T.C.
NİĞDE ÖMER HALİSDEMİR UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF PLANT PRODUCTION AND TECHNOLOGIES

USE OF GENE SILENCING TECHNIQUES IN CONTROL OF COLORADO POTATO BEETLE, *LEPTINOTARSA DECEMLINEATA* (CHRYSOMELIDAE: COLEOPTERA)

MUHAMMAD NADİR NAQQASH

March 2019

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PhD Thesis

Supervisor

Prof. Dr. Ayhan GÖKÇE

March 2019

The study titled "**Use of Gene Silencing Techniques in Control of Colorado Potato Beetle, Leptinotarsa decemlineata (Chrysomelidae: Coleoptera)**" and presented by **Muhammad Nadir NAQQASH** with the help of supervisor **Prof. Dr. Ayhan GÖKÇE,** has been accepted as Doctoral thesis by the jury at the **Department of Plant Production and Technologies,** Niğde Ömer Halisdemir University Graduate School of Natural and Applied Sciences.

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CONFIRMATION:

This thesis has been found appropriate at the date of 18/03/2019 by the jury mentioned above who have been designated by the Board of Directors of Graduate School of Natural and Applied Sciences and has been confirmed with the resolution of Board of Directors dated …./…./20.... and numbered .....).

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THESIS CERTIFICATION

It is certified that I have written this thesis by myself. I further confirm that all information included in this thesis is scientific and is in accordance with the university rules and regulations. Any materials that I have used from external sources as well as help received and all sources used in finalizing this research work and preparing this thesis, all have been acknowledged in the thesis.

Muhammad Nadir NAQQASH

ÖZET

KOLORADO PATATES BÖCEĞİ *LEPTINOTARSA DECEMLINEATA* (CHRYSOMELIDAE: COLEOPTERA) KONTROLÜNDE GEN SUSTURMA TEKNİKLERİNİN KULLANIMI

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Bitkisel Üretim ve Teknolojileri Anabilim Dalı

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Mart 2019, 150 sayfa

Imidaclopride dirençli patates böceği popülasyonunun kontrolünde RNA interferans (RNAi) tekniğinin kullanılma imkânı laboratuar koşullarında araştırılmıştır. Bu amaçla, imidaclopride direnç sağlayan kütikülar protein (CP), sitokrom P450 monoosksigenaz (P450) ve glutatyon sentetata (GSS) genlerinin susturulması hedeflenmiştir. Çalışmada farklı seleksiyon baskısı altında olan iki farklı popülâsyonun doğal artış oranı (r), üreme gücü sınırı(λ), net üreme gücü (R_0) Age-stage, two-sex life table programı ile karşılaştırılmıştır. Tarla popülâsyonun r, λ, R_0 parametreleri 0.12 gün^{-1}, 1.13 gün^{-1}, 71.07 döl/dişi olarak hesaplanmış bu değerler hassas laboratuar popülâsyonu için hesaplanan 0.10 gün^{-1}, 1.10 gün^{-1} ve 38.43 döl/dişi önemli derecede büyük bulunmuştur. dsRNA'ın imidaclopride dirençli patates böceğindeki etkisi beslenme denemeleri ile araştırılmıştır. CP-dsRNA uygulanmış yapraklarla beslenen 1. 2. ve 3. dönem larvalarda yüksek oranda ölümlere neden omuştur. Buna benzer şekilde 2. 3. ve 4. dönem larvalarının canlı kalma oranlarını, ağırlık artış miktarlarını ve gelişim sürelerinide etkilediği sağtanmıştır. dsRNA'ların imidacloprid ile sinerjist etki gösterdiği ve dirençli populasyonda ölüm oranını %100 kadar artırdığı gözlenmiştir. Bu sonuçlar, CP, P450 ve GSS enzimlerini hedefleyen dsRNA imidaclopride dirençli patates böceği popülâsyonlarının kontrolünde kullanılabileceğini göstermektedir.

Anahtar Sözcükler: Patates böceği, direnç yönetimi, age stage, two-sex life table, RNA interferaz, sinerjist

SUMMARY

USE OF GENE SILENCING TECHNIQUES IN CONTROL OF COLORADO POTATO BEETLE, *LEPTINOTARSA DECEMLINEATA* (CHRYSOMELIDAE: COLEOPTERA)

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March 2019, 150 pages

Potential of RNA interference (RNAi) was explored for the control of imidacloprid resistant Colorado potato beetle (CPB) under laboratory conditions. Age-stage, two-sex life table studies were conducted on two popualtions to calculate population parameters. The calculated population parameters for the field population were 0.12 day^{-1} for the intrinsic rate of increase (r), 1.13 day^{-1} for the finite rate of increase (λ), and 71.07 offsprings/female for the net reproductive rate (R_0), and they were significantly higher than the parameters of lab susceptible population (r= 0.10 day^{-1}, λ=1.10 day^{-1} and R_0=38.43 offsprings/female). Three important imidacloprid resistance conferring genes, cuticular protein (CP), cytochrome P450 monoxygenases (P450) and glutathione synthetase (GSS), were targeted with dsRNAs. Feeding bio-assays were conducted on various stages of imidacloprid resistant CPB population. Feeding bio-assays revealed significantly higher mortality in the first three larval stages fed on CP-dsRNA. Survival rate, larval weight and pre-adult duration of insects were also affected by dsRNAs. Synergism of RNAi with imidacloprid caused high mortality in the resistant population. These results showed that dsRNAs targeting CP, P450 and GSS enzymes could be useful tool in management of imidacloprid resistant CPB populations.

Keywords: Colorado potato beetle, resistance management, age-stage, two-sex life table, RNA interference, synergist

ACKNOWLEDGEMENTS

I would like to thank my supervisor Prof. Dr. Ayhan GÖKÇE who gave me the opportunity to pursue my doctoral research under his kind supervision. I also oblige Dr. Allah BAKHSH to provide me an opportunity to work in his laboratory, for all the knowledge in the area of insect biotechnology, and for his very helpful suggestions during my research work. I also wish to express my sincere gratitude to all professors for their support and words of wisdom.

I thank committee members Dr. Halil Toktay and Dr. Emre Aksoy whose knowledge, energy, and enthusiasm were critical to this effort. I am also thankful to Prof. Dr. Hsin Chi for his guidance and contribution regarding Age-stage, two-sex life table program.

My deepest appreciation is conveyed to my family and friends for their constant support and encouragement during all my endeavors. I am heartily grateful to my colleague and friend Dr. Muhammad Salim for his co-operation in my research work.

The present PhD dissertation work was completed as a part of DOĞUŞ TARGE project titled with "Patates Böceği [Leptinotarsa decemlineata (Chrysomelidae: Coleoptera)] Mücadelesinde Gen susturma (RNAi) Tekniğinin Kullanılması". I acknowledge Doğuş Group to support my PhD. research work. I am highly thankful to TÜBİTAK for providing me the scholarship to carry out my PhD. study.

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SYMBOLS AND ABBREVIATIONS

Symbols	Descriptions
LB	Luria-bertani medium
PCR	Polymerase chain reaction
CP	Cuticular protein
GSS	Glutathione synthetase
P450	Cytochrome P450 monooxygenases
CPB	Colorado potato beetle
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
EDTA	Ethylene diamine triacetic acid
FAOSTAT (United Nations)	Food and agriculture organization statistical databases
bp	Base pair
µM	Micro-molar
mM	Milli-molar
mg/ L	Milligram per liter
ml	Milliliter
µl	Microliter
%	Percent
°C	Degree centigrade
RH	Relative humidity

CHAPTER I

INTRODUCTION

Potato, *Solanum tuberosum* Linnaeus (Solanales; Solanaceae) is called as 'The king of vegetables' due to its economic and nutritional importance (FAOSTAT, 2018). Though potato is a non-cereal crop, but it has a significant role in world's food security. It is the only non-cereal crop which is being compared with other cereal crops like rice, wheat and maize owing to its impact to secure the food and nutrition and to control hunger and malnourishment, particularly in developing world (Swaminathan, 2001; Naik, 2005).

Potato crop possesses the ability of producing more food than other crops regarding per unit area and time. It contains more nutrition to sustain escalating world population. Cereal crops, being used as staple food in most of the world areas, can produce 9.1–18.1 kg food/ha/day, while potato has the ability to produce 47.6 kg food/ha/day (Kumar and Pandey, 2008). Potato is a highly nutritious diet consisting of carbohydrates, protein, minerals, dietary fibers, vitamin C, and antioxidants. Due to its versatile nature, a variety of ways can be used to cook and fit it in any meal. Additionally, various products can be prepared from potatoes via processing. Due to upsurging global population, urbanization and consumer's behavior; a sustained alteration in consumption pattern of potatoes has been found in majority of the under-developed and developing areas of the world (Pandey et al., 2005).

Many biotic and abiotic factors decrease the yield of potato every year. Biotic factors mainly include diseases and insect-pests infestation. Insect-pests can reduce potato yield significantly. Various kinds of insect pests belonging to different insect orders infest potato plants e.g. Colorado potato beetle, *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae); Potato leafhopper, *Empoasca fabae* Harris (Homoptera: Cicadellidae); tuber feeders like wireworms, *Agroites* spp. (Coleoptera: Elateridae); insects attacking tubers and also leaves such as black cutworm, *Agrotis ipsilon* Hufnagel (Lepidoptera: Noctuidae); Potato tuber moth, *Phthorimaea operculella* Zeller (Lepidoptera: Gelechiidae); and insect-vectors of plant diseases aphids (Hemiptera: Aphidiidae) (Waters, 2017).

Colorado potato beetle (CPB) is the most devastating insect-pest of potato in America, Asia and Europe. Basically the larvae and adults of CPB are serious defoliators of many members of Solanaceae family including potato, tomato, eggplant and nightshade (Jacques, 1988). Daily foliage consumption by CPB larvae has been calculated to be 9.65 cm^2 while that of an adult has been estimated to be 40 cm^2 in potato (Ferro et al., 1985). Annual yield losses range between 30–50% due to CBP which can increase to no economic yield in some fields (Zhou et al., 2012). Records have proven CPB as the first insect against which first commercial application of insecticides was carried out on a larger scale during 1864 (Gauthier et al., 1981). Heavy reliance on insecticides for the management of CPB since one and a half century is well documented (Casagrande, 1987). This heavy dependence on chemical control and co-evolution of this insect with secondary metabolite rich plant family has led it to develop amazing resistance ability against each insecticide being commercially used to manage it (Bishop and Grafius, 1996). It develops resistance to each new insecticide shortly after its introduction at commercial level (Forgash, 1985; Whalon et al., 2007; Mota-Sanchez and Wise, 2017). Enhanced resistance (> 100-folds) to insecticides can be recorded in only 3 generations (Ioannidis et al., 1992). Decreased susceptibility to neonicotinoids was reported in only 2 years on Long Island, New York, USA (Zhao et al., 2000). It has developed resistance to more than 56 active ingredients (Mota-Sanchez and Wise, 2017). Even with intensive use of a variety of insecticides and high control costs, 20% or higher crop losses were reported in Michigan from 1990–1994 (Grafius, 1997).

Insecticide resistance is the most interesting and attractive evolutionary phenomena for researchers. Excessive reliance on chemicals induces resistance in insects which results in failure of pest management programs of many insect-pests. Thorough studies of these change in genetic basis in resistant insect-pests can help researchers in the devising pest management strategies against them in a wiser manner. Further investigation on the type of genes like co-dominant, recessive, and/or dominant genes on conferring resistance can be helpful in future. It can also guide researchers about time of insecticide rotation and/or replacing one or more insecticides in their pest management programs (Naqqash et al., 2016). Like all other resistant insects, CPB utilizes various resistance mechanisms to withstand insecticide applications. Resistance to a variety of synthetic and natural insecticides has made this mechanism diverse to cope with. However, some mechanisms are common to both synthetic and

bio-insecticides. These tactics may include decreased penetration (Argentine et al., 1994), target site insensitivity (Malekmohammadi and Galehdari, 2016), metabolic detoxification of insecticides (Li et al., 2007), and increased excretion (Dermauw and Van Leeuwen, 2014). Among these different mechanisms, more research has been conducted on metabolic resistance, and is documented that this mechanism arose due to evolution while consuming diet from plants rich in secondary metabolites (Dermauw et al., 2012).

Breakdown of insecticide molecules by detoxification enzymes followed by excretion is termed as metabolic resistance, and is characterized by enhanced activity of detoxification enzymes (Li et al., 2007) and/or xenobiotic transporters (Dermauw and Van Leeuwen, 2014) in the resistant strains of insects. Important kinds of detoxification enzymes which are associated with metabolic resistance include esterases and cytochrome P450s in phase I direct metabolism (Feyereisen et al., 2012; Li et al., 2007); UDP-glycosyltransferases and glutathione S-transferases (Enayati et al., 2005; Ahn et al., 2012; Jancova et al., 2010) in phase II reactions; while ATP-binding cassette transporters commonly known as ABC transporters play role in phase III reactions (excretion) (Dermauw and Van Leeuwen, 2014).

Overexpression of detoxification and ABC transporter genes in resistant populations of insects, usually give rise to above-mentioned proteins (Liu, 2015). Resistance in insects is actually the result of gene-for-gene relationship in insects i.e. up-regulation of detoxification genes and thus the transcriptome level associated with detoxification (Li et al., 2007). The eventual consequence of each insecticide, which is in use or being developed for the future use or either to be considered in future, will be resistance development in its targeted insect population (s). Additionally, this resistance will be characterized by detoxification via enzymes encoded by genes. So, this phenomenon needs exploration (Naqqash et al., 2016).

Resistance in potato plants was established against beetles by insertion of a gene which expressed the *Cry3A* protein isolated from a gram-positive bacterium *Bacillus thuringiensis* var. *tenebrionis*, with the 35S promoter of cauliflower mosaic virus (CaMV 35S). These transgenics provided satisfactory control of the target insect-pest (CPB) which resulted in commercial release of Bt potato cultivars viz. Atlantic, Russet

Burbank, Superior and Snowden by Monsanto in North America during 1995–2001 (Duncan et al., 2002). Though safety of Bt transgenic crops by extensive experiments were established for animals, humans, and the environment, Bt potato cultivars were stopped during 2001 primarily due to commercial reasons, not due to agronomic reasons. Though, transgenics expressing *Bt* endotoxins for management of insect-pests is very successful, but their resistance to insect-pests can be reduced due to evolution of resistance by gene-for-gene relationship. Data for decreased resistance of Bt crops was analyzed from 5 continents established in 41 field by Tabashnik et al. (2009). Additionally, public acceptance was also a hurdle in commercialization of Bt potato (Duncan et al., 2002). The faster development of resistance to insecticides in insects is decreasing the life of transgenics like *Bt* crops which possess endotoxins isolated from the gram positive bacteria, *Bacillus thuringensis (Bt)*. Tabashnik et al. (2013) analyzed the results of around 77 research works collected from 5 different continents, most of which is based on field studies reporting resistance development to *Bt* transgenics and calculation of those reasons which are involved in resistance.

RNA interference (RNAi), an effective gene-silencing tool, has been used in a various organisms as a powerful and quick strategy of functional genomics, especially in living organisms which do not support stable transgenesis, like insects. The dsRNA was first used as a silencing tool by Fire during 1998 in *Caenorhabditis elegans* Maupas (Rhabditida: Rhabditidae) (Fire et al., 1998). Their study upended the established view that antisense RNA can knock down gene transcript by base pairing the bases with its mRNA sense, thus preventing the formation of associated protein. Role of RNA interference (RNAi) in insect-pest management was first studied in 2002 in which pupae of cecropia moth, *Hyalophora cecropia* Linnaeus (Lepidoptera: Saturniidae) were treated with RNAi to silence *Hemolin* gene (Bettencourt et al., 2002). It resulted in less number of larvae emergence from eggs of treated adults. Later on, subolesin gene in ticks was silenced for population control (de la Fuente et al., 2006). However, RNAi gained the attention of researchers in 2007 when Baum et al. (2007) and Mao et al. (2007) worked on dsRNA expressing transgenic plants to target genes in western corn rootworm and cotton bollworm, respectively. Trasngenic plants expressing dsRNA were used to knock-down a gene essential for survival of different insects. Significant mortality of exposed insect-pests was observed when they were allowed to feed on the transgenics (Baum et al., 2007; Mao et al., 2007). Therefore, it has been used for

functional genomics studies and important promising tool for producing insect-proof crops (Zhu et al., 2011). In the past decade, the use of RNAi has gained the importance as a tool of pest management and can be easily used along with other strategies (Gordon and Waterhouse, 2007). RNAi is compatible with many strategies and can be implemented in field mainly given the evidence of its high specificity than any other conventional methods (Baum et al., 2007; Mao et al., 2007). Various studies have demonstrated successful knockdown and significantly higher mortality in RNAi experiments against beetles (Zhao et al., 2011), but less success is reported in lepidopterans due to various reasons (Terenius et al., 2011). However, public acceptance of RNAi-based trasngenics can be a question of great concern and also the biggest disadvantage in the way of dsRNA expressing plants commercialization.

It is proven that RNAi works well in order coleoptera (Tomoyasu et al., 2008; Terenius et al., 2011). Various experiments conducted on coleopterans like the red flour beetle, the western corn rootworm and CPB have shown the impact of RNAi in terms of functional genomics and insect-pest management (Palli, 2012).

Exploration of the mechanisms which are the main contributing factors in producing different kinds of responses in different insect species to RNAi mediated gene silencing, may provide the basis for commercialization of RNAi-mediated insecticides. Some novel studies have proven that alterations in expression of different genes encoding proteins associated with uptake of dsRNA, its spread to target cells and/or tissues, processing of dsRNA, and Risk Complex formation can play significant roles in efficacy of gene silencing via RNAi (Katoch et al. 2013; Spit et al., 2017). Expression of proteins encoded by the genes can be affected by introducing dsRNA in target cells and the variations in the sensitivity to the dsRNA is also a major contributing factor in the success of RNAi-mediated gene silencing. Mechanisms involved in the efficiency of dsRNA in different insects, were uncovered by the preliminary results on dsRNA intake, spread to cells and tissues, its processing and ultimately the formation of Risk-complex in insect's body (Gordon and Waterhouse, 2007).

Mode of dsRNA application like micro-injection and/or feeding is also a major factor effecting success rate of RNAi mediated gene silencing in a variety of insect-pests belonging to approximately all of the insect orders. For example: micro-injections of

dsRNA in locusts successfully decreased the expression of target genes, while there was no significant effect of dsRNA on targeted genes when it was given orally (Luo et al., 2013). Midgut cells were found to secrete dsRNases which play an important role in dsRNA degradation, thus hindering the RNAi effect in such application of dsRNA. Study has proven that both migratory and desert locust show tissue-specific activity when injected with dsRNA (Wynant et al., 2012). Novel studies have shown that degradation of dsRNA is the key factor among many factors in decreasing the efficiency of RNAi mediated gene silencing by oral ingestion in aphids (Christiaens et al., 2014). Polyphagous insects like CPB contain a variety of nucleases which has the ability to rapidly degrade ingested dsRNA and thus can cause a failure of dsRNA applications in many insects. Successful silencing of these nucleases can result in significant mortality and also can act as synergist with dsRNA targeting other genes (Spit et al., 2017). Finally, RNAi technique cannot give adequate control in most polyphagous insects mainly due to lower efficiency, higher production and formulation costs. To date there is no study regarding resistance development against dsRNA and its mechanisms of action. Additionally, less efficacy has been reported in feeding bio-assays (Palli, 2014). Use of RNAi as eco-friendly synergists along with insecticides may not only increase the efficacy and life of these insecticides but it may be a step towards a more environment friendly agricultural pest control strategy.

Neonicotinoids make a share of around 25% in the worldwide insecticide market. They, headed by thiamethoxam and imidacloprid, make an imperious class of new chemistry insecticides in the insect-pests control in various crops (Jeschke *et al.*, 2011). Their selectivity and potency for insect nicotinic acetylcholine receptor (nAChR) depends on the nitro substituent of neonicotinoids. Around 100 different metabolites have been found in plants and mammals from seven commercial neonicotinoids (Ford and Casida, 2006 a, b; 2008).

Imidacloprid is the most common first-generation neonicotinoid. Inhibition of cytochrome P450 family and esterases can significantly enhance the susceptibility of CPB and other insect-pests to the imidacloprid (Mota-Sanchez et al., 2006; Zhao et al., 2000).

The role of detoxification enzymes playing role in conferring metabolic resistance was well-established in the earlier studies (Wilkinson and Brattsten, 1972; Motoyama and Dauterman, 1974). However, identification of specific genes associated with the resistance was not carried out (Wilkinson, 1983). Furthermore, some novel studies have established the role of a variety of genes encoding esterases, cytochrome P450, glutathione S-trasnferases, and ABC transporters in enhancing the resistance of CPB populations (Clements et al., 2016; Zhu et al., 2016). A study conducted on difference between imidacloprid resistant CPB and susceptible CPB regarding transcriptome revealed that there was a difference in 102 transcripts which were coding for various detoxification enzymes and xenobiotic transporters. Among them, 28 transcripts were under-expressed while 74 were over-expressed in the imidacloprid resistant CPB (Clements et al., 2016).

