAACAGCAAA
TTGGCTCCACCAGAACTGTTCTACGAAAAAGCGTCCTCAATCTTCGCTCCGGTAGTCGAGAAAGAATTCCC
AGGAGTCGAAGCGGCTTTGACCGAACCTTTCATAGACCTGCTAACGTCCGGCCGAACAGCCGAAATCCC
GATC

System for computing highly effective dsRNA sequences with maximum target specificity (Table 4.2 and 4.3).

Table 4.2. Primers targeting maximum RNAi target sequence in Juvenile Hormone esterase(A) gene

Oligo	Start	Length	tm GC%	any_th 3'_th hairpin	Sequence
Left Primer	1876	24	59.30 41.67	4.00 2.00	CAGAACCTAATGCTAACAATGTGG
Right Primer	2305	20	59.35 55.00	4.00 1.00	GGAGCGCCATTAGCTTTGAC

Table 4.3. Primers targeting maximum RNAi target sequence in Juvenile Hormone esterase (B) gene

Oligo	Start	Length	Tm	GC%	any_th 3'_th hairpin	Sequence
Left Primer	664	21	59.82	52.38	6.00 2.00	AAACTTCGGTGGGAATCCTGG
Right Primer	1087	20	59.35	55.00	4.00 2.00	GATCGGGATTTCGGCTGTTC

4.2 Synthesis of dsRNA using MEGAscript T_7 RNAi kit

4.2.1 Total DNA isolation

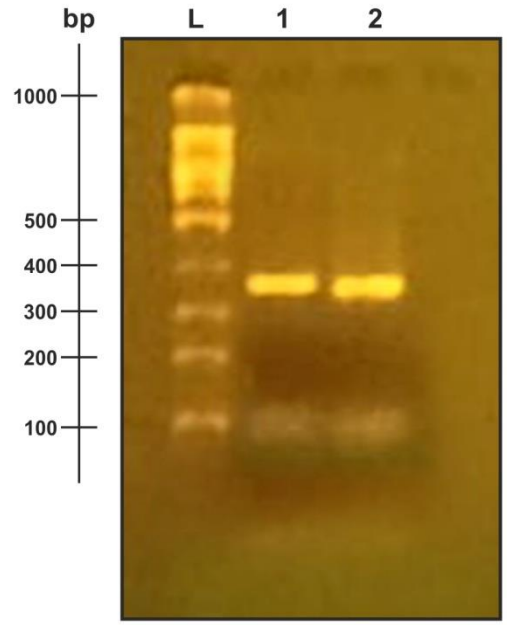
The DNA was isolated successfully from five to six larvae of *P. xylostella* using lysis buffer. To remove the RNA contamination DNA was treated with RNase I (RNase free) for 30 minutes. RNase I treatment has been carried out to achieve proper amplification of gene. The integrity of DNA was checked on 1.2% agarose gel (Plate 2/ A) and quantified using Nanodrop Spectrophotometer. The DNA was stored at -20°C for further use. The DNA was used as the template to amplify both JHE(A) and JHE(B) from *P. xylostella.*

4.2.2 Preparation of template for dsRNA synthesis

In present study, *P. xylostella* gene specific primers were used, (Table 4.2 and Table 4.3). The primers JHE (A) and JHE(B) were designed both manually with help of Primer-3 software. Amplicon of 430 bp size has been obtained from JHE (A). Similarly, amplicons of 424 bp size has been obtained from JHE (B). Depicted in (Plate 2/ B)

4.2.3 Primers for dsRNA with T_7 promoters

The size of T_7 promoter is 400 bp. It was added at 5' end using protocol given in MEGAscript RNAi kit. Size differentiation of template DNA and template DNA along with T_7 promoter. Depicted in (Table 4.4)



Isolated DNA of *P. xylostella* of lane : L-100 bp ladder 1 & 2 - DNA sample

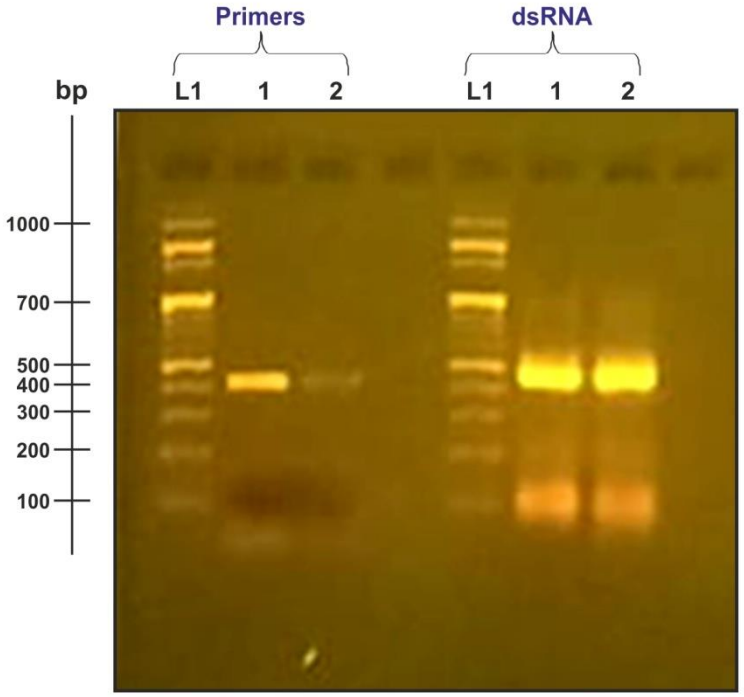


Figure: JHE gene specific PCR on DNA sample.
Lane1: 100bp, 1-JHE(A), 2-JHE(B)
Lane2: 100bp, 1 –dsRNA-JHE(A), 2-dsRNA-(B).