Synergists basically decrease the activity of detoxifying enzymes by physical blockage and/or destroying their structure. These chemicals when added to insecticides can significantly enhance their toxicity; hence result in higher mortality of target insects, with relatively smaller doses (Brindley and Selim, 1984). Synergists first gained the interest of researchers when it was shown that the lethality of pyrethrum was increased due to the addition of natural synergist "sesamin" (Haller et al., 1942). Metcalf (1967) defined the term synergist as "The component of a mixture which is not toxic alone, but enhances the lethality of the insecticide being applied with it."

So this research work has focused the use of RNAi as synergist for imidacloprid resistance management in CPB. Three important genes upregulated in imidacloprid resistant population were targeted to study their effect in decreasing the susceptibility of resistant CPB strains. The targeted three genes involved in expression of cytochrome P450 monoxygeneases (P450), glutathione synthetase (GSS) and cuticular protein (CP). Where, P450 and GSS play crucial role in phase I and phase II reactions during detoxification process while CP is related to growth and penetration resistance to variety of insecticides.

Main aim of the phylogenetic analysis was to find the patterns of species abundance and distribution regarding their ecological mechanisms and evolutionary processes (Whitfeld et al. 2012). Furthermore, phylogenetic analysis of genes can provide

important information about their pattern of evolution. Phylogenetic analysis also provides information regarding the closeness of any gene with the other genes of the same group (Poff et al. 2006). So, such analysis can be useful in prediction of targeting a set of genes in their respective category like P450, GST and/or CP. Phylogenetic tree was constructed to show the pattern of evolution of our targeted genes (CP, P450 and GSS) in relation with other genes in the same category.

Life tables are an important tool to study the development and projection of a population, which are used extensively by ecologists to evaluate and compare the fitness cost of insect populations under differing conditions. Males in insect population and stage difference are important parameters in population data which are ignored by commonly used female-based age-specific life table analysis (Lewis, 1942; Leslie, 1945; Birch, 1948; Carey, 1993). As a result, this limitation has made conventional female-based life tables unable to describe a population's characteristics in the correct way. Thus, female-based life tables can be erroneous and misguiding due to errors in results (Huang and Chi, 2011).

One of the aims of this doctoral work was to study the age-stage, two-sex life table for susceptible CPB populations in comparison with field CPB population. It was hypothesized that a susceptible phenotype may have more impact of abiotic and biotic stress (Mansoor et al., 2013). Resistant insect-pests have various up-regulated genes like P450 monoxygenases, chitin synthase genes, GSTs, ABC trasnporters etc. which do not have direct role in insect growth and development. However, these kinds of genes mediate the expression of important growth related genes (Clements et al., 2017). To highlight the importance of inducing susceptibility in CPB management, fitness parameters of susceptible CPB population were calculated in comparison with normal field populations.

Objectives

The main motive of this study was to bring novel tools in pest management of CPB for sustainable agricultural production especially in the developing world. The approach may be used as a pest management strategy as well as insecticide resistance management.

We have designed a unique, efficient and promising strategy to combat crop losses from this insect pest by deploying RNAi strategy against gene (s) encoding resistance against imidacloprid. Objectives of our studies were:

- To amplify responsible gene(s) for imidacloprid resistance
- Constructing dsRNA plasmid harboring our targeted gene(s)
- To manage the CPB with unique tools i.e. dsRNA in laboratory
- To study the lethal and sub-lethal effects of dsRNA on various life stages of CPB
- To explore possible synergist effects of dsRNA with imidacloprid
- Comparison of life table of the lab susceptible CPB and the field CPB populations

CHAPTER II

REVIEW OF LITERATURE

2.1 Pest Status of CPB

Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Chrysomelidae: Coleoptera), is the most economically important insect-pest of potato crop and also various other solanaceous vegetables in many parts of the World (Alyokhin, 2009; Gokce et al., 2012). It originated from Mexico and south-west America; and gained the importance as a notorious global insect pest (Alyokhin et al., 2008). Jacques (1988) stated about the first outbreak of CPB inn potato crop was recorded in 1859 near Omaha, Nebraska. The successive dispersal to different geographical zones of this insect was quite surprising when these insects reached the Atlantic coastal areas of the Canada and U.S. before 1880 (Casagrande, 1987). In 1922, it was reported in France followed by its dispersal in throughout the Europe in some Asian countries including Iran and west China (Jolivet, 1991; Weber, 2003). Its dispersal is recorded on around 16 million km^2 spread in two continents and is expected to expand to various other regions (Weber, 2003). Possibly, this notorious insect-pest may shift to other geographical zones of Asia, the Africa, South America, New Zealand and Australia (Vlasova, 1978; Worner, 1988; Jolivet, 1991; Weber, 2003). The CPB is a serious defoliator of potato plants resulting in about 30–50% yield reduction per year or in severe cases may result in no economic yield (Zhou et al., 2012).

2.2 Control Strategies for CPB

Different tactics have been employed for the management of CPB viz. mechanical control, biological control, trench traps, mass trapping, push-pull techniques, host plant resistance, genetic control and the most commonly used chemical control (Alyokhin et al., 2008; Casagrande, 2014). Even, CPB resistant potato plants were also used by inserting *Cry3A* protein. Though, these transgenics had provided adequate control of CPB, *Bt* potato transgenics were withdrawn from the market during 2001 due to public acceptance issues. Despite of the fact that various experiments demonstrated the safety of *Bt* crops to non-target animals, humans and the environment (Duncan et al., 2002).

Chemical control is the mostly adopted strategy in the potato growing regions for the control of CBP owing to the fact that other management tactics cannot provide demanded control by the growers (Casagrande, 1987; Gokce et al., 2012; Casagrande, 2014). Currently, chemical control is the most effective way to manage CPB in potato fields. However, high selection pressure in a chemically diverse environment due to insecticides has compelled the CPB to develop resistance to a variety of synthetic insecticides (Jiang et al., 2010; Kim et al., 2007).

2.3 Resistance in CPB Populations

Main reason of developing insecticide resistance against different classes of insecticides is the evolution of CPB with diverse phytochemicals of solanaceous crops which combined with heavy insecticide spray being used each year to control this pest since 1864 (Gauthier et al., 1981). Since 1950s, this notorious insect has become resistant to approximately every chemical used for its control. It has become resistant to 56 different chemicals belonging to all major insecticide classes. Therefore, alternative strategies for CPB management should be explored (Mota-Sanchez and Wise, 2017).

The CPB was first insect on which insecticide sprays were applied on a larger scale in 1864 (Gauthier et al., 1981). Due to more dependence on insecticides for more than 150 years and unique mechanism of resistance in CPB, it has become the most devastating pest in potato growth. Resistance of CPB can enhance to 100-folds against insecticides, under selection pressure, in only 3 generations (Ioannidis et al., 1992). Resistance to organochlorines has been reported to increase up to 220X (Sharif et al., 2007), while the level of resistance to organophosphates was up to 252X (Malekmohammadi et al., 2010). Carbamate resistance has been reported to increase up to 18.7 X. Resistance to pyrethroids can increase to as much as 2749 times in the field strain (Jiang et al., 2010). Resistance to new chemistry insecticides has also been well reported e.g. imidacloprid resistance can develop up to 310X, while spinosad resistance can increase to 7.6X (Mota-Sanchez et al., 2006). Level of resistance to chlorantraniliprole can enhance to 4.89X (Jiang et al., 2012). Additionally resistance to BT Cry 3A has also been reported (Alyokhin and Ferro, 1999).

2.3.1 Genetic basis of insecticide resistance in CPB

Solanaceae plants have higher levels of plant secondary metabolites (glycoalkaloids) and co-evolution of CPB with these plants has naturally enhanced its ability to survive under worst conditions of selection pressure. Like other resistant insect-pests, CPB also uses various mechanisms of resistance to survive the insecticides treatment. Mechanisms of resistance are very diverse because of their exposure to a variety of plant metabolites and synthetic chemical. Metabolic resistance including a complex of detoxification enzymes like carboxylesterases, glutathione-S-transferases (GSTs), UDPs and monooxygenases make the most important component of detoxification in insects like CPB (Clements et al., 2017; Clements et al. 2018). Metabolic resistance has been well studied than other components of detoxification, and is considered to be derivative of an inherited capability to detoxify toxins present in food (Dermauw et al., 2012).

Organophosphate resistant CPB usually contains an α-helix produced due to serine to glycine point mutation in acetylcholinesterase gene (Zhu et al., 1996). Around 45 different kinds of mutations have been reported in four field populations which were contributing to *AChE* insensitivity (Malekmohammadi and Galehdari, 2016). Additionally, particular point mutations like I392T, S291G and R30K found in carbamate and organophosphate -resistant CPB were discovered via site-directed mutagenesis (Kim et al., 2007).

Mutations viz. L1014F and S291G in LdVssc1 and acetylcholine esterase results in resistance to pyrethroids (Shi et al., 2012). Partial resistance of CPB to carbamates has been reported due to mutation *AChE* termed as S291G, while point mutations in the LdVscc1 termed as L1014F confer pyrethroids resistance (Jiang et al., 2011). Due to highly diverse and complex sets of genes to confer resistance, mechanism like RNAi to silence these genes can be a promising tool in resistance management.

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2.4 RNAi Technique

RNA interference (RNAi) is a gene-silencing tool at post-transcription level, which starts after the entrance of double-stranded RNA (dsRNA) in the target cell (Hannon, 2002; Baulcombe, 2004). It is quite popular in plant sciences as "post-transcriptional gene silencing" and is quite similar to regulation of genes at post-transcriptional level via microRNAs (miRNAs), which include hinderance in protein formation by translation (Seggerson et al. 2002). Initial report on RNAi-mediated gene silencing was also published in plants (Napoli et al., 1990), and later, various studies published on various components of RNAi. In animal sciences, RNAi-mediated gene silencing has been well documented in invertebrates, particularly in *C. elegans* (Fire et al., 1998; Timmons and Fire, 1998; Tijsterman et al., 2004) and *Drosophila* sp. (Bernstein et al., 2001, Hammond et al., 2000).

Two RNA silencing paths are reported to exist in insects, which are regulated by micro-RNAs (miRNAs) and small interfering RNAs (siRNAs) (Tomari et al., 2007). Endogenous transcripts and dsRNA structure are used for regulation of developmental processes by the miRNA path. While, the basic function of siRNA pathway is defensive response to exogenous dsRNAs. Target sequence of the RNAi decides the specificity of RNAi which is based on the sequence of either a portion or whole target gene. Introduced dsRNA converts into siRNAs which in turn mediate the mRNA degradation, which is sliced by Dicers (RNase III-like endonucleases). Different kinds of dicers have different functions in insects. In *Drosophila melanogaster* Meigen (Diptera: Drosophilidae), production of miRNAs is primarily associated with Dicer-1; whereas, long dsRNAs are converted into siRNAs by Dicer-2 (Lee et al., 2004). These siRNAs are actually smaller fragment (21-bp) of dsRNA, consisting of 2 base extensions at the 3′ end. This mechanism includes the assembly of RISC in which siRNA is inserted conjugated with the argonaute multidomain protein and an RNaseH-like domain. Translation is stopped following the removal of the passenger strand when the RISC cuts the mRNA (Filipowicz, 2005). An RNA-dependent RNA polymerase (RdRP) acts with the RISC complex to produce new dsRNAs, based on sequence of partly degraded

target template. There are several reports on activity of the RdRPs in plants and also nematodes which increase the efficacy of RNAi-mediated gene silencing by synthesizing endogenous dsRNA of target mRNA (Dalmay et al., 2000; Sijen et al., 2001).

2.5 Delivery Methods of dsRNA

Delivery methods of dsRNA are of profound importance for the overall success of RNAi in insects and are given prime importance in planning the pest control program for use of RNAi. Different delivery systems are reported for different groups of organisms. Given that the cells infected with dsRNAs undergo gene silencing, the key challenge is the delivery method (Terenius et al., 2011). The main dsRNA delivery methods in insects can be broadly categorized into two methods: injection and ingestion.

Micro-injections are efficient tool in functional genomics; however this strategy is not applicable in field for insect-pests management. Also, many other bottlenecks regarding use of microinjections are well documented which include tricky methods of experimentation and difficulty to carry out in smaller-sized insects (Nunes and Simões, 2009; Walshe et al., 2009). The red flour beetle was firstly injected with dsRNA to knockdown genes and to find gene function **(**Baum et al., 2007).

Oral feeding of dsRNA was firstly carried out in nematode, *C. elegans* (Timmons and Fire, 1998), followed by a number of reports in insect species. Feeding assay was conducted on *C. elegans,* in which bacterially expressed dsRNA was fed. Successful knock down of genes via RNAi was carried out in the *Reticulitermes flavipes* Kollar (Isoptera: Rhinotermitidae) and *Diatraea saccharalis* Fabricius (Lepidoptera: Crambidae) by oral feeding (Yang et al., 2010; Zhou et al., 2008). Delivery of dsRNA through oral route by ingestion is relatively attractive as it is easier to carry out, less damaging to the target insect, and a relatively natural way of dsRNA delivery in insect body (Chen et al., 2010). It is especially useful for smaller insects which cannot be injected for introduction of dsRNA. The dsRNA can be introduced by two methods viz. bacteria expressing dsRNA or by in vitro synthesis. Earlier studies depicted that oral delivery of dsRNA may not significantly downregulate the target gene than the delivery

of dsRNA injection in target insect-pests (Rajagopal et al., 2002). However, later reports established the fact that effective down-regulation of genes can be found in many insects, belonging to different orders viz. hemiptera, Lepidoptera, diptera and coleoptera (Mao et al., 2007). Feeding bioassays with dsRNA on larvae of *Epiphyas postvittana* Walker (Lepidoptera: Torticidae) successfully downregulated the transcript level of the carboxylesterase gene *EposCXE1* in the larva midgut and also successfully repressed the transcriptome of the pheromone-binding protein *EposPBP1* in the antennae of target adults (Turner et al., 2006). Additionally, the oral intake of dsRNA significantly decreased the transcript level of the nitrophorin 2 (*NP2*) gene in salivary enzymes of *Rhodnius prolixus* Stal (Hemiptera: Reduviidae), thus resulting in decreased coagulation time of the target insect plasma (Araujo et al., 2006).

2.6 Sites Targeted Via RNAi in CPB

Foliar application of dsRNA targeting actin in CPB was found highly effective for management. It is revealed that dsRNA targeting actin was effective in protecting potato plants for about 28 days in greenhouse conditions. It was also revealed that the dsRNA was not removed by sprinkling of water, if it got dried on the leaves (Miguel and Scott, 2015). Sites which can be targeted due to their synergistic activity with various insecticides are shown in table 2.1.

Table 2.1. Target genes of dsRNA which can be used to synergize insecticide (s)

Class of gene	Target gene of dsRNA	Compatible insecticide
Juvenile hormone pathway	*LdSAHase*	*Juvenile hormone mimics*
Vacuolar ATPases	*LdATPaseE1* and *LdATPaseE2*	Multiple groups of insecticides
20-hydroxyecdysone genes	*LdFTZ-F1-1* and *LdFTZ-F1-2*	*Juvenile hormone mimics*
Ryanodine receptor	*LdRyR*	Chlorantraniliprole
Sclerotization gene	Laccase2	chitin synthesis inhibitor
Juvenile hormone related gene	JHDK	*Juvenile hormone mimics*
Ecdysone related genes	*LdE75*A, B and C	Ecdysteroid agonists
Mevalonate pathway related gene	*LdJHAMT*	*Juvenile hormone mimics*
nAChR genes	*Ldα3*, *Ldα6*, *Ldα10*, and *Ldβ1*	Neonicotinoids
Cytochrome p-450, a cuticular protein, and a glutathione synthetase	Comp115309, Comp105889 and Comp114026	Neonicotinoids
Cytochrome P-450s	*CYP6BQ15*, *CYP4Q3* and *CYP4Q7*	Neonicotinoids
Cytochromes P-450	CYP6BJ, CYP6BJ1v1, CYP9Z25, and CYP9Z29	Neonicotinoids, Plant secondary metabolites
Random	Nucleases	Stomach poisons and other dsRNA
Glutathione synthetase	LdGSTs	Pyrethroids, organophoshate and phenylpyrazole
Carboxylesterase/cholinesterase superfamily	*CCE* genes	Pyrethroids, phenylpyrazole
nAChR subunit genes	Ldα3, Ldα9, Ldβ1, Ldα4, Ldα7 and Ldα9	Neonicotinoids
Basic helix–loop–helix genes	*Ld*bHLH	Hydroprene, Methoprene and Pyriproxyfen
Digestive genes	Cysteine proteases, intestains D, intestains E, cellulases, serine proteases	Plant proteins/Protease inhibitors

Zhou et al. (2013) worked on the production of an inhibitor *S*-adenosyl-*L*-homocysteine (*AdoHcy*) is inversely proportional to juvenile hormone (JH) production during JH biosynthetic pathway. In this work, a putative *LdSAHase* gene was obtained from CPB followed by cloning. Its expression was found in all growth stages. Expression level

was significantly higher in 3rd instar larvae while significantly lower level was found in 4th instar larvae. Feeding bioassay with dsRNA targeting *LdSAHase* significantly down-regulated the expression of *LdSAHase* and *LdKr-h1*mRNA, decreased JH titre, resulted in significant mortality of exposed larvae, and decrease in formation of pupae and of adult emergence. Additionally, silencing of *LdSAHase* also decreased developmental time of larvae, and larval weight. Thus, this research demonstrated that *SAHase* is essential in JH biosynthesis in insects. Concluding, these dsRNA targeting *LdSAHase* can be used as synergist with some Juvenile hormone mimics.

Fu et al. (2014) considered the importance of vacuolar-type ATPases (*vATPases*) in various physiological functions which are crucial for insect survival. cDNA of vATPase subunit E (*LdATPaseE*), encoding a protein of 226 amino acids, was cloned and characterized. Its levels were enhances significantly during immature stages up to the final instar and then started decreasing in pupae and up-regulated again during adult stage. Higher expression was observed in digestive tract than the rest of body organs. Feeding bio-assay using dsRNAs targeting *LdATPaseE1* and *LdATPaseE2*, decreased the expression in larvae by 85% and 55%, respectively. Larval development and survival rate was significantly reduced. Additionally, contact bioassays with cypermethrin, endosulfan, fipronil, and butane-fipronil have been demonstrated to increase the expression of *LdATPaseE*. It depicts that targeting *vATPase* subunit E can be a promising target in management of CPB. Furthermore, dsRNA targeting *vATPase* subunit *E* can be helpful as synergist with various insecticides.

Liu et al. (2014) worked on two 20-hydroxyecdysone (20E) related genes viz. *LdFTZ-F1-1* and *LdFTZ-F1-2* in *CPB*. Both genes have significant role in metamorphosis and growth of each larval instar. Feeding the final instar larvae with ecdysteroid agonist halofenozide significantly enhanced the expression level of both genes. Contrarily, decrease in 20E due to ingestion of dsRNA targeting *LdSHD* decreased the expression. Furthermore, halofenozide (Hal) rescued the level of expression in exposed larvae. *LdFTZ-F1* transcription was induced by the peaks of 20E. Additionally, a conserved sequence of the test genes *LdFTZ-F1-1* and *LdFTZ-F1-2* can effectively silence both the genes, resulting in failure of pupal formation. Concluding, silencing of *LdFTZ-F1*s significantly decreased the level of ecdysteroidogenesis genes, decreased 20E titre, and also down-regulated the 20E receptor genes. Additionally, knocking down the *LdFTZ-*

*F1*s significantly impacted the level of gene involved in JH biosynthesis, enhanced JH titer, however down-regulated the expression of JH early-inducible gene. Finally, they can be used as synergists with JH mimics.

According to Wan et al. (2014), ryanodine receptors (*RyR*s) can be important targets for increasing the life of active insecticide like chlorantraniliprole against CPB. A full length cDNA of *LdRyR*, encoding a protein of 5128-amino acid, was cloned and characterized. *LdRyR* expression level was high at larval stages, especially in 4th instar, and in adults. However, it was also found in all studied tissues viz. epidermal layer, stomodaeum, mesenteron, proctodeum, fat body, nervous system and Malpighian tubules in 4th instar larvae. Feeding bio-assay using double-stranded RNA targeting *LdRyR* down-regulated the target gene in the CPB adults and larvae. This study indicates that *LdRyR* is important in functioning of ryanodine receptor in CPB. Thus this target can be utilized for insecticide resistance management of chlorantraniliprole.

According to Yates (2014), Laccase2 gene, responsible for sclerotization and pigmentation, can be a promising target in control of CPB. Both injection method and feeding bioassay was conducted for inserting dsRNA in the test pests. Significant phenotypic changes were observed in microinjections method than the feeding bio-assay. Illumina high throughput sequencing was used to study the change in gene expression after introduction of dsRNA. There was no any significant change in RNAi genes due to introduction of dsRNA, despite of the fact that various genes associated with the RNAi pathway were over-expressed. Standardizing of the delivery methods for RNAi can be a promising method to study insect-host interactions. Moreover, this study depicted this gene can be useful if used as synergist with chitin synthesis inhibitor (Van Leeuwen et al. 2012).

According to Fu et al. (2015a), degradation of JH is carried out via Juvenile hormone diol kinase (*JHDK*). They cloned a putative JHDK cDNA (*LdJHDK*), obtained from CPB. Expression of LdJHDK can be observed in approximately all body parts during all stages. Oral ingestion of dsRNA to target *LdJHDK* depicted significantly down-regulated the target gene, increase in JH titre, and *LdKr-h1* mRNA level. Adult emergence was significantly affected by silencing of this gene. This research suggested

that this gene is connected with JH degradation and thus can be used in accordance with Juvenile hormone mimics.