Plate 2. Synthesis of dsRNA by using MEGAscript RNAi kit

Table 4.4. Designed primer for dsRNA with T_7 promoter

Sr. No.	Oligo	start	length	tm	GC%	Sequence
1.	Left Primer	1876	24	59.30	42.60	GAATTAATACGACTCACTATAGGGAGAC AGAACCTAATGCTAACAATGTGG
	Right Primer	1305	20	59.35	55.00	GAATTAATACGACTCACTATAGGGAGAG GAGCGCCATTAGCTTTGAC
PRODUCT SIZE: 457, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 4.00						
2.	Left Primer	474	21	59.82	52.38	GAATTAATACGACTCACTATAGGGAGAAA ACTTCGGTGGGAATCCTGG
	Right Primer	663	20	59.35	55.00	GAATTAATACGACTCACTATAGGGAGAG ATCGGGATTTCGGCTGTTC
PRODUCT SIZE: 452, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 4.00						

4.2.5 *In vitro* transcription of dsRNA

4.2.5.1 Template preparation

Nested PCR was carried out using dsRNA specific primers with T_7 promoter. The PCR products were run on 2% agarose gel and thus obtained bands were extracted. The amplicons were extracted using GenElute™ Gel Extraction Kit (Sigma Aldrich). The eluted product was used as a template for synthesis of dsRNA.

4.2.5.2 *In vitro* transcription

The eluted product was used for *in vitro* transcription of the amplified gene with opposing T_7 promoters flanking in the transcription region were carried out using MEGAscript RNAi kit with 6-12 hours incubation at 37°C. The integrity of the dsRNA was checked on 2% agarose gel (plate 2/B) and the transcript product was quantified through Nanospectrophotometer.

4.3 Optimization of nanoparticle

The PLGA nanoparticles prepared using double emulsification solvent evaporation method reported by Stevanović et al., 2007 and modified by Sahin et al., 2017. Nanoparticle size depends on

various parameters include polymer concentration and stabilizer concentration and process variables include stirring speed, stirring time and sonication time.

4.3.1 Chracterization of PLGA nanoparticles by using different techniques.

1. UV Visible spectrophotometric analysis

PLGA nanoparticles were prepared by nanoprecipitation (Barichello et al., 1999). The synthesis was confirmed by UV-Visible spectrophotometric analysis, a typical absorbance peak was observed around 380 nm with absorbance 0.5 indicating the synthesis of PLGA NPs (Fig 3).

2. Particle size analysis

The average particle size of NPs was determined by using a Zetasizer, 0.02% PVA was used as stabilizer during the synthesis of PLGA. On increasing sonication time upto 60 sec particle size decreased. Same trend was also observed with stirring speed and stirring time. 1200 rpm for 15 min was optimized process variables, after that particle size of nanoparticles decreased. It was observed that, on increasing the stirring speed from 2000 to 3000 rpm and increasing the stirring time 4 hr to 5 hr the size of naoparticles was decreased.

During Present investigation, the average size of PLGA nanoparticles was observed to be 244.9 nm and the polydispersity index (PDI) of the nanoparticles was 0.1(Fig 4).

In similar study the average particle size of PLGA nanoparticles was observed between 107.7 nm and 245.7 nm and there all formulations showed uniform particle size distributions (Sahin et al., 2017).

3. Zeta potential

The zeta potential of NPs was determined by using a Zetasizer, the PLGA nanoparticles synthesized by using PVA as stabilizer. The zeta potential was found to be -4.5 mV (Fig 5). The quality

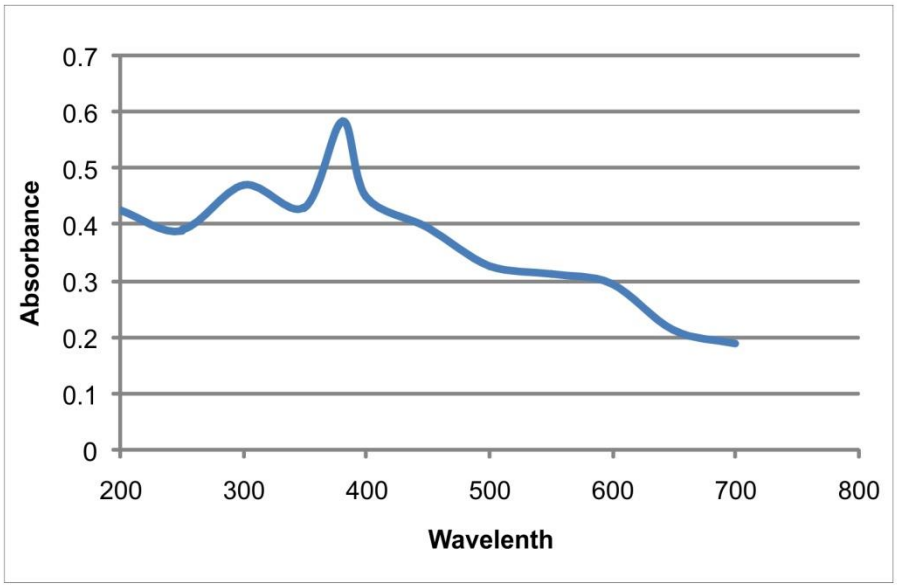


Fig. 3. UV Spectrophotometry analysis of PLGA NPs

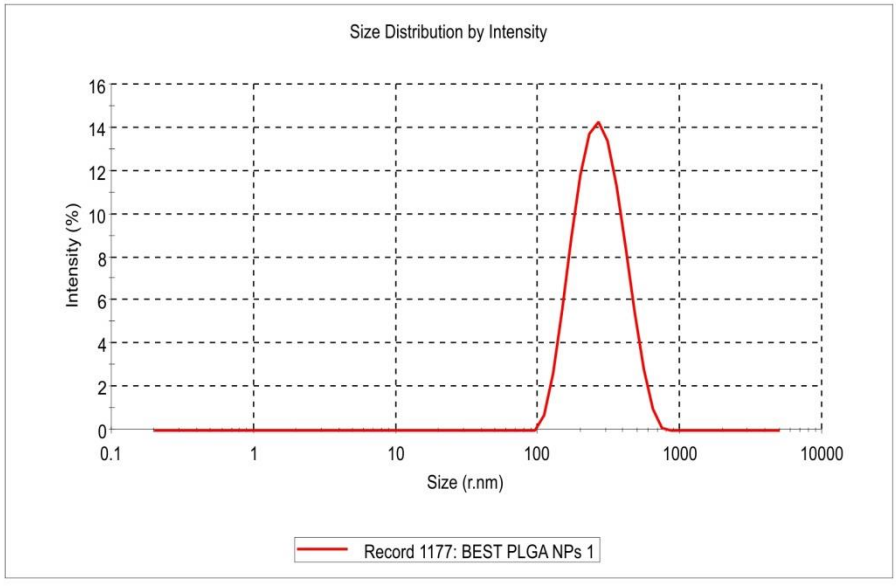


Fig. 4. Particle size analysis of PLGA NPs

of result was good as the zeta potential increases, the repulsive interaction will be larger, leading to the formation of more stable particles with a more with more uniform size distribution.

In same kind of study by Parisa et al., 2018 found zeta potential (mV) of PLGA NPs to be -3.3 mV.

4. FTIR (Fourier transform infrared spectroscopy)

FTIR spectra in the absorbance mode were recorded using FTIR spectrometer (Perkin Elmer). Various peaks in IR spectrum were interpreted for the presence of different groups. An FTIR spectrum in (Fig 6) shows that absorption bands at 3884 cm^{-1} and 3410 cm^{-1} (Alcohol) in the FTIR spectra for lactide and glycolide are attributed to stretching vibrations of OH group, 1780 cm^{-1} (Carbonyl group), 1494 cm^{-1}, and 1666 cm^{-1} (Glycolic acid), 1382 cm^{-1} and 1187 cm^{-1} (Ester) and 1096 cm^{-1}(Vinyl) group during PLGA NPs synthesis.

In similar reports FTIR analysis of PLGA NPs they observed medium intensity bands between 1300 cm^{-1} and 1150 cm^{-1} was recognized to asymmetric and symmetric C-C (=O)-O stretches respectively (Singh et al. 2014). The bands in these regions are useful in the characterization of esters. Bands at 3500 cm^{-1} and 3450 cm^{-1} in the FTIR spectra for lactide and glycolide are attributed to streching vibrations of OH group (Eason, 2007, Kiremitçi and Deniz, 1999).

Table 4.5. FTIR analysis of PLGA nanoparticles

Sr. No.	Range cm^{-1}	Wave number (cm^{-1})	Peak assignments	Functional group
1	4000 -2500	3884 3410	OH – stretching	Alcohol
2	1780-1750	1780	C=O stretch	Carbonyl group
3	1660-1410	1494	CH bending	Glycolic acid
4	1385-1150	1382 1187	C-C(=O)-O	Esters
5	1450-850	1096	O-C-C stretch	Vinyl C-H