Guo et al. (2016) worked on three clones of CPB Ecdysone-induced protein *75*(*LdE75*) viz. *LdE75*A, B and C. Higher expression of the three *LdE75* isoforms was observed just at the termination and initiation of each molt. In fourth larval instar, smaller increase was observed at start while significant enhancement was observed after 40 and 80 h of molting. It has been demonstrated that expression of *LdE75* in 4th larval instars was enhanced with increase in 20E and molting hormone agonist viz. halofenozide (Hal). Contrarily, expression of 20E decreased by feeding the test pest with dsRNA targeting shade gene (*LdSHD*), also suppressed the expression of *LdE75*. Additionally, expression of the three *LdE75*s Hal increased in the *LdSHD*-silenced larvae. Furthermore, ingestion of ds*E75*-1 and ds*E75*-2 containing a conserved sequence of the 3 analogues significantly silenced these *LdE75*s, and ceased development. Knocking down *LdE75*s also affected the expression of gene involved in JH biosynthesis, enhanced JH titre and the expression of gene associated with JH. This research demonstrated that *Ld*E75s have an important role in metamorphosis and thus can be used as synergists with IGRs.

Li et al. (2016) found that mevalonate pathway can be an important target for gene silencing as it has a crucial role in the biosynthesis of various crucial proteins important for insect growth, reproduction, communication and immunity. Genes associated with mevalonate pathway were identified in their study which encode for acetoacetyl-CoA thiolase (*LdAACT1* and *LdAACT2*), mevalonate kinase (*LdMevK*), phospho-mevalonate kinase (*LdPMK*), hydroxymethylglutaryl (HMA)-CoA synthase (*LdHMGS*), farnesyl pyrophosphate synthetase (*LdFPPS*), mevalonate diphosphate decarboxylase (*LdMDD*), HMG-CoA reductase (*LdHMGR1* and *LdHMGR2*) and isopentenyl-diphosphate isomerase (*LdIDI*). Nine of these genes (except for *LdAACT1*) can be found in larvae and adults both. Expression of these 9 genes can be observed at higher levels after each molting. It indicates the involvement of these 9 genes in JH biosynthesis. Additionally, knock down of *LdJHAMT* significantly down-regulated the expression level of these 9 genes. Expression of these 9 genes was also decreased due to the ingestion of JH for activation of JH signaling. Concluding, targeting these genes can be helpful in resistance management of Juvenile hormone mimics.

Qu et al. (2016) cloned the full-length cDNAs encoding *Ldα3*, *Ldα6*, *Ldα10*, and *Ldβ1* (new nAChR subunits) obtained from CPB. They are highly expressed, during all growth stages, in the head, thorax and abdomen. Feeding with double-stranded RNA targeting *Ldα1* (ds*Ldα1*) significantly decreased the expression of *Ldα1* in CPB adults and larvae. Additionally, bioassay conducted on ds*Ldα1* treated adults significantly decreased the susceptibility to neonicotinoids in adults. Concluding, *Ldα1* encoding nAChR has an important role in detoxification of imidacloprid and thiamethoxam against CPB. Hence, it can be used to break resistance and/or tolerance of CPB to neonicotinoids.

Clements et al. (2017) found that CPB has a variety of mechanisms involved in producing resistance to cope with high insecticide pressure, including increased detoxification by metabolic enzymes viz. glutathione S-transferases and cytochrome P450s. A set of three over-expressed imidacloprid resistance conferring genes were selected for RNA interference experiments by injection method. Significant knock-down of genes encoding enzymes viz. cytochrome P450, a cuticular protein, and a glutathione synthetase in a resistant CPB population was carried out. Resistance to imidacloprid was significantly decreased in treated populations, which suggest the utilization of these dsRNA as synergists with imidacloprid and other neonicotinoids.

Kaplanoglu et al. (2017) successfully demonstrated that expression of imidacloprid resistant genes viz. cytochrome P450s (*CYP6BQ15*, *CYP4Q3* and *CYP4Q7*), one ATP binding cassette (ABC) transporter (*ABC-G*), one esterase (*EST1*), and two UDP-glycosyltransferases (*UGT1* and *UGT2*) was decreased by conducting feeding bioassay with dsRNA, successfully targeted the above mentioned genes. Additionally, knock-down of imidacloprid resistance conferring genes (*CYP4Q3* and U*GT2*) decreased the resistance of beetles to imidacloprid, which indicates that these genes can be successfully used for utilizing RNAi as synergist with imidacloprid.

Kalsi and Palli (2017) carried out silencing of four cytochromes P450 genes viz. *CYP6BJ*, *CYP6BJ1v1*, *CYP9Z25*, and *CYP9Z29* playing role in detoxification of both natural and synthetic chemicals. These targets can be utilized to prolong the efficacy of neocnicotinoids and plant defence against CPB.

Spit et al. (2017) described that the nucleases in some polyphagous insects like Colorado potato beetle can be a possible reason of degradation and thus failure of dsRNA in many insects. They worked on the isolation, identification and categorization of two important nucleases specifically found in the gut of CPB. Silencing of nuclease genes in adults decreased the resistance of this insect towards dsRNA, resulting in enhanced protection of plants. Concluding, silencing of nuclease activity can cause synergistic effect for activity of other dsRNA and many stomach poisons (Table 2.1).

2.6.1 Successful examples of RNAi in other insect-pests

Kumar et al. (2009) worked on the use of siRNA targeting acetylcholinesterase (*AChE*) to hinder larval growth in American bollworm, *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae). In this study, siRNA molecules were synthesized chemically and were added to the diet of American bollworm. This feeding assay resulted in downregulation of *AChE* gene and thus caused significantly higher mortality, reduced larval growth, decreased pupal growth, abnormal adult growth and significantly less fecundity with respect to control larvae. This work suggested effective role of *AChE* in larval growth and development and also it is quite effective if orally ingested.

Chen et al. (2010) successfully targeted *trehalose phosphate synthase* (*TPS*) gene in the brown planthopper, *Nilaparvata lugens* Stal (Hemiptera: Delphacidae) via dsRNA based feeding assay. The cDNA of brown planthopper named as *TPS (NlTPS)* was found to be 3235 bp in length encoding a protein of 807 amino acids. Real-time PCR (qRT-PCR) analysis established the fact that *NlTPS* remains expressing with smaller change during the whole life span of the insect. Effective downregulation of the taret gene by dsRNA feeding assay resulted in significant decrease in trasncriptome of *TPS*. Additionally, the growth rate of brown planthopper larvae used in feeding bioassay was altered, resulting in higher mortality lethality where the survival rate decreased to 75.56, 64.44, 55.56 and 40.00% after 2, 4, 7 and 10 days of continuous ingestion of 0.5µg/µl dsRNA.

Zhang et al. (2010) used chitosan/*AgCHS* dsRNA-based nanoparticles to target two chitin synthase genes, *AgCHS1* and *AgCHS2*, in larvae of mosquito *Anopheles gambiae* Giles (Diptera: Culicidae) by feeding assay. There was significant decrease (62.8% and

33.8%) in both targeted genes in the larvae fed on chitosan/*AgCHS1* dsRNA nanoparticles. It was suggested that RNA interference (RNAi) is systemic in mosquito larvae. It was demonstrated that the susceptibility of exposed insects to calcofluor white and diflubenzuron increased when fed on the nanoparticles loaded with *AgCHS1* and *AgCHS2* dsRNAs. This study established the fact that nanoparticle-based RNAi technology can help in devising of novel ways for insect-pest management.

Upadhyay et al. (2011) explored this technique for the management of whiteflies, *Bemisia tabaci* Gennadius (Homoptera: Aleyrodidae). Five different genes viz. ADP/ATP translocase, actin ortholog, ribosomal protein L9 (*RPL9*), *α-tubulin*, and *V-ATPase* A subunit were targeted in this study. A simple feeding bio-assay was conducted for the delivery of ds/siRNA, and efficiency was evaluated. After 6 days of exposure, ds/siRNA caused significant mortality ranging between 29–97%. Downregulation of the *RPL9* and *V-ATPase* A genes resulted in higher mortality. Semi-quantitative PCR revealed adequate downregulation of *RPL9* and *V-ATPase* A transcriptome. It was found that siRNAs were stable in the insect diet for 7 days at the room temperature.

Asokan et al. (2013) used this robust technique for the management of American bollworm, *H. armigera*. Therefore, they conducted a diet bio-assay to knock down *chymotrypsin* (a midgut gene) and a growth associated gene i.e. juvenile hormone acid methyl transferase (*jhamt*) in American bollworm. They found that various applications of dsRNA are more effective in earlier and persistent knockdown of the target gene than single application of dsRNA. Long-lasting systemic knock-down was observed in case of targeting non-midgut gene (*jhamt*) was observed possibly owing to activation of knock-down signals in the target insect. Positive correlation between silencing efficiency of chymotrypsin and dsRNA concentration was found while negative correlation was observed between dsRNA concentrion and *jhamt*. Less silencing efficiency of *jhamt* when exposed to higher concentration of *jhamt* dsRNA was probably due to upregulation of an associated gene, farnesyl diphosphate synthase (*fpps*). This experiment has provided the basis of use of RNAi as a pest management strategy.

Singh et al. (2013) found that dipping larvae in a dsRNA solution for 2 hours can result in RNA interference in the mosquito *Aedes aegypti* Linnaeus (Diptera: Culicidae). The decreased level of trasncriptome of *β-tubulin*, *chitin synthase-1* and *-2*, and *heat shock protein 83* ranged between 30–50% after 3 days post-treatment. A visible dye was added to dsRNA for identification of insects fed on the dsRNA. The main route of dsRNA entry is via gut epithelium in insects. RNA interference phenomenon was found to be systemic in the test insects, resulting in detectable decrease of transcript level of target gene expression in insect cells after passing the gut. Downregulation of the *chitin synthase-1* and *β-tubulin* genes resulted in stunted growth and/or increased mortality of the mosquito larvae. No mortality was observed due to downregulation of *chitin synthase-2* in the test larvae, and decrease in transcriptome of *heat shock protein 83* induced mortality only in the insects which were exposed to heat shock after exposure to dsRNA.

Yu et al. (2014) explored the potential of targeting insect ecdysone receptor (*EcR*) via RNAi-silencing in *N. lugens*, an economic insect-pest of rice. They took a conserved region of two genes viz. *NlEcR-A* and *NlEcR-B* for their experiment. They prepared a 360 base pairs fragment (*NlEcR-c*) dsRNA for oral delivery in their trials which significantly down-regulated the relative expression of targeted genes than the *GFP* control. Additionally, oral delivery of dsRNA also significantly reduced in the fecundity of *N. lugens*. Therefore, *Agrobacterium*- mediated transformation was used to construct transgenic rice lines to express *NlEcR* dsRNA. Results of qRT-PCR and northern blotting verified successful construction of transgenic rice lines expressing ds*NlEcR-c*. Neonates of *N. lugens* were allowed to feed on transgenics. Feeding on all the transgenic lines resulted in significant under-expression of *NlEcR* transcript level than the control. Significantly lower survival rate of the exposed nymphs and decrease in the fertility was observed. Significant decrease in average number of offsprings reached 44.18-66.27% in exposed pairs.

Jin et al. (2015) found that chloroplast genetic engineering was the source of up-regulation of protein at much higher level, so they used it to decrease the expression of three important genes. Chloroplast genome was used to study down-regulation of lepidopteran chitin synthase *(Chi)*, cytochrome P450 monooxygenase (*P450*) and *V-ATPase* dsRNAs in planned hosts. Successful integration of target genes and

homoplasmy were further confirmed by PCR and southern blotting. Abundant copies of dsRNA transcripts of P450 (3.45 million copies) were detected by real-time qRT-PCR and northern blotting. Transgenic plants expressing dsRNA targeting *Chi*, *P450* and *V-ATPase* down-regulated the level of target genes to indiscernible levels in the exposed insect gut. Subsequently, the fitness parameters like weight and growth of larvae; and pupal formation were also significantly decreased due to dsRNA expressed via chloroplasts. Concluding, this experiment has explored the way of higher expression of dsRNA via chloroplast transformation which can decrease the transcriptome to undetectable level. Foremost, chloroplast trasnformation can aid researchers in inactivating genes to induce desirable traits or for production of various biopharmaceuticals especially in case of oncogenic cells.

Malik et al. (2016) used RNA interference (RNAi) to decrease transcriptome level of whitefly genes acetylcholinestrase gene (*AChE*) and ecdysone receptor gene (*EcR*) via dsRNA expressing transgenic plants to control whiteflies and its associated viral disease. They used plant mediated RNAi technique to express *AChE* and *EcR* genes. For this purpose, transgenic tobacco plants expressing dsRNA to target a conserved region of both *AChE* and *EcR* were constructed. Significantly higher mortality (>90%) in exposed whiteflies was recorded than non-transgenic plants within 3 days of feeding. Real time PCR verified the results that mortality occurred due to decrease in expression level of target genes. This research proved that silencing of genes involved in neural transmission and/or genes involved in developmental process, can be potentially used as bio-pesticide (s) to decrease whitefly infestation in the field and its associated virus.

According to Guan et al. (2017), *chymotrypsins* (*CTP*) make the most important category of protein digesting enzymes in lepidopterans. They may have vital role in degradation of Bt endotoxins in some insect-pests, which results in increased insect resistance to Bt toxins. In this experiment, 7 different *CTP* enzymes were isolated via transcriptome analysis of Asian corn borer, *Ostrinia furnacalis* Guenée (Lepidoptera: Crambidae). All of the selected enzymes were highly expressed in the saliva and midgut especially during larval stage. DsRNA from the selected genes were prepared and diet bioassay was conducted with them at the different larval instars. dsRNA targeting the selected seven *CTPs* significantly increased the larval mortality up to

third instar, while dsRNA down-regulating *CTP8* increased the mortality of 4th instar. Diet comprising dsRNA and Bt significantly increased the mortalities (up to 100%) in all cases except for dsRNA from *CTP5*. This study has proven that *CTP*-like genes can be a promising target for RNAi based insect-pest management. Additionally, these genes can significantly enhance the susceptibility of larvae to Bt toxins.

According to Qiu et al. (2017), *Bt* rice is a major source of management of lepidopterans like striped rice stemborer, *Chilo suppressalis* (Walker) (Lepidoptera: Pyralidae). Exploration of interaction between host and these is very important to understand their mode of action. This research dealt with silencing of two important aminopeptidase genes viz. *APN1* and *APN2* which enhanced the resistance of Bt rice genotype TT51 to stemborer larvae. This research explored the way of resistance management of Cry1A and Cry1C toxins in borers by targeting aminopeptidase genes.

According to Luo et al. (2017), RNAi is not much successful phloem feeders/sucking insect-pests i.e. hemipterans due to poor RNAi efficacy due to a variety of nucleases and lack of RNAi core machinery. They conducted an experiment to explore the hurdles in the way of success of RNAi in the *B. tabaci* reared on tomato plants. This study using transgenic tomato lines expressing *GFP* (green fluorescent protein) traced out that dsRNA is phloem-mobile, oral ingestion by the insect-pests, and degradation occurred in the insect body. They found that some nucleases in whitefly known as *dsRNase*s, as found in other insect-pests, were responsible for degradation of dsRNA. So, nuclease genes were also targeted with dsRNA with two insect genes named sucrose *SUC1* and aquaporin *AQP1*, which have a vital role in protecting whiteflies to osmotic collapse. Increase in mortality (50%) was observed in merely 6 days of oral intake by stacking the dsRNA constructs of target genes (*AQP1* and *SUC1)* with dsRNA targeting nucleases *(dsRNase1* and *dsRNase2)*. Additionally, stacking resulted in significant down-regulation of the targeted genes in exposed insects. This research has proved that combining the dsRNA targeting an essential gene with a relevant nuclease targeting dsRNA can significantly enhance the efficiency of RNAi in insect-pests.

2.7 Possible Targets for Resistance Management

There are many promising targets for CPB resistance management which can be further considered. Larvae detoxify protease inhibitors (PIs) being synthesized in potato leaves via substitution of inhibitor-sensitive digestive cysteine proteases with inhibitor-insensitive cysteine proteases. Enzymes involved in digestion are very important in this regard. They are the initial barrier to all kinds of plant defense mechanisms and also insecticides. They are key players in detoxification of food related toxins. Important digestive enzymes include cysteine proteases, intestains D, intestains E, cellulases, serine proteases, and an endopolygalacturonase (Petek et al., 2012).

Insect Cytochrome P450 (CYPs) genes has significant role in making insects able to survive in chemically diverse ecology. Some of these CYPs detoxify a large variety of plant metabolites chemicals, synthetic insecticides and/or other environmental chemicals (Liu and Zhu, 2012; Schuler, 1996). While some of them have very crucial role in physiological functions of insects like the synthesis of juvenile hormone and ecdysone hormone (Liu et al. 2015). CYPs can also degrade pheromones in some insects, which are a source of alteration in insect behavior and chemical communication (Yang et al., 2015; Xi et al., 2015). Furthermore, catalyzation and hydroxylation of fatty acids for synthesis of physiologically important biochemical is carried by insect CYPs (Helvig et al., 2004).

Around 70 novel carboxylesterases and 2 acetylcholinesterases were isolated from the CPB genome. These 72 sequences were members of a multifunctional carboxylesterase/cholinesterase superfamily (CCE). Dietary/detoxification, hormone/ semiochemical processing and neurodevelopmental classes are the three main phylogenetic groups of CCEs. Numbers of CCEs in CPB reported till now are: 52 (dietary/detoxification enzymes), 12 (hormone/semiochemical processing enzymes) and 8 (neurodevelopmental enzymes). The dietary/detoxification class can be further divided into two categories viz. α-esterase type and coleopteran xenobiotic metabolizing CCEs. The hormone/ semiochemical processing enzymes include: β- and pheromone CCEs, exoskeleton related CCEs and juvenile hormones. Acetylcholinesterase, neurotactin, neuroligin, gliotactin, glutactin, and many others are the main neurodevelopmental CCEs. Among the 70 novel *CCE* genes, *KM220527, KM220538*,

KM220541, *KM220542*, *KM220554*, *KM220561*, *KM220564*, *KM220566*, and KM220578 cyhalothrin-inducible while KM220527, KM220530, KM220541, KM220566 and *KM220576*, were fipronil-inducible (Lu et al., 2015). Pyrethroid resistant insects showed higher expression of nine *CYP* genes (Wan et al., 2013). Three insecticides i.e. fipronil, cyhalothrin or endosulfan resulted in the upregulation of *LdGSTe2a*, *LdGSTe2b*, *LdGSTo5* and *LdGSTt1* transcripts (Han et al., 2016).

Three non-target genes named *Ldα3*, *Ldα9*, and *Ldβ1* were over-expressed in test larvae. *Ldα4*, *Ldα7* and *Ldα9* are the *nAChR* subunit genes playing role in producing resistance to neonicotinoids (Li et al., 2014).

Peaks in imidacloprid resistance have been reported due to various mechanisms of resistance like mixed-function oxidases (Zhao et al., 2000), cytochrome P450 and cuticular protein transcripts (Clements et al., 2016).

According to Fu et al. (2015b), basic helix–loop–helix (bHLH) transcripts have important roles in functions in cell proliferation, differentiation, determination, maintenance of cell cycle and ability to withstand different kinds of stresses. The categorization and characterization of *bHLH* members is the initial step. By transcript analysis, 49 bHLH members have been isolated. All *LdbHLH* members were described according to their names and families by various phylogenetic analyses with *bHLH* homologues of fruitfly, honeybee, silkworm and the red flour beetle. These results have provided the base for using them in combination with a few JH mimics targeting bHLH members (Table 2.1).

2.7.1 Use of RNAi as synergists

Billions of dollars are being spent in insecticide discovery and development each year. Over-reliance on insecticides also causes human health hazards and environmental pollution. Every chemical insecticide either being used for the CPB or will be used in the future will ultimately result in resistance. As a matter of fact, such resistance involves diverse genes and metabolic enzyme systems associated with them. Instead of consuming time and money on discovery and development of new chemicals, older chemicals can be used with eco-friendly plant-based or RNAi-based synergists against

resistant insect-pests. Though some possible challenges can be faced regarding RNAi uptake by the insects or their stability with insecticides can be question but further research on these aspects and possible implementation in the field can significantly decrease our cost of development of new insecticides.

RNAi technique cannot give adequate control in field conditions in most of the polyphagous insects, mainly due to lower efficiency, higher production and formulation costs, practically no study regarding resistance development against dsRNAi and lack of adequate scientific literature on mechanisms of action. Additionally, less efficacy has been reported in feeding bio-assays (Palli, 2014). Use of RNAi as eco-friendly synergists along with insecticides will not only increase the efficacy and life of these insecticides but it will be a step towards a less polluted environment.

Synergists has significant role in hindering metabolic resistance by inhibiting detoxification enzymes. Synergists/chemicals which are added to the insecticides to increase their toxicity significantly thus resulting in enhanced lethality of insecticides when given in sub-lethal dose (Brindley and Selim, 1984). Synergists have been utilized in different ways since the first report on enhanced insecticidal activity of pyrethrum after the addition of natural synergist "sesamin" (Haller et al., 1942). Metcalf (1967) defined the term synergist as "The component of a mixture that is which is not toxic alone, at the rate of treatment, but enhances the lethality of the insecticide being applied with it."

For environmentally safer management of resistance in insect-pests, selection of eco-friendly synergists is very important. These eco-friendly synergists can be divided into two main categories viz. plant-based synergists and RNAi based synergists. Use of plants and RNAi technique in insect-pest control is getting importance due to less environmental hazards. The synergistic approach of the use of RNAi against Colorado potato beetle can be considered as potential eco-friendly insect management strategy. Highly diverse mechanism of resistance in CPB to approximately all the introduced insecticides for its control is the key reason to explore new ways of control. In this regard, synergists can help researchers in eco-friendly management of CPB with little use of insecticides. A simple graphical representation regarding the mechanism of resistance and how synergists will help in breaking that resistance is shown in figure 2.1.

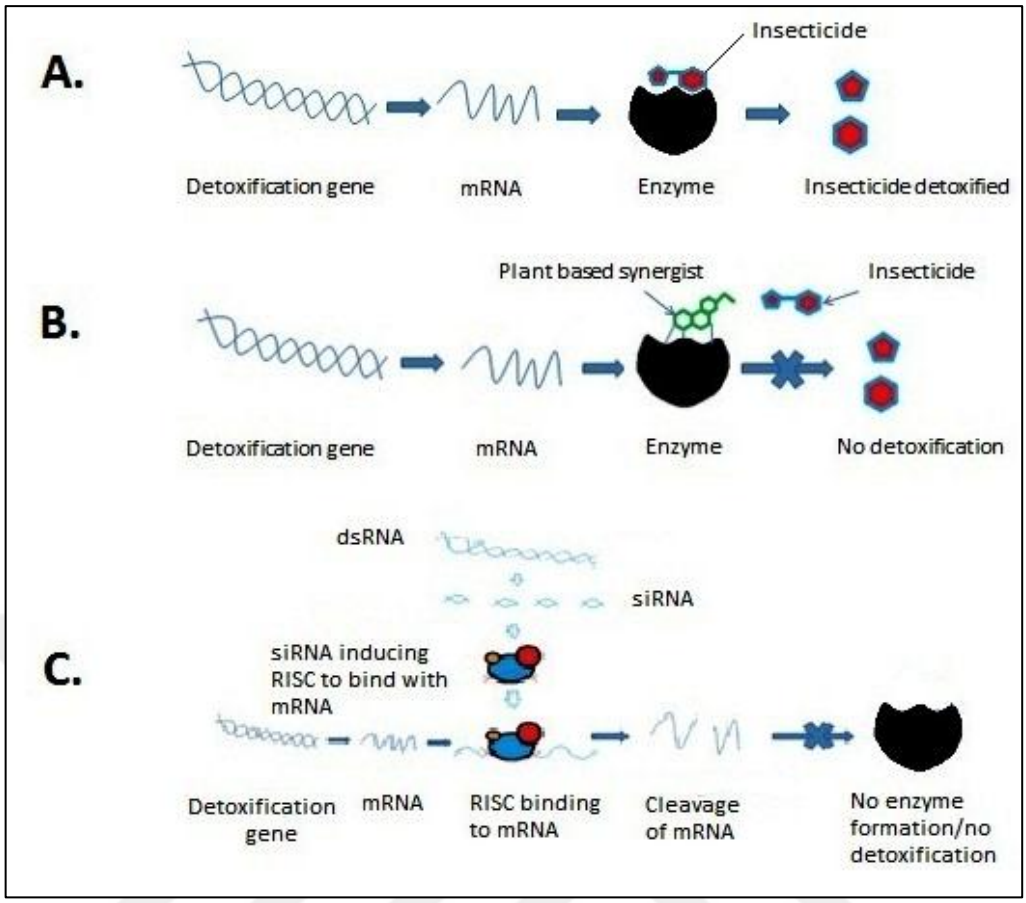


Figure 2.1. Normal detoxification process (a), enzyme binding site due to plant-based synergist resulting in no detoxification (b), dsRNA inducing RISC to bind with mRNA thus cleavage in mRNA results in decreased transcriptome (c)

2.8 Targeted Genes

2.8.1 Cytochrome P450 monooxygenases

The cytochrome P450 monooxygenase (CYPs), an ancient enzymatic system, comprises of a number of phase I and phase II detoxifications enzymes and can be found as primary detoxification enzyme super-family in approximately all living organisms (Scott and Wen, 2001; Feyereisen, 2006). CYPs of insects has important role in biochemical reactions like metabolizing endogenic chemicals viz. juvenile hormones, ecdysteroids, pheromones and fatty acids, and exogenous chemicals of synthetic origins like insecticides and/or natural origin like plant secondary metabolites (Guo et al., 2012; Zhou et al., 2012; Wang et al., 2015).

Enhanced resistance to insecticides in insects can be associated with CYPs. Increased expression of P450 enzymes has been proven in insecticide-resistant populations, in many studies (Scott and Wen, 2001; Feyereisen, 2006; Yang et al., 2008). Oxidative metabolism of insecticides by P450 enzymes has been found to be key resistance tactic to insecticides of different classes in arthropods (Feyereisen, 2006; Li et al., 2007; Yang et al., 2008). Eight field populations, resistant to diazinon, out of 15 different strains of housefly, *Musca domestica* Linnaeus had higher expression of *CYP6A1*. Diazinon resistance resulted in upregulation of the *CYP6A1* in *M. domestica* (Carino et al., 1994). Also, two CYP genes (*CYP9A12* and *CYP9A14*) were found over expressed in *H. armigera* resistant to pyrethroids (Yang et al., 2008).

2.8.2 Glutathione synthetase (GSS)

Glutathione synthetase has an imperative role in the regulation of cell defense to various biotic and abiotic stressors (Stohs et al., 2000). It is of prime importance in the stress and in detoxification especially the regulation of important metabolic enzymes glutathione S-transferases (GSTs). The majority of scavenging activities occur due to reactive oxygen species consisting of complex glutathione system. Glutathione synthetase is a key member of this system. It is also documented that peculiar stress-inducible response in the glutathione pathway due to exposure to stress conditions to regulate the homeostasis in insect's body. The increase in activity might indicate induction of de novo glutathione biosynthesis by glutathione synthetase; and, is likely to play a crucial role (Kolawole and Kolawole, 2014). Where, GSTs have a vital role in detoxification of endogenous chemicals like insecticides, drugs and other toxins (Labade et al., 2018; Pavlidi et al., 2018; Reid et al., 2019). GSTs, distributed in all living organisms, primarily conjugate the exogenous compounds to tripeptide glutathione (GSH) via an electrophilic center, which in turn enhances the solubility of exogenous chemicals and thus help in excreting them from the cell (Panini et al., 2016; Zhang et al., 2016; Dang et al., 2017).

2.8.3 Cuticular protein

Glycine-rich proteins (GRPs) constitute a chemically diverse group of enzymes distinguished by glycine rich nature. They are distributed well in diverse groups of

living organisms ranging between prokaryotes and eukaryotes. These enzymes contain little number of conserved regions excluding some short glycine-rich repeats (Ruiter et al. 1997; Deshoux et al. 2018; Muthukrishnan et al. 2018), which puts a question on their classification system in a single family (Sachetto-Martins et al., 2000). A type of GRPs named as silk proteins isolated from insects and spiders; elastin and collagen proteins are also placed in this enzyme family (Xu and Lewis, 1990; Qin and Waite, 1998; Zhong et al., 2006). Glycine-rich regions on C-terminus and/or N-terminus connected with a peptide are the main constituents of the insect structural cuticle GRPs (Andersen et al., 1993, 1995). Basically, it contains cuticle proteins and chitin fibers assembled together.

Immediately after molting, cuticle is soft and paler in start which quickly becomes hard, followed by melanization during tanning procedure. The process in which cuticle is hardened is called sclerotization, and is associated with cross-linking of components of cuticle viz. proteins and chitin via derivatives of dopamine (Noh et al., 2016; Balabanidou et al., 2018). Kind of cuticle viz. soft/pliant vs. hard/rigid are main factors effecting the degree of sclerotization. Type of precdysial cuticle can significantly effect and act as limiting factor in sclerotization (Hopkins et al., 2000). Additionally, sclerotization may or may not occur in post-ecdysial cuticle. Peptide hormones originating from peripheral peptidergic cells and/or peripheral nervous system control melanization and sclerotization of cuticle; and also the ecdysis related stereotyped behavior (Arakane et al., 2016; Noh et al., 2016; Zhu et al., 2016). Different amounts of ecdysteroids are the factor affecting the bio-synthesis and discharge of these peptide hormones (Zitnan et al., 2007).

Epidermal layer is the main expression site of Insect cuticular GRP protein genes (Arakane et al., 2016; Noh et al., 2016). It may express at different growing stages of insect-pests and is associated with ecdysis (Willis, 1996). For example, the *BmGRP*s is over-expressed at 4^{th} ecdysis in silkworm (Zhong et al., 2006) while in Yellow mealworm during the process of post-ecdysial cuticle deposition in adults, *ACP*-20 is over-expressed (Charles et al., 1992). Decreased in permeability of cuticle is the most important tactic of penetration resistance in insects to a variety of insecticides (Motoyama et al., 1992; Medina et al., 2002; Ahmad et al., 2006).

Penetration resistance to an insecticide reduces the rate of penetration of a specific insecticide/toxin thus preventing its target site from exposure to lethal dose which in turn allows adequate time to detoxifying enzymes to act. Cuticle is also a hurdle in the way of rapid moisture loss from insect body due to harsh environmental conditions (Motoyama et al., 1992; Pelletier, 1995; Balabanidou et al., 2018). Alteration in cuticular composition may help insects in decreasing dehydration and thus make it able to withstand dry weather. Alterations in permeability of cuticle can be associated with ability of insects and other animals of different ecological zones (Cloudsley-Thompson, 1975).

More specifically, epicuticle's wax layer is the key hurdle in the way of penetration (Beament, 1959; Locke, 1961). Difference in the rate of penetration among susceptible and resistant insect strains persists. Penetration resistance to fenvalerate was halved due to removal of wax layer in diamondback moth. It also indicated that some other factors are also involved in enhancing the penetration resistance (Motoyama et al., 1992). Epicuticle also contains lipids in bound to proteins which are involved in enhancing penetration resistance to organic substances like insecticides (Hadley, 1982). The covalently associated lipo-proteins provide an imperative barrier to lipid-soluble chemicals like toxins. The external protein layer of corneocytes can also provide resistance to penetration of water (Downing, 1992).

2.9 Life Table Analysis

Life tables are important for ecologists, researchers and pest managers due to the fact that they provide better understanding of ecology of any insect-pest (Chi, 1988). Male individuals, sex ratio and stage differentiation are not the components of traditional female-based age-specific life tables (Lewis, 1942; Leslie, 1945; Birch, 1948; Carey, 1993). Owing to this disregard, they are not able to describe various aspects in insect ecology like growth, survival, and stage differentiation (Huang and Chi, 2012, Huang et al., 2018). Age-stage, two-sex life table gave a solution to these solution to these shortcomings by taking these aspects viz. stage differentiation along with male population into consideration. It was developed by Chi and Liu (1985) which was further refined by Chi (1988). Age-stage, two-sex life table have gained special attention of researchers (Chen et al., 2018; Liu et al., 2018). It can be utilized in various

studies involving insect ecology (Huang et al., 2018), physiology (Peng et al., 2016, Zanardi et al., 2016), and insecticides' susceptibility (Huang and Zhang, 2015). Some studies have been conducted on female biased life table analysis of CPB.

Yas and Güngör (2005) worked on life table analysis of CPB on the 5 different potato cultivars viz. Agria, Caspar, Marfona, Granola and Pasinler. Shorter developmental time of CPB was observed on the Caspar as a longer developmental time of CPB was found on the Granola cultivar. Less number of hatching in CPB eggs was observed on the Marfona cultivar, while the more hatching efficiency was calculated on the Pasinler cultivar. Less of survival rate for immature stages of CPB was calculated on the Marfona cultivar and the higher survival rate was found on the Caspar cultivar. More daily fecundity was recorded on the Granola cultivar and the highest total fecundity was found on the Pasinler cultivar. However, the least values for daily fecundity and total fecundity were calculated on the Agria cultivar. The increasing order of intrinsic rate of increase (r) was Granola (0.028), Marfona (0.035), Agria (0.043), Caspar (0.047) and Pasinler cultivar (0.051). The lowest net reproductive rate (R_0) was found on the Granola cultivar while highest was observed on the Caspar. The generation (T) time was the shortest in the Pasinler and longest in the Caspar. It was concluded that the Marfona potato cultivar was relatively resistant to CPB than the rest of the tested cultivars.

Fathi et al. (2013) investigated the life-table parameters of CPB on 7 different potato cultivars viz. namely Aozonia, Agria, Cosmus, Diamant, Morene, Kondor, and Savalan under controlled conditions. It was revealed that the developmental period of larvae was significantly more on Savalan (18.3 days) than other potato cultivars. Significantly lower survival rates were calculated on Savalan and Morene than other cultivars used in their study. A lower fecundity (286.3 eggs/female) was observed in the CPB reared on Savalan cultivar than the rest. Savalan and Diamant also supported a relatively shorter oviposition period of females than other cultivars. Savalan showed lower intrinsic rate of natural increase i.e. 0.055 $day^{-1}$and lower population growth rate i.e. 1.056 day^{-1}, respectively. Mean generation time (69.5 days) and doubling time (12.7 days) were significantly longer on Savalan than the rest of cultivars. They concluded that Savalan was most resistant cultivar for CPB than the other cultivars tested.

Iranipour et al. (2016) conducted a life table study on CPB on four potato cultivars viz. Agria, Savalan, Satina and Marfona under controlled conditions (62 ± 10% RH, 23 ± 3 ºC, and natural photoperiod). Development period and generation time was shortest on Agria i.e. 27.8 days and 35.99 days, respectively. However, longest development period and generation time was found on Savalan viz. 31.07 days and 42.72 days, respectively. Mortality during immature stages was lower on Marfona i.e. 22.5% and higher on Satina i.e. 47.5%. Intrinsic rates of increase were 0.129 day^{-1} in Agria; 0.127 day^{-1} in Marfona; 0.129 day^{-1} on Satina, and 0.104 day^{-1} on Savalan. The lower net reproductive 81.18 offspring/female was on Savalan and higher net reproductive rate was observed on Marfona i.e. 145.26 offspring/female. It was concluded that the Savalan cultivar was relatively resistant to CPB, resulting in the less growth of the beetle.

All above studies were carried out with the female-biased life table of CPB. However, , a study on two-sex life table analysis of CPB was required to fill the knowledge gap due to shortcomings of female-biased life-table. Additionally, there is no available literature on comparison of susceptible and resistant populations of CPB. Therefore, keeping in view the importance of two-sex life table analysis, life table parameters of lab susceptible and field CPB population were calculated.

CHAPTER III

MATERIALS AND METHODS

3.1 Plant Material

Pesticide free tubers of *S. tuberosum* cultivar Agria were obtained from a local potato farm (Niğde). This cultivar is grown in approximately all of the potato growing regions of Turkey due to its better quality and more yield. Tubers were sown in 1 L capacity plastic disposable pots (Senkap, Konya, Turkey) containing soil media, consisting of mixture of peat, perlite and vermiculite (mixed in equal proportion of 1:1:1) (Klasmann-Deilmann Gmbh Germany). Plants were watered regularly according to the requirement. These plants were grown without any pesticide application inside a growth chamber (Delta, Konya, Turkey) at 25±1°C and 60-65% relative humidity (RH) with a 16: 8 light (L) –dark (D) photoperiod. When the plants reached 4-6 leaf stage, they were used for rearing of different CPB populations. Potatoes tubers were sown every 2nd week for continuous supply of fresh potato foliage to CPB populations.

3.2 Imidacloprid

Imidacloprid (IUPAC name: 2-chloro-5-{[2-(nitroamino)-4,5-dihydro-1H-imidazol-1-yl]methyl} pyridine), discovered and patented by Bayer Crop science, is a neonicotinoid, a chemical analogue of nicotine. Chemical structure of imidacloprid is shown in Figure 3.1 (Kumar et al., 2013). The main aim of imidacloprid development was to introduce an effective chemical against insects with low mammalian toxicity. It is considered as ‘‘moderately toxic’’ regarding animal toxicity studies (class II by WHO and toxicity category II EPAV). In 1994, it was registered by United States Environmental Protection Agency (USEPA) for the first time (Hovda and Hooser, 2002). It is quite effective against a broad range of insects especially due to its systemic action (Tomizawa and Casida, 2005). For control of CPB, imidacloprid was first used in 1995 due to its novel mode of action (Mullins, 1993). Formulation of imidacloprid used in this study was Confidor® 350 SC (Bayer Crop Science, Germany).

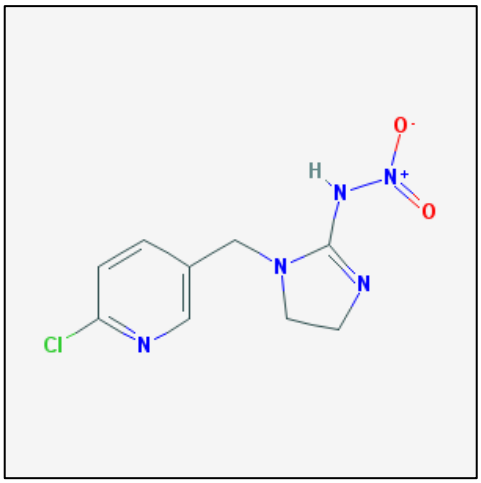


Figure 3.1. Chemical structure of imidacloprid

3.3 CPB Populations

3.3.1 Lab susceptible CPB population

The CPB population has been maintained in Entomology laboratory of Department of Plant Production and Technologies since June 2015. For starting the colony, about 40 CPB adults were collected from the pesticide free potato fields and brought into the laboratory. They were placed into a wood and mesh made rearing cages (100cm×100cm×100cm) with 4 potato plants as food source in one cage. Since then the population was reared in the insect growth chamber (Devpet, Konya, Turkey) at 28±1°C with 16: 8 h light–dark photoperiod and relative humidity (50-60%). Fresh potato plants were provided as food according to the requirement. Daily eggs obtained were separated in 90 mm Petri plates (VWR, USA) on filter paper (Whatman®, Sigma-Aldrich, US). Filter paper was moistened daily to prevent eggs from desiccation. The eggs were incubated at above conditions. After hatching, larvae were transferred into rearing boxes prepared from plastic boxes (90mm×180mm×120mm) until they entered pupation (Figure 3.2). Before pupation, 4th larval instars were shifted to 147.85 ml volume plastic cups (Yöm Plastik Company, Istanbul, TR) filled with soil to provide them a medium (prepared as described in 3.1) for pupation. Cups containing the pupae were monitored and moistened daily until the adults emerged from them. After adult emergence, they were sexed according to the ventral tips of their abdomen i.e. male CPB adults contain a smaller depression on posterior ventral tip of the abdomen (Rivnay, 1928; Gelmen et al., 2001) and were divided into two sets one set was reared in reared in cups while rest

were put in the bigger cage rearing cages (100cm×100cm×100cm) with 4 potato plants as food source. Around 4 adults (2 female:2 male) were shifted to a set of two cups combined upside down two 90 mm rearing cups containing 3 leaflets of potato. Leaflets were provided in the cup by dipping its stalk in glass vial containing water. Adults were monitored daily for mating and egg-laying. To avoid inbreeding depression, adults from pesticide free potato fields were also introduced in the susceptible population during different times. These insects were reared in laboratory without any insecticide application to maintain susceptibility to insecticides. They were reared in laboratory for about 4 years before conducting all the experiments

Figure 3.2. Rearing of CPB larvae in growth chamber

3.3.2 Field population

During February 2018, hibernated CPB adults (40-50) were collected from previous year potato field, used in every season for potato growing, at the Faculty of Agricultural Sciences and Technology. Adults were collected from the field and brought to the laboratory. Under controlled conditions (28±1°C, 50-60% and 16: 8 h light–dark photoperiod), they came out of the hibernation stage. After breaking the hibernation, CPB adults were sexed and 10 adults (5 female: 5 male) were placed into a wood and mesh made rearing cage (100cm×100cm×100cm) with 4 potato plants as food source in one cage. These adults were provided with fresh leaflets daily. While, daily eggs obtained were separated in petri plates on the filter paper. Filter paper was moistened daily to prevent eggs from desiccation. Daily eggs obtained were separated in 90 mm Petri plates (VWR, USA) on filter paper. Filter paper was moistened daily to prevent

eggs from desiccation. After hatching of eggs, larvae were transferred into rearing boxes prepared from plastic boxes (90mm×180mm×120mm) and kept there until they entered pupation. Soil medium was prepared according to 3.1 for pupation. Pupae were incubated until the adults emerged from them. After adult emergence, adults were divided into two sets and these two groups were reared as described in 3.3.1. Adults were monitored daily for mating and egg-laying (Figure 3.3). These insects were used after one generation (F_1) for imidacloprid resistance bioassays. While, F_2 from this field CPB was used for age-stage two-sex life table analysis.