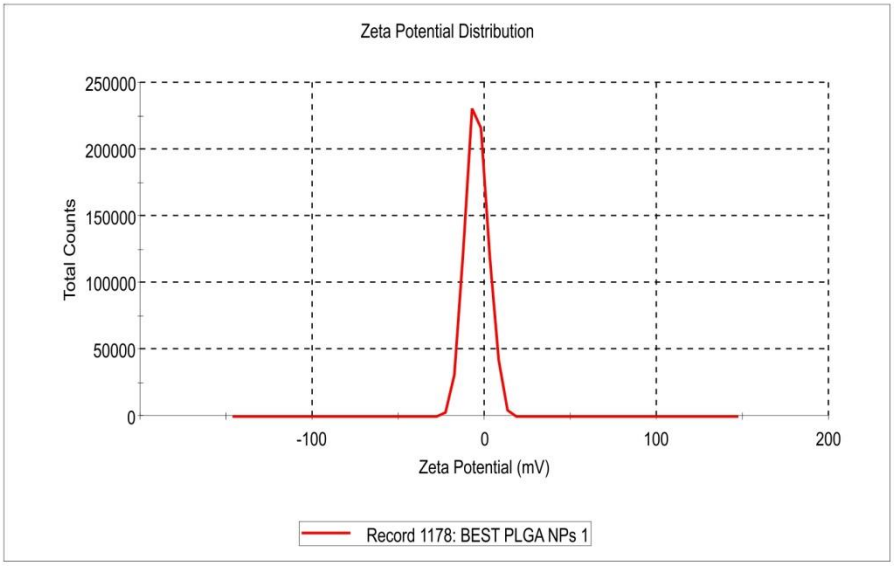


Fig. 5. Zeta potential analysis of PLGA NPs

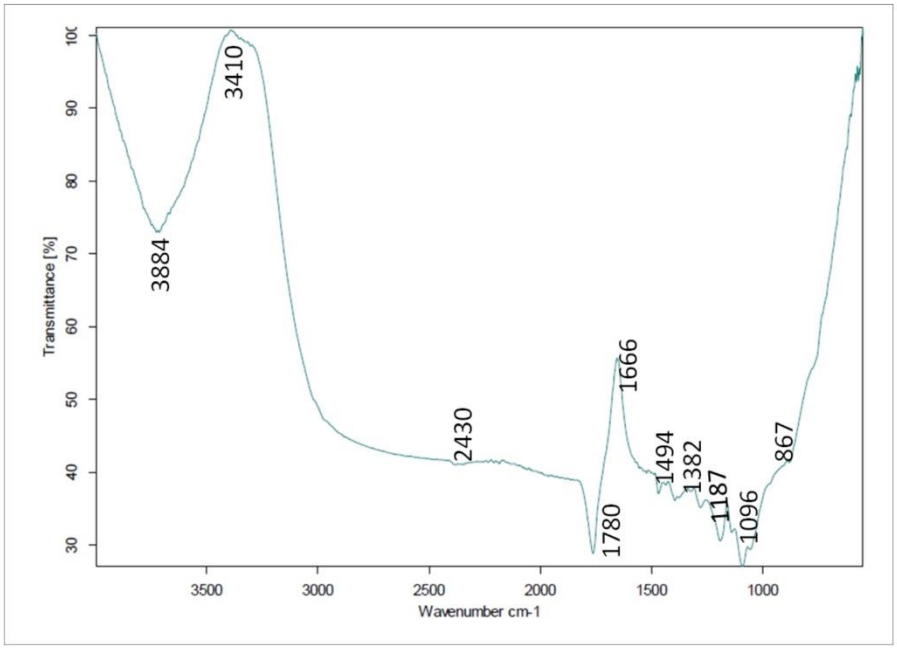


Fig. 6. FTIR Analysis of PLGA NPs

4.3.2 Encapsulation of dsRNA with PLGA nanoparticles

The process of encapsulation was done by following the protocol given by Pantazis *et al.* (2012) with some modifications. Prior to analysis dsRNA-encapsulated particles were collected by Centrifugation (Eppendorf) at 12,000 ×g for 10 min. The supernatants were discarded, and nanoparticles stored at -20°C.

4.3.3 Characterization of encapsulated nanoparticles

The particle size of the resulting both encapsulated particles were determined. The average size of encapsulated dsRNA-JHE(A) was 199.4 nm (Fig 7) and encapsulated dsRNA-JHE(B) particle size was 205.2 nm (Fig 10) respectively. Zeta potential of the samples was measured by using a Zetasizer (Malvern Nano ZS, Malvern Instruments Ltd., UK) and the zeta potential values were found to be (Fig 8) and -4.31mV (Fig 11) for dsRNA-JHE(A) and dsRNA-JHE(B), respectively.

Table 4.6. FTIR analysis of encapsulated dsRNA-JHE(A) nanoparticles

Sr. No.	Range cm^{-1}	Wave number (cm^{-1})	Peak assignments	Functional group
1	3550-3200	3376 3210	OH	Alcohol/Phenol O-H Stretch
2	1750-1725	1729	C=O	Ester C=O Stretch
3	1680-1500	1510	C=C	Alkynyl C≡C Stretch
5	1450-850	1421	CH	Aromatic C-H Bending, Vinyl hydrogen
6	1300-1150	1374	C-C(=O)-O	Esters
7	1040-100	1091 945	PO_2	PO_2 streching in RNA and DNA

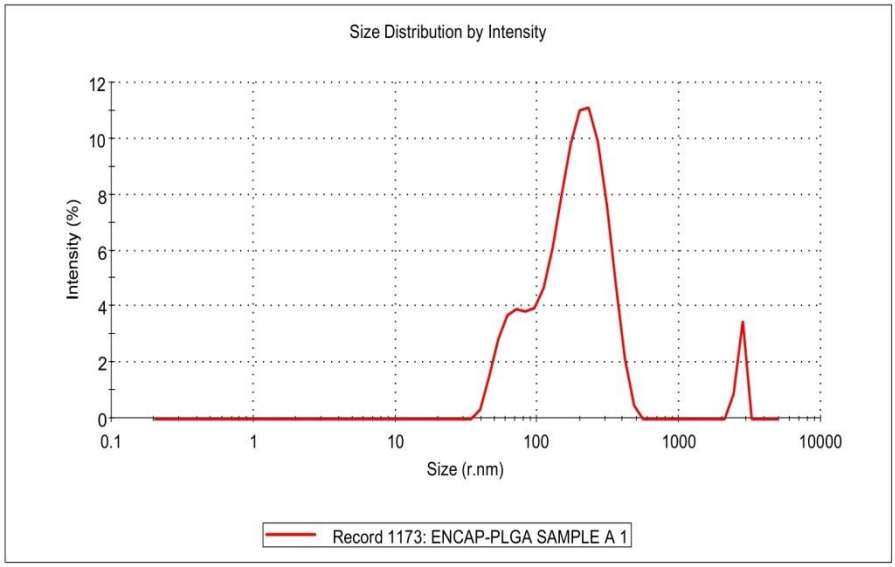


Fig. 7. Particle size analysis of ENCAP-PLGA NPs (A)

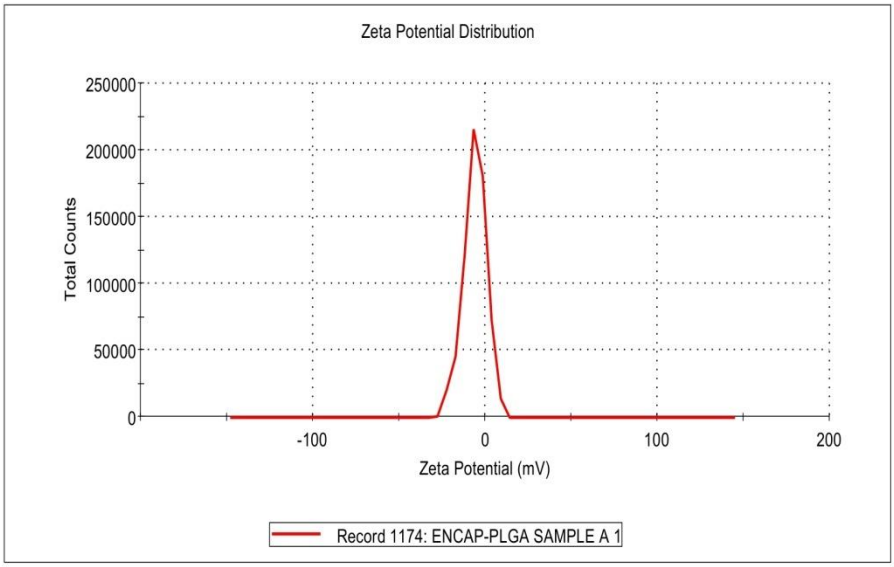


Fig. 8. Zeta size analysis of ENCAP-PLGA NPs (A)