Figure 3.3. Rearing of CPB adults on Agria cultivar in growth chamber

3.3.3 Lab resistant CPB population

Hibernated CPB adults (40-50) were collected from previous year potato field at the Faculty of Agricultural Sciences and Technology during December 2017. Adults were collected from the field and brought to the laboratory. Around 25 collected adults were put in transparent plastic cups (147.85 ml, Yöm Plastik Company, Istanbul, TR) containing fresh potato leaflets. Under controlled conditions (28±1°C, 50-60% and 16: 8 h light–dark photoperiod), they came out of the hibernation stage. After breaking the hibernation, CPB adults were sexed as described in 3.1.1. About 10 adults (5 female:5 male) were placed into a wood and mesh made rearing cages (100cm×100cm×100cm) with 4 potato plants as food source in one cage. These adults were provided with fresh potato plants when needed. Daily eggs obtained were separated in petri plates on the filter paper. Filter paper was moistened daily to prevent eggs from desiccation. To provide imidacloprid selection pressure, 1µL of imidacloprid at 300µg/ml dose was

topically applied on 2nd instar reared from F_1 of the field collected adults. Surviving larvae were collected and transferred into rearing boxes prepared from plastic boxes (90mm×180mm×120mm) at 28±1°C, 50-60% and 16: 8 h light–dark photoperiod until they entered pupation. The pupation medium was prepared and the pupae were incubated as described in 3.3.1, until the adults emerged from them. After adult emergence, 4 adults (2 female:2 male) were shifted to a set of two cups combined upside down two 90 mm rearing cups containing 3 leaflets of potato. Leaflets were provided in the cup by dipping its stalk in glass vial containing water. Adults were monitored daily for mating and egg-laying. In this way, eggs were separated and 2nd instar larvae of each generation were topically treated with imidacloprid and surviving larvae were used for next generation. After consecutive generations, resistance level of lab resistant population was compared with the field and lab susceptible populations as described in Wang and Wu (2012).

3.4 Resistance Study with Different CPB Populations

Comparison of imidacloprid resistance level of three different CPB populations (the lab susceptible, field and lab resistant) was carried out under laboratory conditions. Bioassays were conducted with 2nd instar larvae of CPB populations according to the methodology of Mota-Sanchez et al. (2006). For the field population, the F_1 generation was used for bioassays. The similar size 2nd instar larvae were collected from the colonies. The dose range used in the bioassay was based on preliminary assessment with all three populations. Serial dilutions of imidacloprid were prepared in distilled water. The doses for the lab susceptible population were 23.43 µg mL^{-1}, 46.87 µg mL^{-1}, 93.75 µg mL^{-1}, 187.5 µg mL^{-1} and 375 µg mL^{-1}; for the field population were 187.5 µg mL^{-1}, 375 µg mL^{-1}, 750 µg mL^{-1}, 937.5 µg mL^{-1} and 1135 µg mL^{-1}; and for the lab resistant population were 187.5 µg mL^{-1}, 468.75 µg mL^{-1}, 937.5 µg mL^{-1}, 1875 µg mL^{-1} and 3750 µg mL^{-1} in water. The doses were applied in ascending order and the syringe was cleaned with ethanol (96%) and water three times between treatments. One µl insecticide solution using a 50 µL micro-syringe (Hamilton Company, Reno, NV) connected to a micro-applicator (Burkard Scientific Limited, UK) was applied on the dorsal side of each larva. After the treatment, CPB larvae were allowed to dry for half an hour under laboratory conditions. They were then placed in petri dishes (90 mm) and provided with fresh potato leaves, and kept at controlled conditions at 28±1°C with 16:

8 h light–dark photoperiod and relative humidity (50-60%). In the control group, each larva was treated with 1 µL distilled water by a separate micro-syringe connected to the micro-applicator. Data were recorded 24 hour intervals for three days after treatment. Larvae unable to respond on stimulus when disturbed were counted as dead. Randomized block design was used in the study. Each block consisted of all tested concentrations of insecticides and a control group for each colony. The experiment was repeated three different times. For each concentration and the control group, total 30 second instar larvae of equal size were treated. The LD_{50} of susceptible CPB colony was used as susceptibility/base-line data (Busvine, 1980).

3.5 Life Table Studies

Life table parameters of the lab susceptible population were calculated after 3 years of rearing without any pesticide pressure. While, the field CPB population F_2 was reared for conducting the life table study.

3.5.1 Rearing method

Numbers of eggs taken to study the susceptible CPB population were 72, while 59 eggs were taken to study the field population. These eggs were kept in petri plates on filter paper which was kept moist to avoid desiccation until hatching. Total 46 insects hatched from the lab susceptible population eggs, while 57 eggs hatched from the eggs of the field CPB population which were used to calculate age stage two sex life table parameters. These eggs were observed daily and after hatching each neonate larva was transferred to separate 90 mm Petri plate containing fresh potato leaflet as food. The neonate larvae were kept at controlled conditions at 28±1°C temperature with 16: 8 h light–dark photoperiod and relative humidity (50-60%). Potato leaflets were replaced by fresh ones daily. Larval growth and development was observed daily until the formation of pupae. Change in larval instar was confirmed by the presence of exuvium. Before pupation, 4^{th} larval instars were shifted to 147.85 ml volume plastic cups (Yöm Plastik Company, Istanbul, TR) filled with soil to provide them a medium for pupation. Cups containing the pupae were monitored and moistened daily until the adults emerged from them. Newly emerged adults were sexed according to the ventral tips of their (Rivnay, 1928; Gelmen et al., 2001). After sexing, each pair (1 male: 1 female) was separated to

an egg laying apparatus (two 147.85 ml plastic cups combined with a parafilm band Figure 3.3). There were four holes on each cup for aeration containing four fresh leaflets of each cultivar for oviposition. Leaflets were provided in the cup by dipping its stalk in glass vial containing water. Adults were monitored daily for mating and egg-laying. Number of eggs laid by each female were separated and counted daily. Pre-oviposition period, oviposition period and adult longevity were recorded for each adult until the death of all the adults.

3.6 Targeted Gene Amplification and Cloning in *L4440* Vector

3.6.1 Total RNA extraction from resistant CPB

For extraction of total RNA, the TRIzol method was used with some modifications (Simms et al., 1993). The 2nd instar larvae of resistant CPB population were collected. Larvae were properly ground to fine powder with mortar and pestle by the addition of liquid nitrogen. Then, the insect powder was transferred into 1.5 mL of eppendorf tube, and 1 mL of TRIzol (Sigma) was added. Afterwards, samples were mixed in vortex and kept at room temperature for 10 minutes. Then, the tubes were incubated for 20 min at 70 ºC. Later, the tubes were centrifuged at 14,000 rpm at 4°C for 10 minutes, and the supernatant was taken and mixed with equal amount of chloroform after keeping at room temperature for 5 minutes. The tubes were passed through centrifugation process again at 14,000 rpm for 15 minutes at 4°C. Then, upper phase was shifted into a clean eppendorf tube, and 500 µL of cold isopropanol was added. After a brief mixture of the solution in the tubes, they were kept at -20°C for 10 minutes. Then, they were centrifuged at 14,000 rpm at 4°C for 20 minutes. Finally, the predicated pellet was washed with 1 mL of 75 % of ethanol and was dried at room temperature for 10 minutes. Pellet was dissolved in 30 µL of DEPC-treated sterile distilled water.

3.6.2 Agarose gel electrophoresis

Agarose gel electrophoresis was carried out to check the quality of RNA. About 0.5% agarose gel was prepared using 0.5X TAE buffer. Around 0.5 µg/mL of ethidium bromide was added to the gel. Gel was run at 80V for 30 minutes to resolve the total RNA.

3.6.3 Quantification of Total RNA

RNA was quantified by spectrophotometer (BioSpec-nano manufactured by SHIMADZU Biotech). 1 µL of each sample was put to find out the quantity and quality of RNA was recorded.

3.6.4 cDNA synthesis

RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) was used for the synthesis of cDNA. Total RNA samples were treated with DNase I (Thermo Fisher) to remove genomic DNA contamination according to the manufacturer's manual. Simply, 10 µg of total RNA was mixed with 10 µL of 10X reaction buffer, 10 U of DNase I and DEPC-treated water to make a final volume of 100 µL and incubated at 37°C for 30 minutes. Then, the reaction was stopped by addition of 10 µL of 50 mM EDTA and incubation at 65°C for 10 minutes To synthesize cDNA, the reagents used were: RNA 1µg (DNase treated), 20 µM of anchored oligodT primers (1µL) and DEPC-treated water (12µL). Incubation of reaction mix was carried out at 70ºC for five minutes followed by chilling treatment on ice. Afterwards, 4µL of 5X reaction mix, 2µL of dNTP mix (10mM) and 1µL of ribonuclease inhibitor (20 U/µL) were added and incubated for 5 min at 37ºC. In the last step, 1µL of H minus M-MuLV reverse transcriptase (200µ/µL) was added and mixture was incubated at 42 ºC for 60 min. Finally, heat shock at 70ºC was applied for 10 min to stop the reaction.

3.6.5 Primer design for *L4440*

The *L4440* plasmid (Addgene, USA) was selected for the preparation of dsRNA of all the genes. *L4440* plasmid contains two T7 promoters. In between those T7 promoters, fragments of our targeted genes were copied to produce dsRNA. Primers used earlier were modified with restriction sites of *Kpn*I and *Bgl*II according to the map of *L4440* (Figure 3.4). Possible plans to copy our gene fragments are shown in Figure 3.5 (A, B and C).

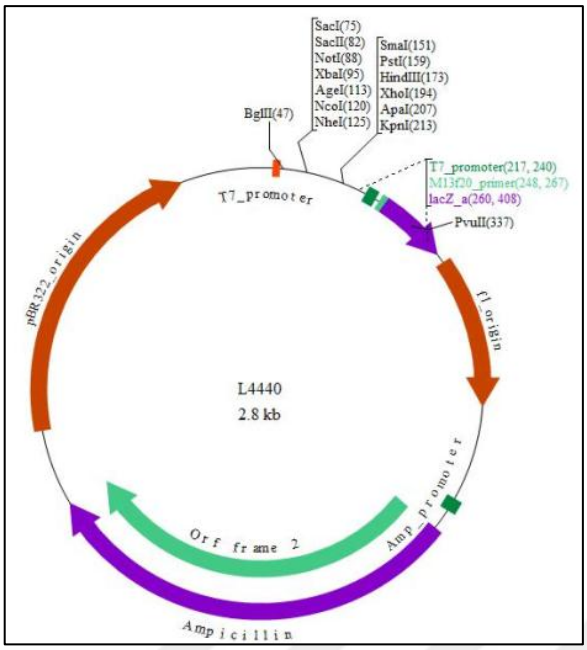


Figure 3.4. Map of *L4440* showing its available restriction sites and two promoters

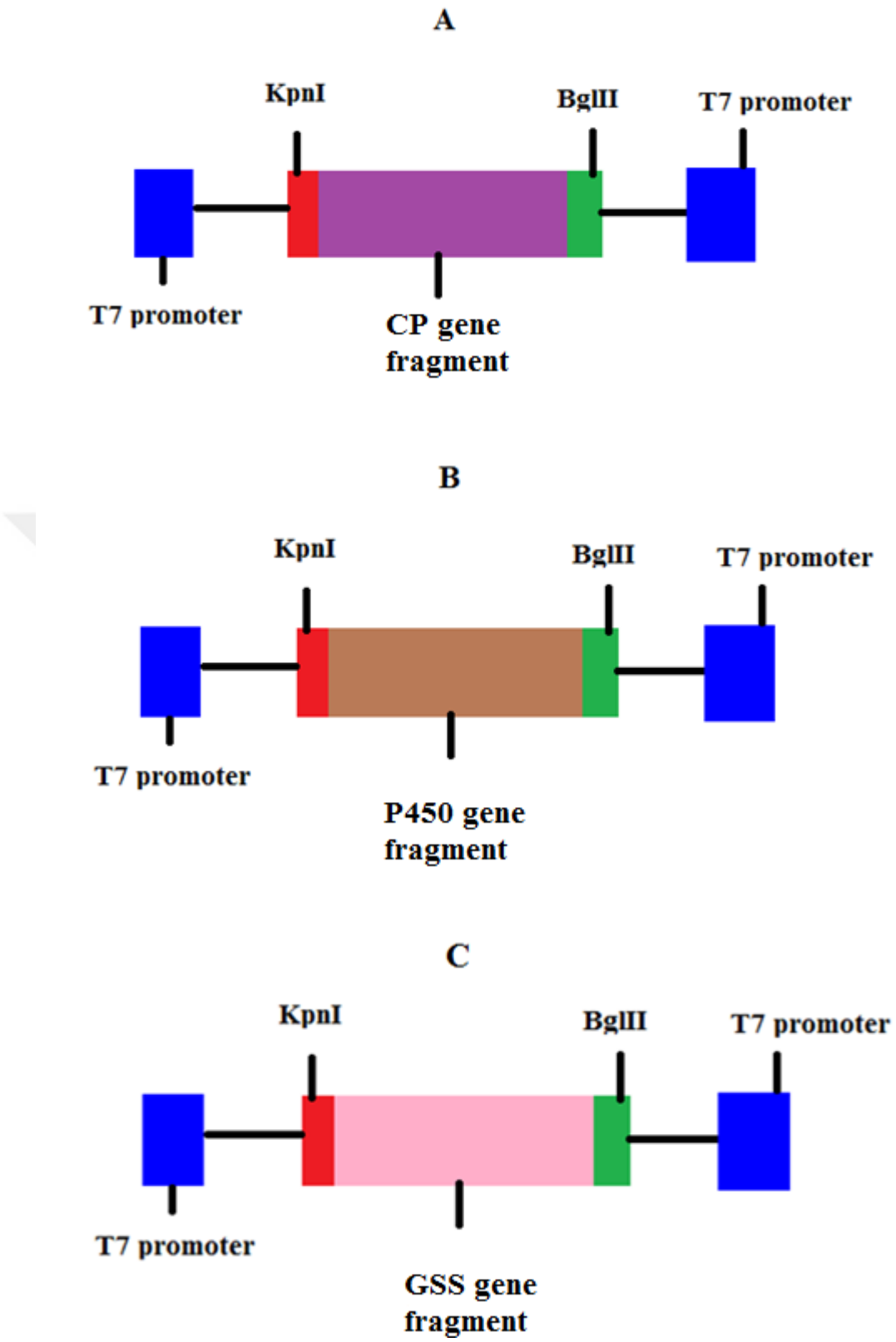


Figure 3.5. Planned ligation of CP (a), P450 (b) and GSS gene fragment (c) between T7 promoter in *L4440* plasmid

3.7 Preparation of dsRNA

3.7.1 PCR for amplification of targeted genes' fragments

The cDNA prepared earlier was used to amplify the fragments (portions) of targeted genes (CP, P450 and GSS). To carry out PCR, reaction volume was adjusted to 20 µL containing the forward and reverse primers 50 pM each, cDNA template (20 ng), 2X PCR Buffer (50 mM KCl, 1.5mM MgCl2 and 10mM Tris-HCl), 200 µM dNTPs, Taq Polymerase 1 unit (Thermo Scientific) and nuclease free water. First a gradient PCR using 4 different temperatures viz. 50 ºC, 55 ºC, 60 ºC and 65 ºC was conducted to determine the optimum annealing temperature. Afterward, the PCR was performed using denaturation temperature 94ºC for 4 minutes, annealing temperatures was optimized at 60ºC for 1 min while extension was carried out at 72ºC for 1 minutes. Final extension was carried out for 7 min. Reaction was set at 35 cycles. The amplified PCR fragments were resolved on 1% agarose gel and observed under UV light. List of primers is given in Table 3.1.

Table 3.1. Sequences of primers modified according to the sites of *L4440*

Gene	Primer sequence	Product size
P450 F	5´-ACAGATCTAAAGATCAGGAGCCACGAAAA-3´	306
P450 R	5´-CAGGTACCGACCTATTCTCAGCCCATCG-3´	
CP F	5´-ACAGATCTCGCTCAACGTAACGGAGTCT-3´	393
CP R	5´-CAGGTACCTGTCCGTCTGGTCCGATTTG-3´	
GSS F	5´-ACAGATCTTGCTTGATCGCCGGATTCAT-3´	407
GSS R	5´-CAGGTACCTTGCCGACCAGAGATTCCAT-3´	
L4440 F	5´-AGCGAGTCAGTGAGCGAG-3´	290
L4440 R	5´- TGTAAAACGACGGCCAGT-3´	

3.7.2 Purification of genes' fragments from gel

The amplification products of fragments of targeted genes were further used for gel elution. Respective gel band was excised and purified using GeneJET Gel extraction and DNA cleanup micro Kit after some modifications (Cat# k0832). Around 200 mg of gel slice (for each gene) containing the PCR product was excised by a clean scalpel and put in separate eppendorf tubes. Gel was excised from the nearest portion of gel to

reduce the volume of extra gel. Around (200 µL) of extraction Buffer was added and mixed by inverting. Gel mixture was heated at 55°C for 15-20 minutes until the gel was completely dissolved. About 200 µL of ethanol (96%) was added and mixed by pipetting. The mixture was transferred to the DNA Purification Micro Column containing a collection tube. After waiting for 20-30 minutes, centrifugation of column was carried out at 14,000 rpm for 60 seconds. After discarding the flow-through, DNA Purification Micro Column was put back on the collection tube. Prewash Buffer (200 µL) was added to the DNA Purification Micro Column followed by centrifugation at 14,000 rpm for 60 seconds. After discarding the flow-through, DNA Purification Micro Column was put back to the collection tube. Wash Buffer (700 µL) was added to the DNA Purification Micro Column followed by centrifuge process at 14,000 rpm for 60 seconds. Addition and removal of wash buffer was carried out again. Flow-through was discarded and DNA Purification Micro Column was put back on the collection tube. An extra spin was carried out at 14,000 rpm for 1 min to completely remove residual Wash Buffer from the empty DNA Purification Micro Column. The DNA Purification Micro Column was transferred to a clean 1.5 mL microcentrifuge tube. Elution Buffer (20 µL) was added to the DNA Purification Micro Column. Finally, buffer was centrifuged for 1 minute at 14,000 × g to elute DNA.

3.7.3 Quantification of eluted gene

Agarose gel of 1% strength was prepared using 0.5X TBE buffer. Around 0.5 µg/mL of ethidium bromide was added to the gel. About 2 µL of PCR products were run on the gel for quantification along with 3 µL of marker. Gel was run at 90V for 30 minutes. After running, the concentrations of genes' fragments were observed under UV light. Concentration of PCR products of each gene were also verified using spectrophotometer (BioSpec-nano manufactured by SHIMADZU Biotech).

3.7.4 Plasmid extraction

The *DH5α* cells containing the *L4440* vector were inoculated in 10 ml of LB media containing 40 µL of ampicillin (25 µg/µL). It was allowed to grow overnight and then plasmid was extracted using Thermo scientific plasmid miniprep kit (#K0503). Bacterial media was first centrifuged to get pellets of bacteria. The pelleted cells were

re-suspended in 250 µL of the resuspension solution and transferred to the cell suspension to a microcentrifuge tube. Lysis Solution (250 µL) was added to the solution followed by thorough mixing via inverting the tube (4-6 times) till a slightly clear viscous color appeared in the solution. Neutralization solution (350 µL) was added followed by thorough mixing via inverting the tube (4-6 times). Mixture was spun for 5-10 mins to pellet cell debris and chromosomal DNA. Supernatant was shifted to the supplied GeneJET spin column by pipetting very carefully without disturbing the precipitates. After waiting for 20-30 minutes, mixture was centrifuged for 1 min. Flow-through was discarded and the column was kept back into the same collection tube. Wash Solution (500 µL) was added to the GeneJET spin column and centrifuged for 60 seconds. Flow-through was discarded and the column was kept back into the same collection tube. This step of adding wash solution was carried out twice. Column was centrifuged for an additional 1 min to remove residual Wash Solution. GeneJET spin column was transferred to a fresh 1.5 mL microcentrifuge tube. Pre-heated elution Buffer, at 65 °C (50 µL), was added to the center of GeneJET spin column membrane to elute the plasmid DNA.

3.7.5 Quantification of plasmid

Agarose gel of 1% strength was prepared using 0.5X TBE buffer. Around 0.5 µg/mL of ethidium bromide was added to the gel. Around 2 µL of plasmid was run on the gel for quantification along with 3 µL of marker. Gel was run at 90V for 30 minutes. After running, the concentration of plasmid was observed under UV light. Concentration of plasmid fragment was also verified using spectrophotometer (BioSpec-nano manufactured by SHIMADZU Biotech).

3.7.6 Digestion of plasmid and genes

The purified gel-eluted fragments of targeted genes (CP, GSS and P450) and *L4440* vector were digested with *Kpn*I and *Bgl*II restriction enzymes (Thermo Scientific) to create sticky ends in desired fragments of the gene fragment and vector. Following reaction mixture combination were used as given in Table 3.2. The samples were incubated for 30 minutes at 37^{o}C followed by deactivation of enzyme activity at 65^{o}C for 5 minutes to deactivate enzyme activities. The digested genes' fragments and vector

was run on 1% agarose gel at 80V for 45 min and observed under UV-light. Digested plasmid (100 ng/µL) and 25 ng/µL, 40 ng/µL and 50 ng/µL of CP, GSS and P450, respectively were eluted according to the above mentioned procedure using GeneJET Gel extraction and DNA cleanup micro Kit after some modifications (Cat# k0832).