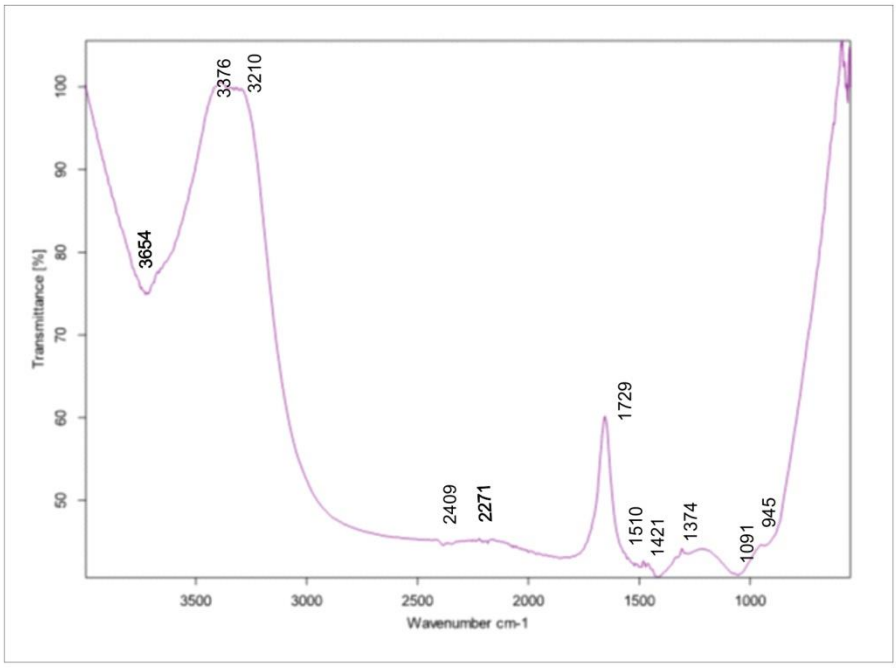


Fig. 9. FTIR analysis of ENCAP-PLGA NPs (A)

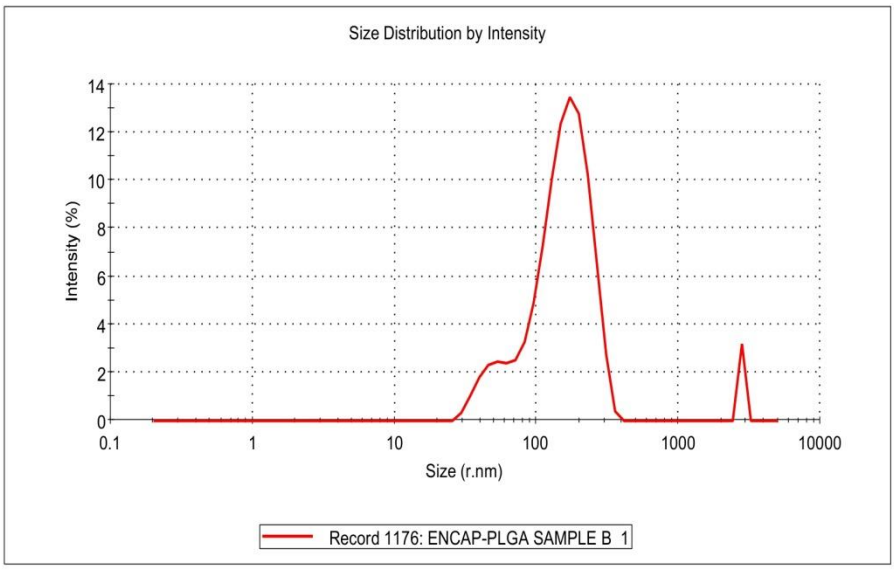


Fig. 10. Particle size analysis of ENCAP-PLGA NPs (B)

FTIR analysis of encapsulated dsRNA-JHE(A) and encapsulated dsRNA-JHE(B) were also performed. The FTIR of encapsulated dsRNA-JHE(A) data in (Fig 9) shows the characteristic absorption peak at 3376 cm^{-1} and 3210 cm^{-1} (Phenol), 1729 cm^{-1} (Ester), 1510 cm^{-1} (Alkynyl), 1449 cm^{-1} (Aromatic C-H Bending and Vinyl hydrogen), 1374 cm^{-1} (Esters), 1091 cm^{-1} and 945 cm^{1} (Symmetric PO^{2} streching in RNA and DNA).

The FTIR data of encapsulated dsRNA-JHE(B) in (Fig 12) shows the characteristic absorption peak at 3442 $cm^{-1\ 1}$ and 3281 cm^{-1} (Alcohol or phenol), 1833 cm^{-1} (Anhydrides), 1696 cm^{-1} (Alkenes), 1431 cm^{-1} (Carboxylic acid), 1312 cm^{-1} (aromatic amines), 1042 cm^{-1} and 963 cm^{-1} (Symmetric PO^{2} streching in RNA and DNA).

Table 4.7. FTIR analysis of encapsulated dsRNA-JHE (B) nanoparticles

Sr. No.	Range cm^{-1}	Wave number (cm^{-1})	Peak assignments	Functional group
1	3550-3200	3442 3281	OH	Alcohol/Phenol O-H Stretch
2	1830-1800	1833	C=O stretch	Anhydrides
3	1690-1630	1696	C=C stretch	Alkenes
4	1440-1400	1431	O-H bend	Carboxylic acids-COOH.
5	1342-1307	1312	C–N	Aromatic amines
6	1042-100	1042 963	PO_2	PO_2 streching in RNA and DNA

In FTIR analysis spectrum of dsRNA-JHE(A) encapsulated nanoparticles and dsRNA-JHE(B) encapsulated nanoparticles observed peaks showing presence of dsRNA and which were not to be found in PLGA nanoparticles FTIR analysis.

In the earlier, study during FTIR analysis 1,667 and 1,596 cm^{-1} belonging to the amides of CS, and the C–O stretching vibrations peaks of pyranose in CS molecules located at 1,150–1,000 cm^{-1}. The

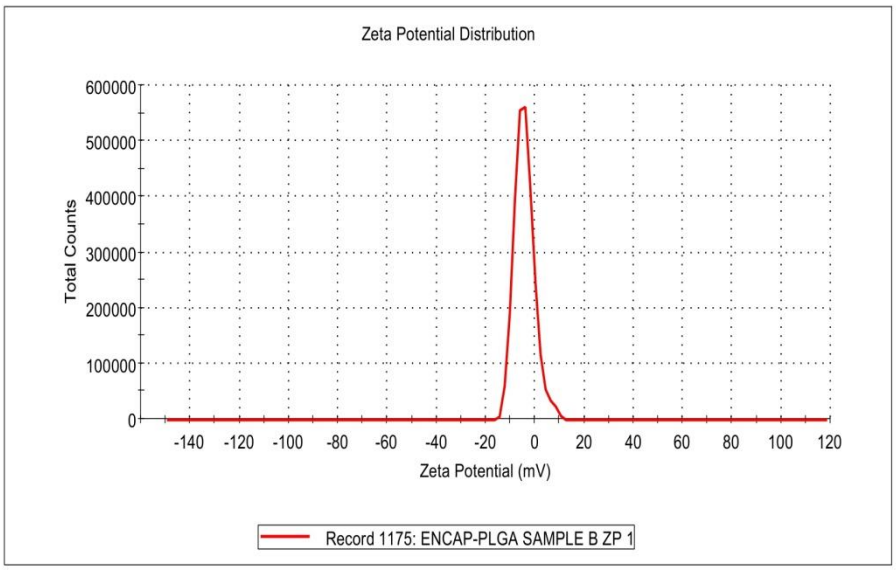


Fig. 11. Zeta size analysis of ENCAP-PLGA NPs (B)

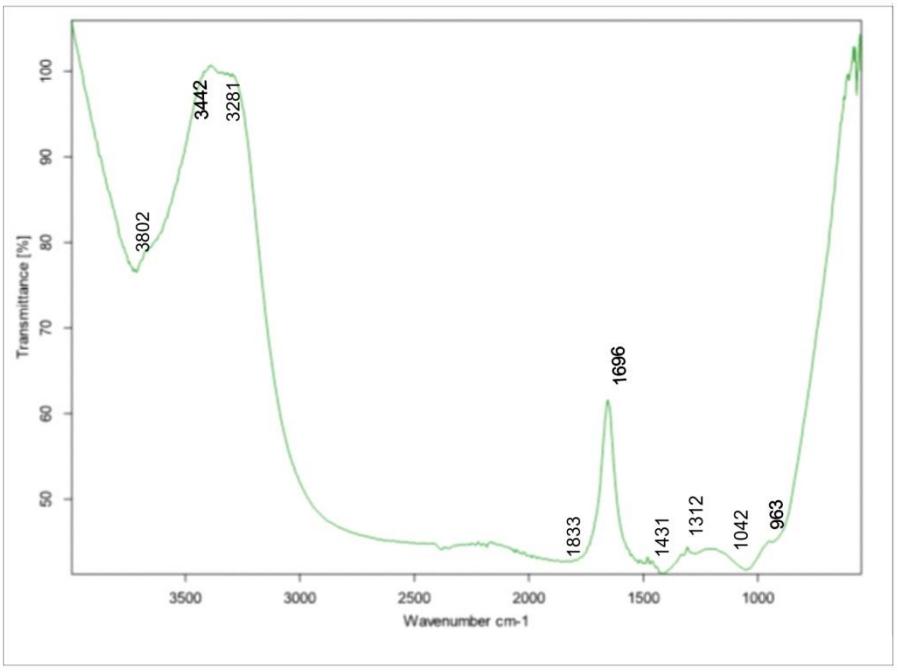


Fig. 12. FTIR analysis of ENCAP-PLGA NPs (B)

spectrum of PEG had the characteristic peak at 3,446 $cm^{-1}$and 1,733 cm^{-1}, which corresponded to the stretching vibrations of hydroxyl (−OH) and the stretching vibrations of carbonyl group (C=O) of carboxyl group, respectively. The peaks at 2,888 cm^{-1} and 1,113 cm^{-1} refer to the stretching of the alkyl group (−CH_2) and stretching vibration of the ether group (C–O) in PEG, respectively. The FTIR spectra shows PEG-grafted CS, the characteristic peaks at 2,884 cm^{-1} (C–H stretching) and 1,105 cm^{-1} (C–O stretching) belong to PEG. The peaks at 1,595 cm^{-1} (amide II) and 1,150 cm^{-1}–1,000 cm^{-1} (pyranose) belong to CS. The results suggested that PEG was successfully conjugated to the CS (Rajeswari et al. 2016 and Preethi et al., 2014).

Movasaghi et al. (2015) found the 1040–100 cm^{-1} Symmetric PO_2^- stretching in RNA and DNA, Rising ribose content (1121 cm^{-1}) is seen to correlate with rising 996 cm^{-1}/966 cm^{-1} ratio (another index of RNA/DNA) was observed in Andrus 2004 FTIR analysis study.

4.4 Insect bioassay

Considering the disrupting growth and molting processes in insects, JHE has been proven insecticidal inhibitory effects. After performing the feeding leaf bioassay on 3rd instar larvae of *P.xylostella* significant mortality was observed due to silencing of candidate genes. The mortality rate was recorded at three intervals viz. 2, 4, and 6 days in comprising of five treatments of dsRNA and encapsulated dsRNA viz. 20µg/µl (Plate 9)

Insect mortality study suggests that, dsRNA-JHE(A) showed 36% mortality of DBM after 6 days. Similarly, dsRNA-JHE(B) shows 48% mortality. Encapsulation of dsRNA with PLGA nanoparticles was carried out to see the possibility of protecting ds-RNA from degradation by insect digestive enzyme, other conditions and thereby increasing the efficiency of dsRNA.