Table 3.2. Reaction mix for digestion of fragments of genes and vector

Ingredients	CP gene fragment	P450 gene fragment	GSS gene fragment	Vector
Gene fragment/Plasmid	0.5 µg	0.5 µg	0.5 µg	1 µg
10x FD Buffer	2 µl	2 µl	2.0 µl	2 µl
*Kpn*I (1 U/µl)	0.5 µl	0.5 µl	0.5 µl	1 µl
*Bgl*II (1 U/µl)	0.5 µl	0.5 µl	0.5 µl	1 µl
Water	2.0 µl	4.0 µl	6.0 µl	8.5 µl
Total	20µl	15µl	15µl	20 µl

3.7.7 Ligation

Digested fragments of targeted genes and plasmid were ligated using T_4 DNA Ligase (Promega) following the instructions of manufacturer's protocol. For ligation, equal concentration of vector and insert (1:1) were added. Ligation buffer (1.5 µL), T4 ligase (1 µL) and water were added to adjust the volume to 15 µL. Ligation temperature was 22^{o}C for 2 hours, 15 ^{o}C for 3 hours and 4^{o}C overnight. Vector's concentration was 100 ng/µL, while the concentrations of inserts were 25 ng/µL, 40 ng/µL and 50 ng/µL in CP, GSS and P450, respectively. Reaction mix used for ligation is given in Table 3.3.

Table 3.3. Reaction mix for ligation of targeted genes fragments

Ingredients	CP gene fragment	GSS gene fragment	P450 gene fragment
Insert	100 ng	100 ng	100 ng
Plasmid	100 ng	100 ng	100 ng
10X Ligation buffer	1.5 µl	2.0 µl	2 µl
T4 ligase	1.0 µl	1.0 µl	1.0 µl
Water	2.5 µl	6.0 µl	0.0 µl
Total	15 µl	15 µl	15 µl

3.7.8 Bacterial transformation

The *Escherichia coli* strain *DH5α* was used for transformation with constructed recombinant vector in ligation mixture. From the ligation mixture, 4 µL of the ligated products of CP, GSS and P450 were transformed in *DH5α* chemically competent cells (50µL). These cells were kept on ice for 20-30 minutes and were subjected to heat shock at 42 ^{o}C for 1 minute. LB broth medium (400 µL) was added on the cells in laminar flow (CHC Biolus) followed by incubation at 37 ^{o}C for 1 hour on thermo-shaker at 500 rpm. After 1 hour, transformed cells were spread on LB plates containing ampicillin (100 µg mL^{-1}) and incubated at 37 °C overnight.

3.7.9 Colony PCR

To carry out colony PCR, reaction volume was adjusted to 20 µL containing the forward and reverse primers 50 pM each, 200 µM of dNTPs, 2X PCR Buffer (Thermoscientific) (50 mM KCl, 1.5mM MgCl2 and 10mM Tris-HCl), 1 unit of Taq Polymerase and nuclease free water. Total of 10 colonies were randomly selected from one plate of each targeted gene. One colony was inserted from culture plates in each tube. cDNA prepared from the mRNA of resistant CPB was used as positive control. While, negative control did not include any bacterial colony. The PCR was performed using denaturation temperature of 94ºC for 4 minutes, optimized annealing temperature of 60ºC for 1 min, followed by extension at 72ºC for 1 minutes. Final extension was carried out for 7 mintutes. Amplification was done for 35 cycles. The amplified PCR fragments were resolved on 1% agarose gel.

3.7.10 Plasmid extraction

Positive colonies were inoculated in different tubes containing 10 ml LB media and 40 µL of ampicillin (25 µg/µL). They were allowed to grow overnight and then plasmid was extracted using Thermo Scientific plasmid miniprep kit (#K0503). Plasmid extracted was further quantified on 1% agarose gel followed by observing over the UV lamp.

3.7.11 Bacterial expression system

The *E. coli* strain (*HT115*) was ordered from Caenorhabditis Genetics Center (CGC). Bacterial strain *HT115* does not contain RNase-III that's why it is capable of producing dsRNA without degradation. It starts producing dsRNA by insertion of *L4440* plasmid and induction by IPTG (50 µM).

3.7.12 Preparation of HT115 chemically competent cells

Bacterial strain *HT115* (*DE3*) was inoculated overnight in 10 ml of liquid LB medium in a shaker (90 rpm) at 37°C along with appropriate antibiotic (Tetracycline 5 µg/mL). From overnight cultured bacteria, 1 ml was inoculated into 100 ml fresh LB along with tetracycline. Afterwards, cells were grown in sterile flask (500 ml), and grown for 5 hours to get OD_{595}= 0.4. Cells were collected at the bottom of a falcon tube by centrifugation at 3000 rpm for 10 min at 4°C. Pellet was resuspended in sterile 50 mM $CaCl_2$. Volume of 50 mM $CaCl_2$ used at this step was 0.5X of the original volume (100 ml) of bacteria used. Resuspended pellet was incubated on ice for 30 min. It was centrifuged again at 3000 rpm for 10 minutes at 4 °C. Keeping cells cold, pellet was again resuspended in 0.1X original volume (100 ml) of 50 mM $CaCl_2$. Resuspended cells were aliquoted into eppendorf tubes, and stored at -80 °C. Cells (50 µL) were used latter for the transformation.

3.7.13 Transformation of *HT115* (*DE3*):

In 50 µL of bacterial aliquot, 2 µL of each plasmid containing the gene fragment was added on ice in 3 different eppendorf tubes. The mixture was incubated on ice for 30 min and tube was immersed in water bath for 1 min at 37 °C. Later, the tube was incubated on ice for 2 min. Next, 1 ml of sterile LB broth was added in tube and incubated at 37 °C for 1 hour on thermoshaker at 500 rpm. Bacterial culture (three replicates of 50, 100 and 200 µL) were then spread on LB plates containing antibiotics (Ampicillin: 100 µg/µL; Tetracycline: 5 µg/mL) for selection and incubated overnight at 37 °C.

3.7.14 Colony PCR

Colony PCR was carried out using 10 µL of 2 X PCR master mix (Thermo Scientific), forward and reverse L4440 primers 50 pm each (Table 3.1) and 9 µL of water in final volume of 20 µL. Ten colonies were randomly selected from one plate of each gene. One colony was inserted randomly from culture plates into each tube. Negative control did not include any bacterial colonies. While, the empty *L4440* vector was used as control. Then, the PCR was performed using denaturation temperature of 94ºC for 4 minutes, optimized annealing temperature of 60ºC for 1 min, followed by extension at 72ºC for 1 minutes. Final extension was carried out for 7 minutes. Amplification was done for 35 cycles. The amplified PCR fragments were resolved on 1% agarose gel and observed over UV lamp.

3.7.15 Confirmation via digestion

Successful ligation was confirmed via digestion as given in section 3.7.6. The ligated plasmids were digested with *Kpn*I and *Bgl*II restriction enzymes to get a band of 393, 407 and 306 bp for CP, P450 and GSS gene fragments. The samples were incubated for 30 minutes at 37^{o}C followed by deactivation of enzyme activity at 65^{o}C for 5 minutes to deactivate enzyme activities. The digested vector was run on 1% agarose gel at 80V for 45 min and observed over UV lamp.

3.7.16 Storage of bacterial stock containing dsRNAs

Positive colonies from each gene's fragment were inoculated in 2.5 ml of LB broth along with antibiotics and were grown to stationary phase. For storage, mixture was made by pipetting 0.25 ml of 80% glycerol and 0.75 ml of bacterial culture into a sterile screw-caped tube. Mixture was quickly put on ice and stored at -80 °C to avoid heat shock. Schematic representation of recombinant plasmids containing fragments of our targeted genes (CP, P450 and GSS) are given in Figure 3.6 (A, B and C).

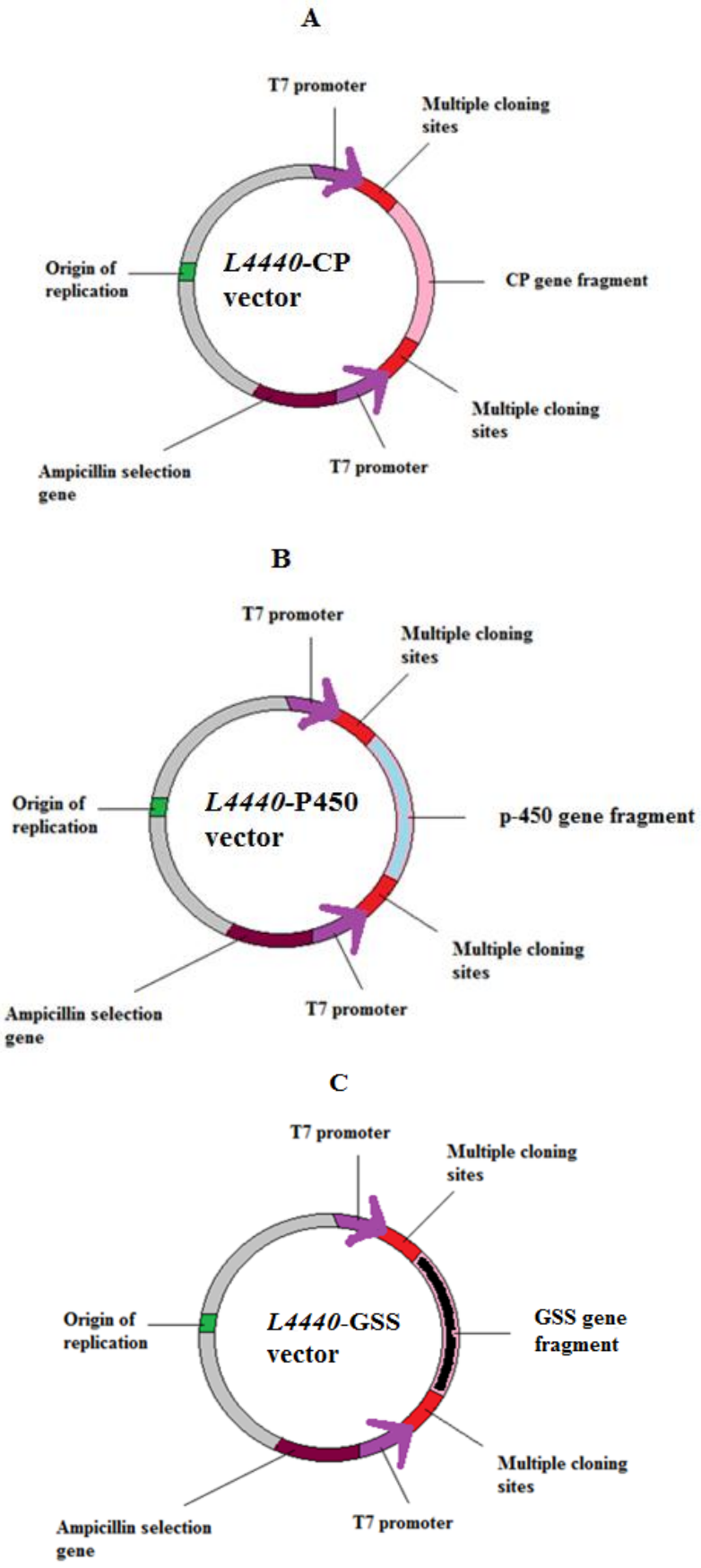


Figure 3.6. Schematic representation of CP (a), P450 (b) and GSS (c) recombinant plasmids

3.8 dsRNA Synthesis in Bacteria

Transformation of cloned *L4440* vectors was performed using competent cells of *HT115* (DE3) strain. Single positive colony of *HT115* containing vector *L4440* with CP, GSS and P450 was inoculated in LB broth (10 mL) along with 40 µL of ampicillin (25 mg/µL) and cultured overnight. The bacterial culture was diluted to 100X with LB medium and was grown to OD600 = 0.4. For dsRNA induction, IPTG (50 µM) was added to concentration of 1mM and culture was incubated at 37 °C for about 5 hours on shacking. At the end solution was given heat shock at 80 °C for about 20 min and saved at -20 °C.

3.8.1 Identification of dsRNA produced in bacteria

Total RNA from bacteria was extracted to analyze the proper synthesis of dsRNA in bacteria, TRI reagent was used for RNA extraction. Extracted total RNA was treated with DNase-I to degrade the DNA. Prepared pellet was dissolved in double distilled water (50 µL) and concentration was checked by loading 4µg of extracted RNA on the 1% agarose TBE gel, ethidium bromide was used for staining of the gel. Volume of samples was the normalized accordingly.

3.9 Multiple Sequence Alignment and Phylogenetic Analysis

All the relevant gene sequences of targeted genes' family viz. CP (30 sequences), P450 (17 sequences) and GSTs (15 sequences) were retrieved from National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/Genbank/index.html), and were subjected to Clustal Omega to compute percent identity matrix. GSS sequences of CPB were not available on NCBI so closely related gene family was used to study percent identity matrix and phylogentic relationships. Sequences were aligned these sequences using MUSCLE (Edgar 2004) integrated in Mega 7. The alignment was used to construct a tree using the Neighbor-Joining method according to Saitou and Nei (1987) trees using Mega 7 to select the best-fitting model i.e. Maximum Composite Likelihood (Kumar et al. 2016). The reliability of tree topology was evaluated by bootstrap method using 1000 replicates.

3.10 dsRNA Feeding Bioassays of CPB Larvae

Effects of dsRNAs on survival, development duration and weight gain were studied under laboratory conditions with the lab resistant CPB population. All the dsRNA feeding bioassays were performed using the modified methodology of Baum et al. (2007) and Zhu et al. (2011).

3.10.1 Feeding bioassay on CPB larvae

Same aged larvae were collected from the lab resistant CPB population and pre-starved for 6 hours before initiation of feeding bioassay. All CPB larval instars i.e. 1^{st}, 2^{nd}, 3^{rd} and 4^{th} instar were used for this experiment. Three similar size fresh potato leaflets were selected and kept on top of filter paper in a 90 mm plastic Petri plate (VWR, USA). Each potato leaflet was treated with 500 µL of bacteria expressing dsRNA containing one of the targeted gene's fragment. After applying, dsRNA was spread on surface of leaflets by the help of glass spreader to equally distribute the dsRNA and the leaflets were left to dry under the laminar flow. Each time the glass spreader was cleaned with ethanol and rinsed with water to avoid any contamination. To avoid contamination, all the procedure of dsRNA application and drying was carried out in laminar flow (CHC Biolus, Korea). After drying in laminar flow, the larvae were shifted to each petri plate. The larvae were incubated in insect growth chamber at 28±1°C under a 16: 8 h light–dark photoperiod and 50-60% relative humidity. Each day, the larvae from one petri dish were gently transferred with soft forceps to a new petri dish containing fresh potato leaves treated with dsRNA. The 1^{st} and 2^{nd} instar larvae were fed on dsRNA treated leaves for 6 days. While, 3^{rd} and 4^{th} instar larvae were fed for 3 days. In the control each potato leaflets were treated with 500 µL of LB media and supplied to the control group larvae. The mortality was recorded after 3 and 6 days. Randomized block experimental design was used and 3 replications were performed on different days for each dsRNA. Total 30 larvae were used for each dsRNA and the control group.

3.11 Effect of dsRNA on Survival of Different CPB Larval Instars and Development Period

As result of feeding bioassays, the survival rate of different CPB larval instars (2^{nd}, 3^{rd} and 4^{th}); and larval and pupal duration were calculated until the emergence of adults from pupae. Methodology followed for this experiment was according to the group rearing method of life table (Chi and Liu, 1985; Chi, 1988). Due to higher mortality in the 1^{st} instar CPB larvae, this stage was not included in this experiment. In initial 3 days, the larvae were reared on dsRNA treated potato leaflets as described in 3.12.1. The treatment of potato leaflets and incubation of the larvae were performed as described in 3.12.1. After three days, the larvae were transferred into new Petri plates and fed with non-treated fresh potato leaflets in the insect growth chamber at 28±1°C under a 16: 8 h light–dark photoperiod and 50-60% relative humidity. In the control group, the larvae were fed with non-treated potato leaflets during whole experiment. Survival of 2^{nd}, 3^{rd} and 4^{th} instar was recorded until the emergence of adults from pupae. Duration of larval and pupal periods was also calculated. The larval period was calculated until all the larvae pupated in a treatment. Similarly, pupal duration was calculated according to the emergence of adults from pupae. Pupal period was recorded for 20 days and adults which were not able to emerge were considered as dead. While, less number of adults emerged from the treated 2^{nd} instar CPB larvae so pupal duration was not recorded for this stage. The experiment was set up in randomized block design and repeated on three different times. Total 30 larvae were used for each stage and each treatment.

3.12 Feeding Effects of dsRNA on CPB Larval Weight Gain

Effect of the dsRNA treatments on weight gain of CPB larvae (3^{rd} and 4^{th} instar) was tested under laboratory conditions. Due to higher mortality and less feeding at 1^{st} and 2^{nd} instar CPB larvae, these stages were not included in this experiment. Prior to the feeding of larvae with the dsRNA treated leaflets, the initial weight of each CPB larvae was measured with a sensitive balance (Model: ATX224; SHIMADZU). The larvae were transferred to the petri plates containing one of the dsRNA treated leaflets. The treatment of the potato leaflets and incubation of the insects were carried out as

described in 3.12.1. Weight of 3rd and 4th instar larvae was measured after 3 days of feeding (Sintim et al., 2009). Increase in weight was calculated by using formula:

$$WG = FW-IW \quad (3.1)$$

Where, WG is the weight gained; FW is the final weight; and IW is the initial weight

3.13 Synergistic Effect of dsRNA with Imidacloprid

The second instar larvae of equal size were collected from the lab resistant population and pre-starved for 6 hours before initiation of feeding bioassay. As described in 3.12.1 the equal-sized fresh leaflets of potato were selected and transferred on top of filter paper in a petri plate (90 mm). Each leaflet was treated with reduced dose of 300 µL of bacteria containing one of the dsRNA. The leaflets were left to dry under the laminar flow cabinet and then the larvae were shifted to the petri plates. The larvae were incubated at controlled conditions viz. 28±1°C temperature under a 16: 8 h light–dark photoperiod and 50-60% relative humidity and fresh leaflets treated with dsRNA were provided daily for 3 days. After feeding of dsRNA for 3 days, the dead larvae were recorded and discarded from the plates. The remaining larvae were topically treated with imidacloprid at 187.5 µg mL^{-1} dose with the help of micro-syringe attached to a hand micro-applicator as described in 3.4. The larvae were then transferred to the new petri plates containing the untreated fresh potato leaflets as food. The larvae were incubated at 28±1°C temperature under a 16: 8 h light–dark photoperiod and 50-60% relative humidity. There were two control groups (positive and negative control) in the experiment. In the positive control group, the insect were fed on non-treated potato leaflets for the initial period and then they were treated with imidacloprid at 187.5 µg mL^{-1} dose after 3 days. In the negative control, the larvae were fed on non-treated potato leaflets and also there was no imidacloprid application for this group. Mortality of larvae was recorded 24 h intervals until pupation. Randomized complete block experimental design was used and 3 replications were performed on three different days for each ds RNA and positive and negative control. Total 30 larvae were used for each group.

3.14 Real-Time Quantitative PCR (qRT-PCR)

It was hypothesized that mortality in targeted CPB larvae will occur due to downregulation of all the genes' mRNA levels after feeding on dsRNA-treated leaves. To check the hypothesis, mRNA levels in target insects' larvae was measured by quantitative real-time PCR (qRT-PCR). Three replications were performed for these experiments.

For this purpose, collection of larvae was done to perform the extraction of total RNA after feeding on the dsRNAs for designated time period for qRT-PCR analysis. Total RNA was extracted by TRIzol method as discussed in section 3.7.1. First stranded cDNA was made from the RNA (1µg) primed by oligo dT using MMLV reverse transcriptase. The primers for all genes were designed by using NCBI primer blast tool and given in Table 3.4. Furthermore, qRT-PCR was performed in 20 µl volume by using cDNA gene specific primers (0.5 µM), 10 µl of SYBR Green Master Mix (Biorad, USA) and water. In order to determine the best internal control gene that is going to be used for data normalization, amplification and melting curves of ribosomal protein gene (EB76117) of CPB were analyzed. For comparison of down-regulation in insects, gene expression in control insects was measured with the primers of each gene and was set at 1 as reference. Analysis of data was done using Real-Time PCR Detection System (Qiagen, Netherlands). Real time expression was calculated according to the equations given by Livak and Schmittgen (2001):

$$\text{Amount of target} = 2^{-\Delta\Delta CT} \quad (3.2)$$

$$\Delta\Delta CT = (CT,\text{Target} - CT,\text{housekeeping})_{\text{Time x}} - 2\,(CT,\text{Target} - CT,\text{housekeeping})_{\text{Time0}} \quad (3.3)$$

Table 3.4. List of primer for qRT-PCR to detect down-regulation in targeted insects

Oligo name	Scale (µmol)	Primer sequence (5´-3´)
CRT-F	0.05	AAAGTTGCCAGTCGTCCAGG
CRT-R	0.05	TGTCCGTCTGGTCCGATTTG
PRT-F	0.05	ATCAGGAGCCACGAAAAATCCT
PRT-R	0.05	CTGCCTTTGGTGTGAAAGTCG
GRT-F	0.05	AACAGTCTGGCGTCCCATTC
GRT-R	0.05	GTGATAGAGGCAGGATCAGCG
CPBRP-18F	0.05	TAGAATCCTCAAAGCAGGTGGCGA
CPBRP-18R	0.05	AGCTGGACCAAAGTGTTTCACTGC

3.15 Statistical Analysis

3.15.1 Life table analysis of susceptible and field CPB population

The life history raw data viz. developmental period of immature stages, survival, pre-oviposition, oviposition, post-oviposition period and adult longevity of both susceptible and field CPB populations were analyzed according to age-stage two-sex life table methods (Chi and Liu 1985; Chi 1988). The population parameters like development duration, TPOP, APOP, oviposition, and longevity were calculated according to the two-sex life table theory (Chi and Liu, 1985) in the computer program TWO-SEX-MS Chart (Chi, 2017). The standard errors and variances of the life table statistics were computed by the bootstrap technique (Efron and Tibshirani 1993). For precise and stable estimation, 100,000 bootstraps were used to compare the variances after running the computer program TWOSEX-MSChart (Chi 2017). Paired bootstrap was performed to compare the difference between developmental time of immature and mature stages, adult longevity, APOP, TPOP, oviposition days, and fecundity. Population parameters like r, λ, R_o and T were also compared using paired bootstrap test.