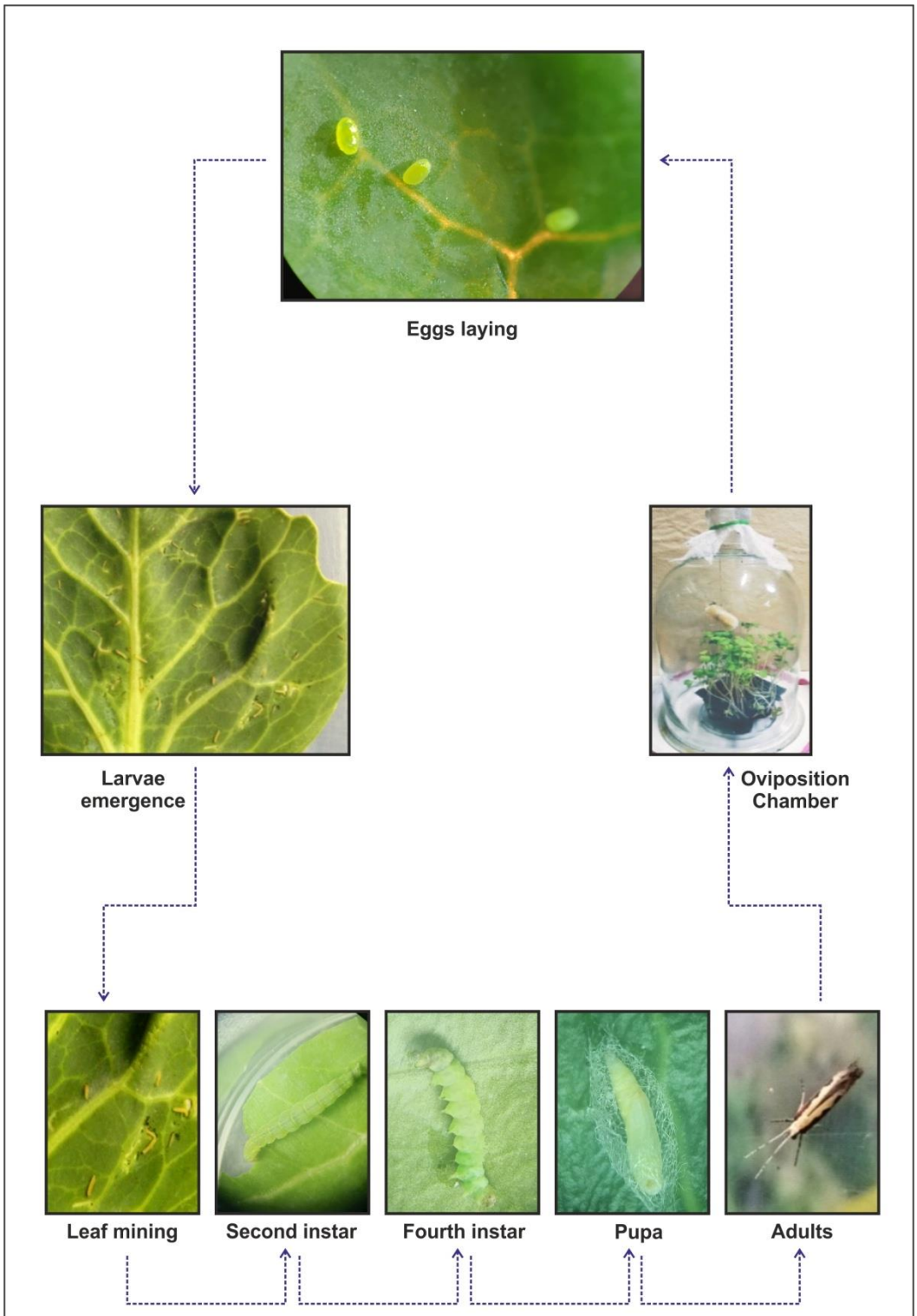


Plate 3. Rearing of Diamondback moth

Results endorse the hypothecation and showed 60% mortality after 6 days in encapsulated dsRNA-JHE(A) treatment and 72% mortality after 6 days in encapsulated dsRNA-JHE(B) treatment (Table 4.8). Increased percent mortality suggests the utility of PLGA nanoparticle as vehicle of delivering dsRNA.

Table 4.8. Mortality percentage of *P. xylostella* with treatments

Sr. No.	Treatments	Mortality (%)		
		2 days	4 days	6 days
1	Control	0	0	0
2	dsRNA-JHE(A)	12	24	36
3	dsRNA-JHE(B)	24	36	48
4	Encapsulated dsRNA-JHE(A)	12	36	60
5	Encapsulated dsRNA-JHE(B)	24	48	72
		Total analysis – C. D. -5.432		
		SE (m)- 1.384		
		C. V. – 5.612		

In similar kind of experiment, during insect bioassay experiment (Chaitanya et al., 2017) resulted after post treatment with ds-PxJHEH showed 66.66% mortality with 20 µg/µl at 96 h; likewise, with respect to post ds-PxEcR treatments; the mortality chronicled was 53% with 20 µg/µl dsRNA concentrations at 96 h. The mortality in off-target control (LacZ) and untreated control significantly changed. In either the treatments (ds-PxJHEH and ds-PxEcR), the mortality increased with increase in dsRNA concentration.

Yu et al. (2014) reported that on days 2, 4, 6 and 8 after ingestion of dsNlEcR-c at 0.1 µg/µl and on days 1, 2, 6 and 8 after ingestion of dsNlEcR-c at 0.5 µg/µl, the relative mRNA expression levels of NlEcR were significantly decreased compared with those in the dsGFP control (t-test, $p < 0.01$). In addition, some nymphs exhibited difficulty in molting. The survival rate of BPH nymphs was significantly decreased in 2 days after ingestion of 0.1 µg/µl dsRNA, while a significant reduction in

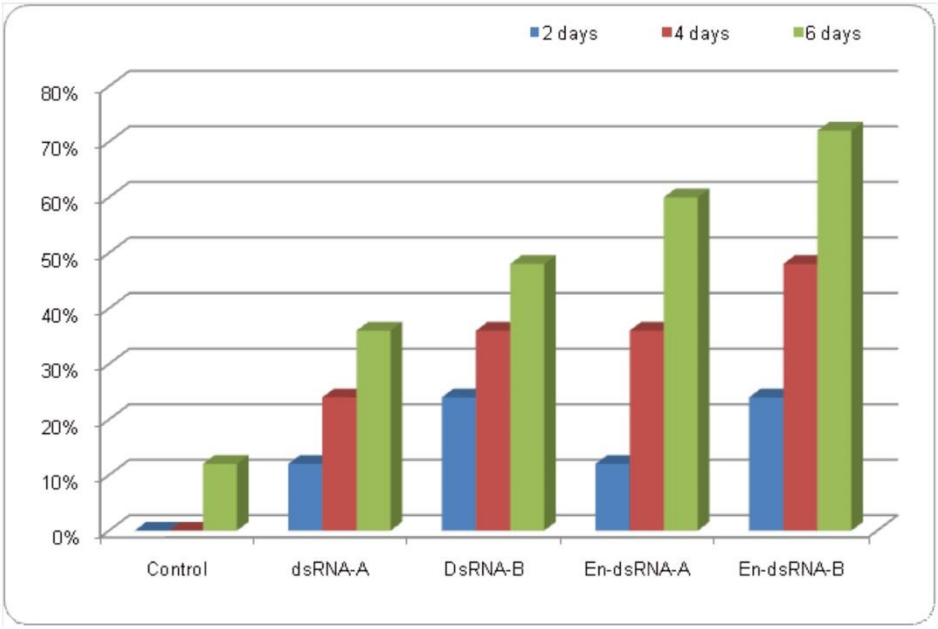


Fig. 13. Extent of JHE silencing in the larvae treated with various treatments (The expression levels of JHE(A) and JHE(B) were analyzed on the 6th day of feeding with various treatments. Error bars indicate the standard error of the mean of 3 replicates)

Control dsRNA (A) dsRNA (B)

Encapsulated dsRNA with NPs (A) Encapsulated dsRNA with NPs (B)

Insect bioassay with different treatments

Miscroscopic view of dead larvae

Plate 4. Insect bioassay with different treatments

the survival rate occurred only 1 day after ingestion of 0.5 µg/µl dsRNA. Likewise, mortality increased with an increase in dsRNA concentration (Fig 9).

The most important finding of this study is the discovery that chemically synthesized and modified dsRNA targeting *P. xylostella* encapsulated JHE(A) and JHE(B) genes caused significant mortality of the insect in the lab, which provide a novel strategy to control *P. xylostella* and to develop bio-pesticides based on RNAi and nanotechnology. However, some significant challenges must be overcome before RNAi-based pest control can become a reality. One of these challenges is the complexity of selecting a good target for RNAi.

CHAPTER V

SUMMARY AND CONCLUSIONS

The present investigation entitled “Nanoencapsulation of dsRNA of metamorphosis related genes and its insecticidal potential against Diamondback Moth” was carried out to study the silencing of specific genes of *P. xylostella* by RNAi through effective PLGA nanoparticles. The present investigation aim to develop dsRNA Encapsulated with PLGA nanoparticles has been suitable as a carrier to deliver dsRNA into the *P.xylostella.* It is efficient method for inhibition of gene as means of controlling plant pest have been successfully demonstrated in laboratory.

During Present investigation, general RNAi technology has facilitated the identification of insect gene function and thereby insect pest management by inhibiting the metamorphosis related genes which are essential for insect growth and development. JHE genes of *P. xylostella* has metamorphosis related function, has been targeted during Present investigation. Primer designed to amplify the selected regions and these primers tailed with T_7 RNA polymerase primer for synthesis of dsRNA. The dsRNAs synthesized by using MEGAscript T_7 RNAi kit.

During present study, the PLGA nanoparticles synthesized using double emulsification method and carried out its characterization. The results showed particle size 244.9 nm and -4.5 mV zeta potential. Moreover, the stability of the nanoparticles is a crucial parameter for efficient delivery. However, insecticidal potential of JHE(A) and JHE(B) dsRNA found to be increased when it encapsulated with PLGA nanoparticles. by adding PEG, inanoparticles shows high stablility inside the *P. xylostella* without altering their biological activity and without any toxic effects. These novel nanoparticles are promising for *in-vivo* systemic delivery and will create new perspectives for future gene silencing treatments.

In insect bioassay, we found the mortality infested by encapsulated JHE(A) and JHE(B) dsRNA was 60% and 72%, respectively as compared to control (only water) and dsRNA JHE(A) and JHE(B) having least effects. Based on these results showed protection of dsRNA from digestive degradation and delivering it on target site, because of PLGA encapsulation is highly responsible for increasing effectivity of dsRNA.

This safe and nontoxic method could be alternative against transgenic technology for controlling insect pest. RNAi with nanoencapsulation shows potential as an agricultural technology for management of insects.

This new information will facilitate the future refinement of insect pest control methodologies based on RNAi and will continue to inspire discoveries of new strategies, which will provide new solutions to many of the existing and emerging problems related to the management of insect pests. There is a necessity for an environmentally friendly strategy to control crop insect pests and the use of dsRNA coupled with PLGA nanoparticles in order to form self-assembling nanoparticles came as a safest approach. This strategy can also be applied to other crop insect pests.

CHAPTER VI

IMPLICATIONS

The present investigations attempts for Nanoencapsulation of dsRNA of metamorphosis related genes and its insecticidal potential against DBM through encapsulating PLGA nanoparticles. dsRNA delivery could be environmentally safe, low toxic, transmembrane abilities, and possibilities to target nanoparticles to the specific organs or cells and have broad prospects of immense application.

- The diamondback moth is the polyphagous pest and chemically synthesized insecticides are only main strategy for *P. xylostella* due to their easy application and cost-effectiveness. The massive application of these insecticides leads to the development of high magnitude resistance to these insecticides by *P. xylostella*. RNAi mechanism provides a potential novel approach to control insect pest.
- PLGA nanoparticles will act as a Trojan horse for the intact delivery of dsRNA to the insect midgut and this is the novel method used for insect pest management.

CHAPTER VII

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