The age-stage-specific survival rate of CPB (S_{xj}, where x is the age in days while j represents the stage), age-specific survival rate (l_x), age-stage-specific fecundity (f_{xj}), age-specific fecundity (m_x), age-stage reproductive value (v_{xj}), age-stage life expectancy (e_{xj}), and other important life table parameters like net reproductive rate (R_0); intrinsic rate of increase (r); finite rate of increase (k); and the mean generation (T) were also

calculated. According to age-stage, two-sex life table theory the age-specific survival rate (l_x), age-stage-specific fecundity (f_{xj}), age-specific fecundity (m_x) and their product $l_x m_x$, were calculated as:

$$\sum_{x=0}^{\infty} e^{-r(x+1)} l_x m_x = 1 \tag{3.4}$$

$$lx = \sum_{j=1}^{k} S_{xj} \tag{3.5}$$

The parameter S_{xj} is the probability that a newborn will survive to age x and stage j. Where k is the number of stages of the study cohort.

The net reproductive rate (R_o) is defined as the average number of offspring that a female produces during her lifetime is called the net reproductive rate (R_0). It is the cumulative summation of $l_x m_x$ from birth to death.

$$R_0 = \sum_{x=0}^{\infty} l_x m_x \tag{3.6}$$

The T is the length of time that a population needs to increase to R_0-fold of population size (CPB) at stable age distribution:

$$T = (\text{In } R_0) / r \tag{3.7}$$

The exponent r is the difference between the birthrate (b) and the death-rate (d) in the population (r = b - d). In this study, the r was estimated using the iterative bisection method from the Euler–Lotka formula with age indexed from 0 (Goodman, 1982) as shown in Equation:

$$\sum_{x=0}^{\infty} e^{-r(x+1)} l_x m_x = 1 \tag{3.8}$$

The finite rate is calculated as*:*

$$\lambda = e^{r} \tag{3.9}$$

The e_{xj} is defined as the length of duration or time that an individual or insect of x and j is predictable to living. According to life table theory, individuals of the same age may have different life expectancies, hence it can be calculated by the method of (Chi and Su, 2006) as:

$$e_{xj} = \sum_{i=x}^{\infty} \sum_{y=j}^{k} s'_{iy} \tag{3.10}$$

Where S'_{iy} is defined as the probability that individuals of x and j will survive to age i and stage y, and is found by assuming S'_{iy} =1 (Tuan et al., 2014).

The v_{xj} is defined as different individuals have different "value" in terms of their contribution to future generations is called their reproductive value (Fisher 1930). It represents the share of an individual of age x and stage j towards the up-coming population or in other words it is the scale of population forecasting. The reproductive value (v_{xj}) and was calculated as:

$$v_{xj} = \frac{e^{-r(x+1)}}{S_{xj}} \sum_{i=x}^{\infty} e^{-r(x+1)} \sum_{y=j}^{k} s'_{iy} f_{iy} \tag{3.11}$$

3.15.2 Population projection

Survival rate and fecundity data of both the lab susceptible and field CPB populations were used to draw population projection curves using the TIMING-MSChart program for 120 days (Chi and Liu, 1985; Chi, 1990; Chi, 2017). The potato growth from sowing to harvest takes about 70-120 days, so that this projection was based on the longest growing period (Dam et al. 1996).

3.15.3 Analysis of variance of feeding bioassay, developmental time and weight change

The mortality data recorded for different stages of CPB fed on dsRNA were corrected with Abbott's formula (Abbott, 1925). The data were then subjected to arcsine transformation for normalization (Zar, 1999). The transformed data was analyzed with one-way analysis of variance (ANOVA) at the 5% significance level ($P \leq 0.05$) and then the Tukey multiple comparison test was used to find the difference between treatments ($P \leq 0.05$). Similarly, the data regarding survival rate for the 2^{nd}, 3^{rd} and 4^{th} instar larvae were first transformed to arcsine and then subjected to ANOVA at the 5% significance level. The difference between treatments were analyzed with the Tukey multiple comparison test ($P \leq 0.05$). The effect of dsRNA on larval and pupal duration time and weight gain was simply subjected to ANOVA without arcsine transformation. Means were compared by the Tukey multiple comparison test at $P \leq 0.05$. Paired t-test was used for the analysis of qRT-PCR results. All the data were analyzed using Statistix 8.1 (Analytical Software, 2005).

3.15.4 Analysis of dose-response bioassays

The dose-response data were corrected with Abbott's formula (Abbott 1925) before analysis. The probit analysis was used to estimate Lethal Dose 10 (LD_{10}), Lethal Dose 50 (LD_{50}) and Lethal Dose 90 (LD_{90}), their fiducial limits (FL), the slope and other related parameters. Pairwise comparison of LD_{50} values was carried out and if two treatments (at 95% FL) were not overlapping, they were taken as different at 5% significance level (Litchfield and Wilcoxon 1949). Resistance ratios were calculated according to formula described by Roush and Tabashnik (1991). Resistance ratio was calculated as:

$$\text{Resistance Ratio} = LD_{50} \text{ resistant strain}/LD_{50} \text{ susceptible strain} \quad (3.12)$$

The dose response data were analyzed with POLO-PLUS® software program (Leora software, 2003).

CHAPTER IV

RESULTS

4.1 Life table Analysis of Lab Susceptible Population

The developmental duration for pre-adult stages and longevity of adult females and males are given in Table 4.1. Egg hatching duration viz. 4.11±0.01 and 4.15±0.01 days in the lab susceptible and field population was similar to each other. Duration of 1st instar was significantly longer in the lab susceptible population i.e. 2.22±0.06 days than 2.00±0.02 days in the field population. Similarly, a longer duration of 2nd instar (1.63±0.06 days) was calculated in the lab susceptible population than the duration for the field population (1.40±0.07 days). Significantly more time was required by the lab susceptible population i.e. 2.56±0.08 days in 3rd instar than the 2.00±0.09 days by CPB larvae of the field population. However, the 4th instar of both populations was similar regarding duration. A longer pupal duration (11.07±0.25 days) was recorded in pupal stage for the lab susceptible population compared to the field population (9.83±0.07 days). The adult pre-ovipositional periods (APOP) viz. 6.58±0.26 days in the lab susceptible population was significantly longer than the field population. Similarly, the total pre-ovipositional period (TPOP) in the lab susceptible population was 31.25±0.30 days. Adult longevities of both male (49.58±2.93 days) and female (52.94±2.36 days) were significantly shorter in the lab susceptible population than the field population. The total fecundity in the lab susceptible population was 104.00±20.01 eggs per female which was significantly lower than the field population as it was 184.14±34.90 eggs. While, the oviposition period (4.00±0.41 days) in the lab susceptible population were also significantly less than the field population (5.90±0.89 days).

Table 4.1. Different biological parameters of the lab susceptible and field CPB populations

Parameters	CPB population	
	Lab susceptible	Field
Egg hatching (days)	4.11±0.01	4.15±0.01
1st instar (days)	2.22±0.06*	2.00±0.02
2nd instar (days)	1.63±0.06*	1.40±0.07
3rd instar (days)	2.56±0.08*	2.00±0.09
4th instar (days)	3.47±0.09	3.77±0.19
Pupal duration (days)	11.07±0.25*	9.83±0.07
Male adult longevity (days)	49.58±2.93	71.58±2.36*
Female adult longevity (days)	52.94± 0.23	69.32±2.93*
APOP/APRP (days)	6.58±0.26*	5.10±0.39
TPOP (days)	31.25±0.20*	27.75±0.36
Oviposition (days)	4.00±0.41	5.90±0.89
Fecundity (total eggs/female)	104.00±20.01	184.14±34.90*

Note: Standard errors were analyzed using 100,000 bootstraps replicates. Means followed by different letters in the same row indicate significantly different values

4.1.1 Population parameters

Population parameters calculated for the lab susceptible and field population are presented in Table 4.2. The values for the intrinsic rates of increase (r), finite rates of increase (λ) and net reproductive rates (R_0) are important in assessment of population projection of any insect population. The r, λ and R_0 values calculated for the lab susceptible population were 0.10±0.01 day^{-1}, 1.10±0.01 day^{-1} and 38.43±10.31 offsprings/female respectively which were significantly lower than the field population for which the intrinsic rates of increase, finite rates of increase and net reproductive rate were 0.12±0.01 day^{-1}, 1.13±0.01 day^{-1} and 71.07±17.79 offsprings/female respectively. However, the mean generation time (T) was 36.61±0.42 days and it was significantly longer than the field population (34.00±0.93 days).

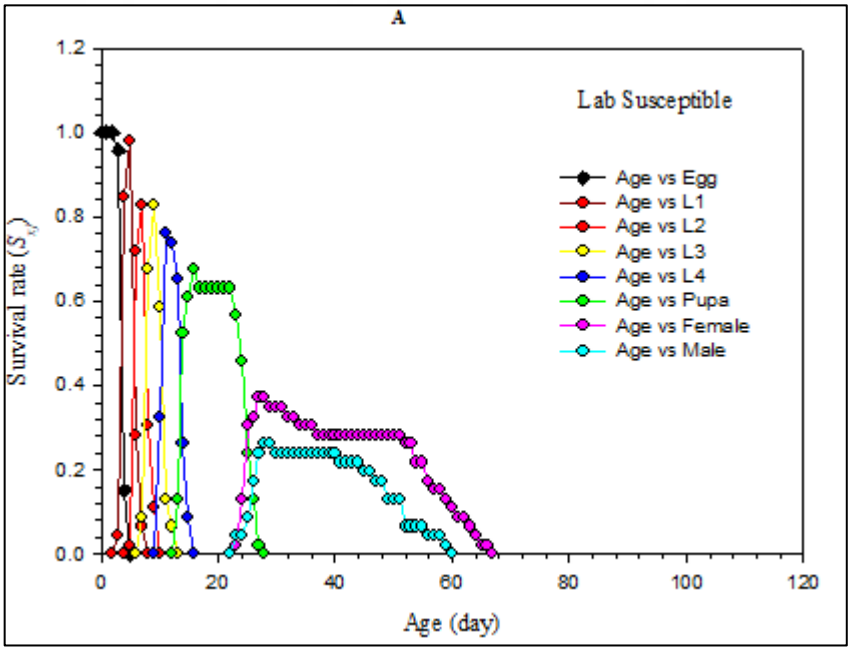
Table 4.2. The intrinsic rates of increase (r), finite rates of increase (λ), net reproductive rates (R_0) and mean generation time (T) of the lab susceptible and the field population of CPB

Parameters	CPB Population	
	Lab Susceptible	Field
r (per day)	0.10±0.01	0.12±0.01*
λ (per day)	1.10±0.01	1.13±0.01*
R_0	38.43±10.31	71.07±17.79*
T (days)	36.61±0.42*	34.00±0.93

Note: Standard errors were analyzed using 100,000 bootstraps replicates. Means followed by different letters in the same row are significantly different

4.1.2 Age-stage survival rates

Calculated age-stage survival rates (S_{xj}) of the lab susceptible and field resistant population are given in Figure 4.1 (A and B). Overlapping in S_{xj} curves indicates the variation in developmental rate between different individuals. A higher survival rate was observed in the field population than the lab susceptible population.



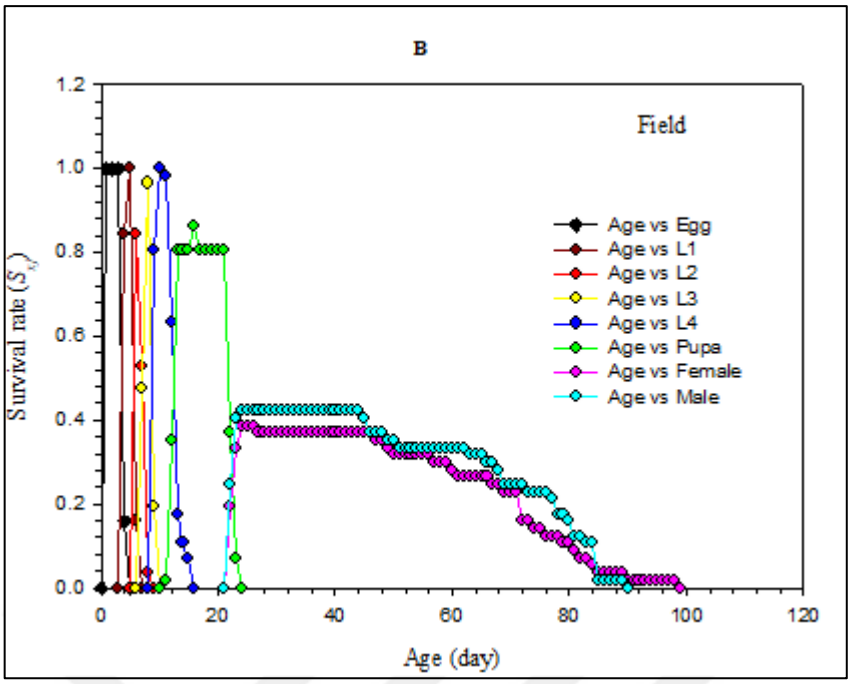


Figure 4.1. Age-stage-specific survival rate (S_{xj}) of the lab susceptible (A) and field (B) CPB populations. L1= 1st instar, L2= 2nd instar, L3= 3rd instar, L4= 4th instar

4.1.3 Age-specific maternity ($l_x m_x$)

Age-specific survival rate (l_x), age-specific fecundity of whole population (m_x) and their product viz. age specific maternity ($l_x m_x$) in two different CPB populations are presented in Figure 4.2 (A and B). Age-stage specific fecundity (f_x) approached the peak value of 22.62 on day 29, which was higher than the value (22.43) calculated for the lab susceptible population on day 34. These graphs show that age-specific survival rate (l_x) decreased more rapidly in the lab susceptible population than the field population. However, the peak in the lab susceptible CPB population declined earlier than the field CPB population. The age-specific fecundity (m_x) started at 25th day and it ended at 61st day in the field population while in the lab susceptible population it started on day 29 while ended on day 49. The peak value of the field population (10.56 offsprings/day) was observed on day 29, while the peak value in the lab susceptible population was 12.56 offsprings/day on day 34. For age-specific maternity ($l_x m_x$) the highest peak (8.33 offsprings/day) was observed on day 29 regarding in the field population while the peak value in the lab susceptible population was only 6.83 offsprings/day on day 34.

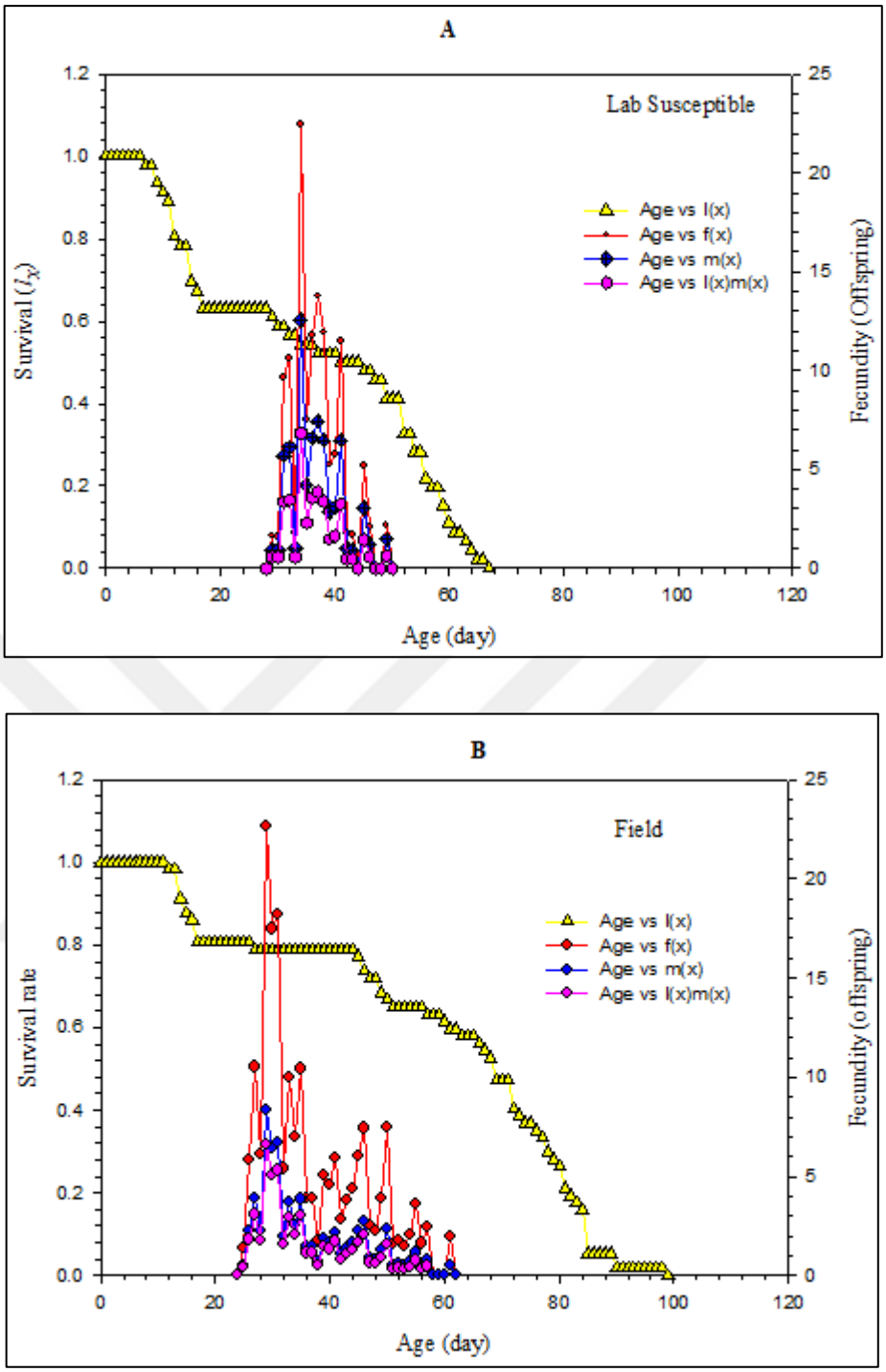


Figure 4.2. Age-specific survival rate (l_x), female age-specific fecundity (f_x), age-specific fecundity (m_x), and age-specific maternity ($l_x m_x$) of the lab susceptible (A) and the field (B) CPB populations. L1= 1st instar, L2= 2nd instar, L3= 3rd instar, L4= 4th instar

4.1.4 Age-stage life expectancy

The age-stage life expectancy (e_{xj}), varied among the two tested populations. The difference in the age-stage-specific life expectancy of two different CPB populations is

shown in Figure 4.3 (A and B). The life expectancy from age zero (e_o) was maximum 66 days in the lab susceptible population, which was less than the field population (98 days). Where, life expectancy of males in susceptible population was maximum 59, in the field population male life expectancy was maximum 89 days. Similarly, for females in the lab susceptible population life expectancy was maximum 66 days and 98 days for field population.

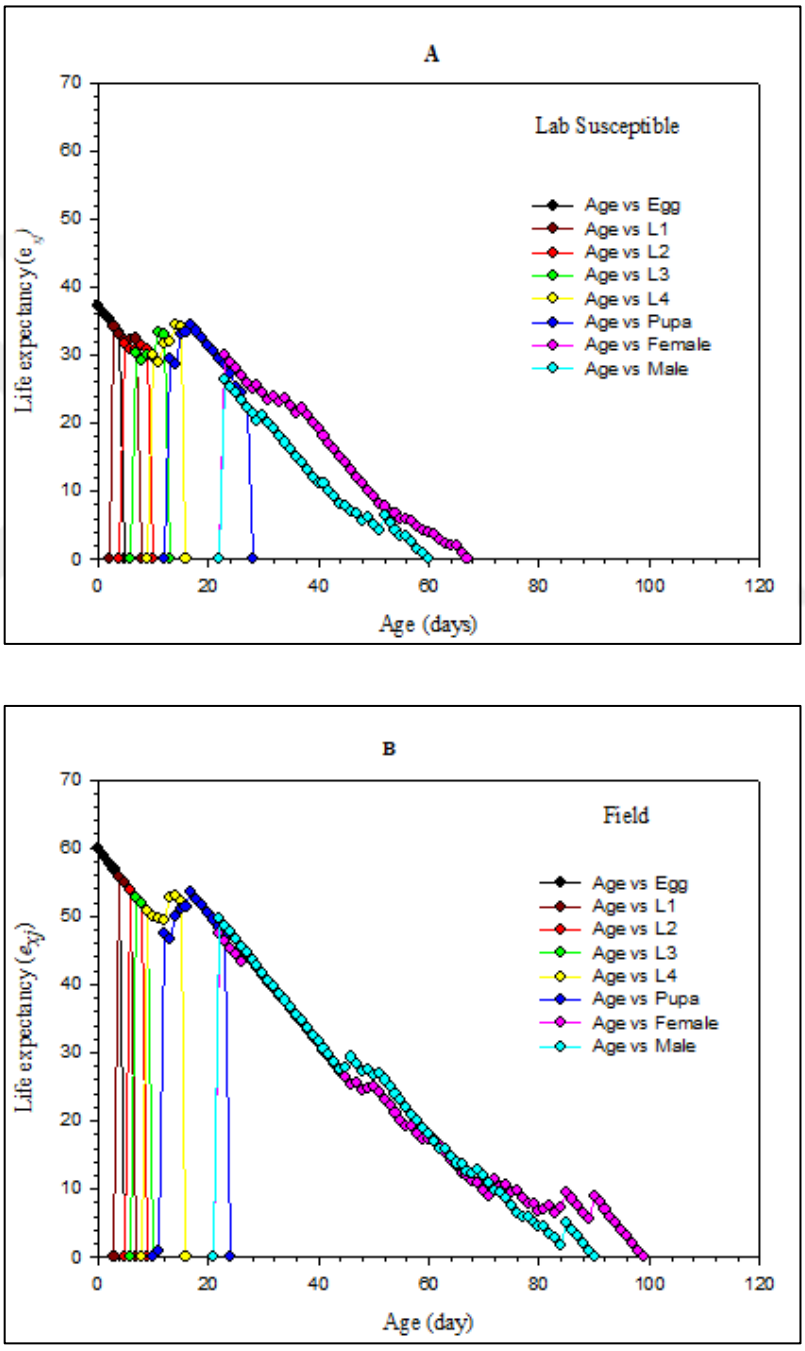


Figure 4.3. Age-stage-specific life expectancy (e_{xj}) of the lab susceptible (A) and the field (B) CPB population. L1= 1st instar, L2= 2nd instar, L3= 3rd instar, L4= 4th instar

4.1.5 Age-stage reproductive value

Age-stage reproductive values (v_{xj}) for the both populations are shown in the Figure 4.4 (A and B). These curves show that age-stage reproductive value increased with the start of reproduction and reached to a peak 80.96 value offspring/day at 30^{th} day and then it started to decline gradually after 62 days in field population. Similarly, in the lab susceptible population the peak value was 70.99 offsprings/day at 34^{th} day and decreased to 0 offsprings/day at 50^{th} day.

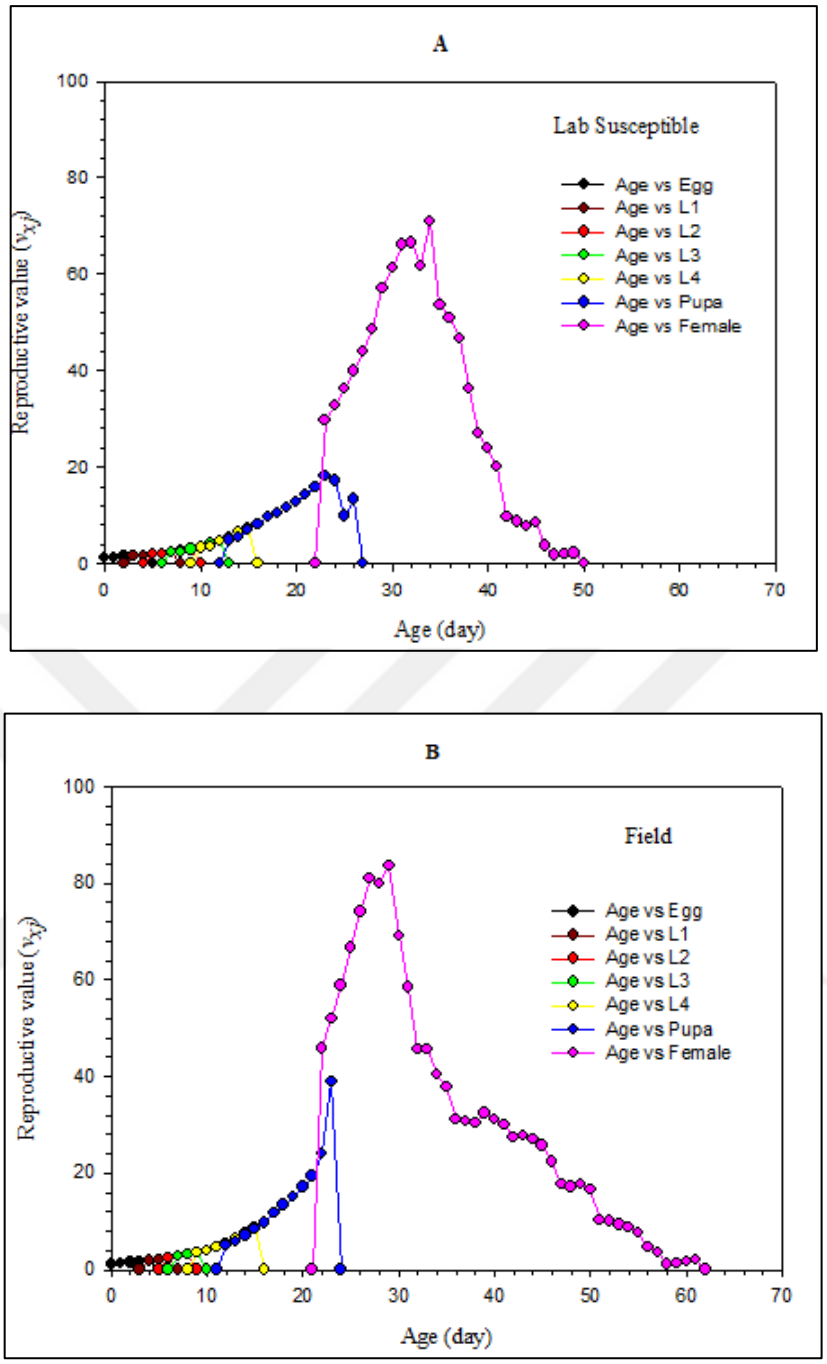


Figure 4.4. Age-stage reproductive value (v_{xj}) of the lab susceptible (A) and field (B) CPB populations. L1= 1st instar, L2= 2nd instar, L3= 3rd instar, L4= 4th instar

4.1.6 Projection results

The initial population size to calculate the population projection was 10 eggs for each population, and the projection was based on population parameters like mean generation time, fecundity, intrinsic rate, survival rate etc. The population projection figures of the lab susceptible and the field populations are presented Figure 4.5 (A and B). The CPB

population of field reached to 8036143 individuals including all stages after 120 days, while number of individuals in the lab susceptible population was 437489. This result shows that the number of individual in the field population can be 18.36 fold higher than that of the lab susceptible population just after 120 days.

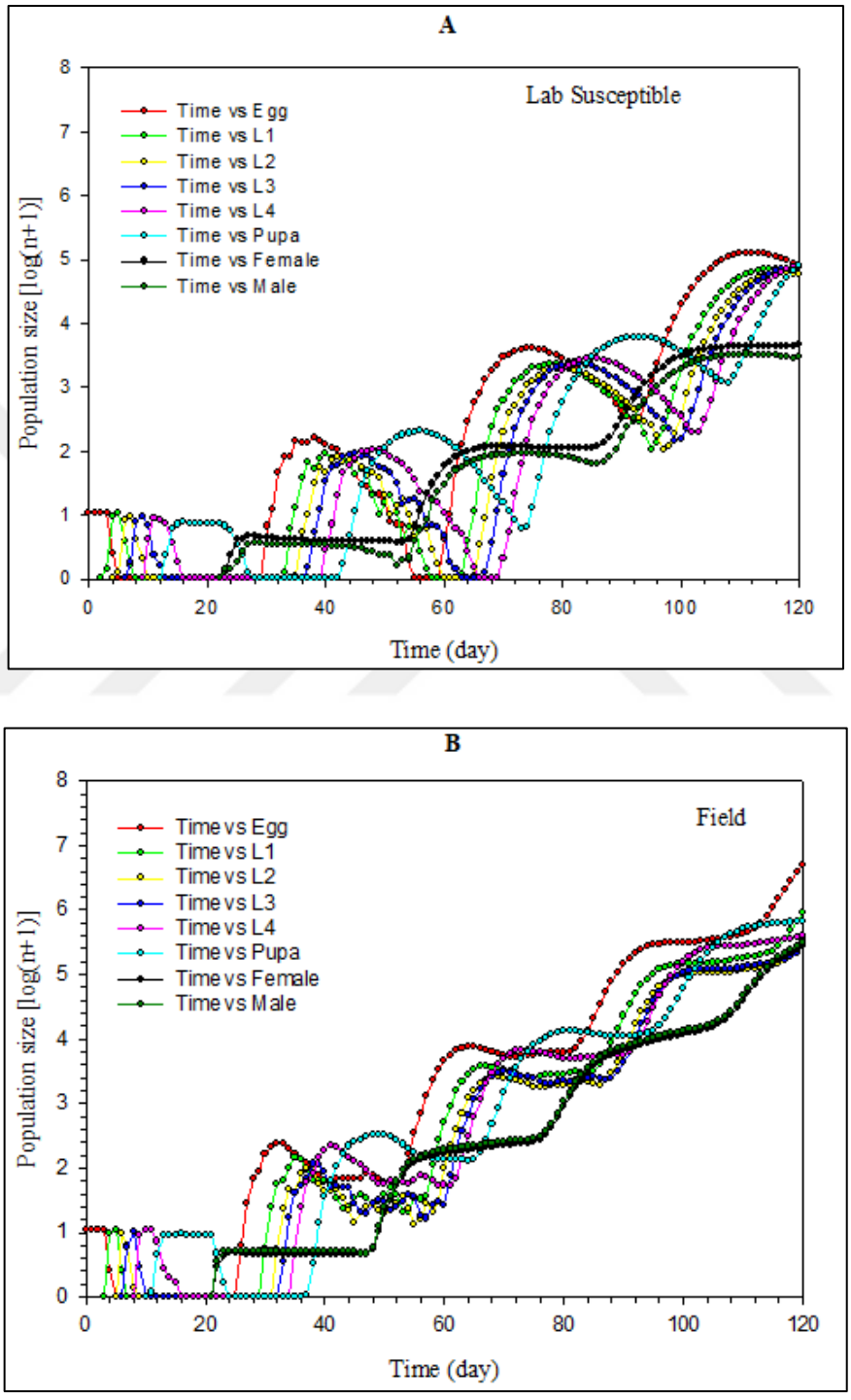


Figure 4.5. Population projection showing the change in population of the lab susceptible (A) and the field (B) CPB populations after 120 days. L1= 1st instar, L2= 2nd instar, L3= 3rd instar, L4= 4th instar

4.2 Dose-Response Analysis of Three Different CPB Populations

The dose response bio-assays results for three different CPB populations are shown in Table 4.3. The LD_{10} value for the lab susceptible population was significantly lower 36.28 µg mL^{-1} (23.62-47.80 µg mL^{-1}) than the LD_{10} values of the field population and the lab resistant CPB population. The LD_{10} value for the field population was 215.38 µg mL^{-1} (102.45-309.33 µg mL^{-1}), which was between LD_{10} values of the lab susceptible and the lab resistant CPB populations. The LD_{10} value for the lab resistant population was 535.08 µg mL^{-1} (280.21-774.95 µg mL^{-1}) and it was significantly higher than the lab susceptible population. The LD_{50} value for the field population was 804.16 µg mL^{-1} (642.60-1083.93 µg mL^{-1}), which was between LD_{50} values of the lab susceptible 102.52 µg mL^{-1} (83.06-128.86 µg mL^{-1}) and the lab resistant CPB populations in which the LD_{50} was 2628.51 µg mL^{-1} (1903.50-4340.26 µg mL^{-1}) and it was significantly higher than the LD50 calculated for other two populations. The LD_{90} value for the lab susceptible population was significantly lower 289.72 µg mL^{-1} (212.20- 478.05 µg mL^{-1}) than the LD_{90} values of the field and the lab resistant CPB population. The LD_{90} value for the field population was 3002.5 µg mL^{-1} (1883.1-8129.7 µg mL^{-1}), which laid between LD_{90} values of the lab resistant and lab susceptible CPB populations. However, significantly higher LD_{90} value was observed in case of the lab resistant population viz. 12912.00 µg mL^{-1} (6832.10-46006.00 µg mL^{-1}). Slope was more steep with 2.94±0.39 value in the lab susceptible CPB population than the other two tested CPB populations. The slope was less steep in the field population than the lab-susceptible population i.e. 2.24±0.43, laying between the lab susceptible and the lab resistant CPB populations. The calculated slope value in the lab resistant population was 1.85±0.33 and that is the shallowest among the tested population. The calculated resistance ratio for the lab resistant population was 25.64 folds greater than the lab susceptible population while for the field population it was 7.84 folds greater than the lab susceptible population.

Table 4.3. Dose-response results for 2nd instar Larvae of 3 different CPB colonies treated with imidacloprid after 72 hours

Population	n	LD_{10} (95% FL) (µg mL^{-1})	LD_{50} (95% FL) (µg mL^{-1})	LD_{90} (95% FL) (µg mL^{-1})	Slope (±SE)	χ^2	DF	RR* (folds)
Lab-Susceptible	180	36.28 (23.62-47.80)	102.52 (83.06-128.86)	289.72 (212.20-478.05)	2.94(±0.39)	10.19	13	-
The Field	180	215.38 (102.45-309.33)	804.16 (642.60-1083.93)	3002.50 (1883.1-8129.7)	2.24(±0.43)	2.65	13	7.84
Lab resistant	180	535.08 (280.21-774.95)	2628.51 (1903.50-4340.26)	12912.00 (6832.10-46006.00)	1.85(±0.33)	7.00	13	25.64

**RR* Resistance ratio, calculated as LD_{50} of resistant population (field/lab)/LD_{50} of susceptible population

4.3 . dsRNA Preparation

4.3.1 Target genes

All three targeted gene fragments selected to test feeding RNAi in CPB were important in imidacloprid resistance. Cuticular protein (CP) (Accession number: GEEF01064138) participates in penetration resistance against a number of insecticides, whereas cytochrome P450 monoxygenases (P450) (Accession number: GEEF01131148) belongs to a group of detoxification enzymes, Glutathione synthetase (GSS) (Accession number: GEEF01119768) is enzyme playing role in detoxification and phase II reactions. CPB larvae were used for the extraction of total RNA followed by conversion to cDNA. This cDNA was further used to amplify the fragments (portions) of 3 targeted genes (CP: 393 bp; P450: 306 bp and GSS: 407 bp). A gradient PCR on 4 different temperatures was conducted which revealed that 60 °C was the optimum temperature for amplification of fragments of 3 targeted genes. Therefore, another PCR was carried out to amplify fragments of targeted genes (Figure 4.6). Gel elution of the amplified sequences was carried out via Thermo Scientific Gene JET Gel Extraction Kit (Cat. No. K0692). Quality of eluted genes was measured by running them on 1% gel. Eluted fragments were further used for cloning purpose.

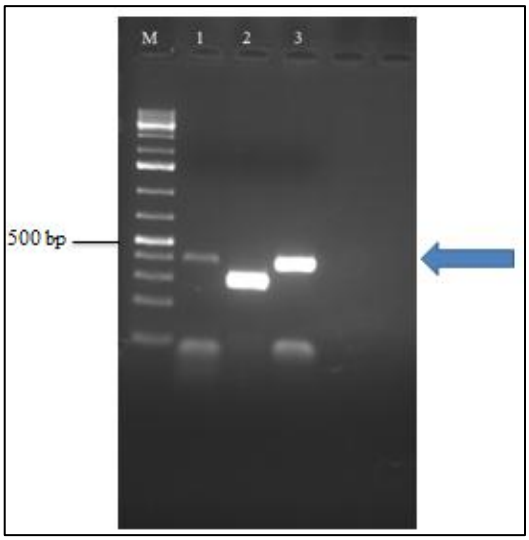


Figure 4.6. PCR assay to amplify gene fragments (portions) of cuticular protein, P450 monoxygenases and GSS from cDNA
Lane 1: 1 Kb plus DNA ladder (Thermo scientific); lane 2: CP gene fragment: 393 bp; lane 3: P450 gene fragment (306 bp); lane 4: GSS gene fragment (407 bp)

4.3.2 . Digestion

For proper cloning of desired genes with *L4440* plasmid, digestion was required. PCR products of gene fragment (CP, P450 and G) amplified by gene specific primers were digested using *Kpn*I and *Bgl*II according to the concentration of genes. The original vector was also digested with the *Kpn*I and *Bgl*II restriction enzymes for the cloning of fragments of targeted genes. Gel eluted amplified fragments were used and ligation was preceded

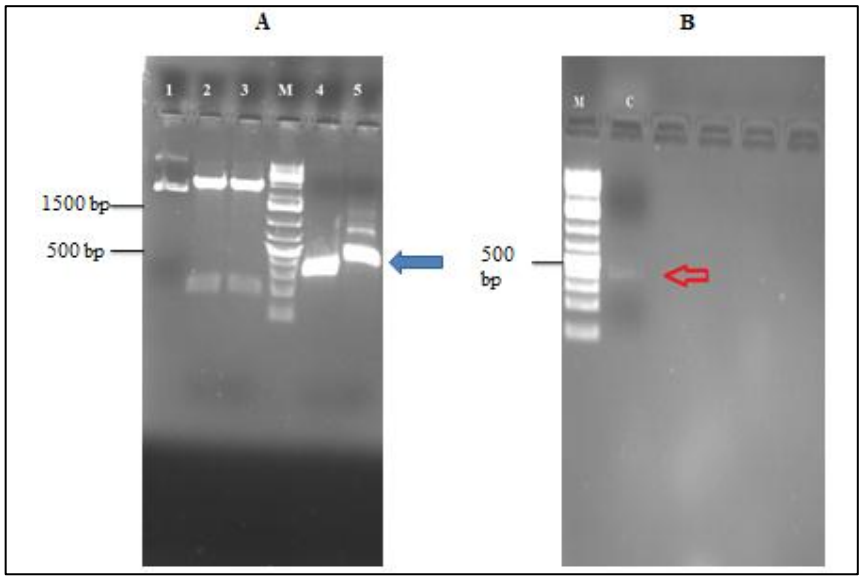


Figure 4.7. Results of restriction of genes and plasmid (A and B)
Left image is showing undigested plasmid in the first lane while next two lanes are showing digested plasmids followed by 1 Kb plus DNA ladder (Thermo scientific) and P450 (306 bp) and GSS gene fragments (407 bp). Right image is showing 1 Kb plus DNA ladder (Thermo scientific) in first lane while digested C fragment (393 bp) in the 2nd lane

4.3.3 Ligation and bacterial transformation

Prior to ligation, concentration of all gene fragments and vector were determined. For ligation, the vector to insert ratio was taken as 1:1. After successful ligation, the *E. coli* strain *DH5α* was used for ligated plasmid transformations. Four µL of ligation product was used for transformation. After transformation, media plates were kept at 37 °C for 24 hours and a colony PCR was conducted using gene specific primers to obtain the desired gene fragments (CP: 393 bp; P450: 306 bp and GSS: 407 bp) (Figure 4.8, 4.9 and 4.10). Positive colonies (three colonies for CP gene; three colonies for for GSS; two colonies for P450) were further inserted in LB media with antibiotic. Plasmid was extracted from the positive colonies and was used for further transformation into *HT115* strain.

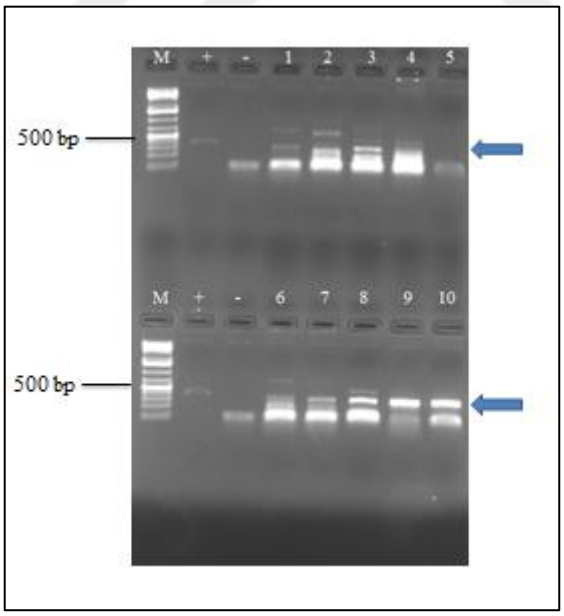


Figure 4.8. Amplification of the P450 fragments (306 bp) via colony PCR
Lane 1: 1 Kb plus DNA ladder (Thermo scientific); lane 2: positive (Gene fragment); lane 3: negative (No template); lane 4-8: P450 gene fragment (10 colonies)

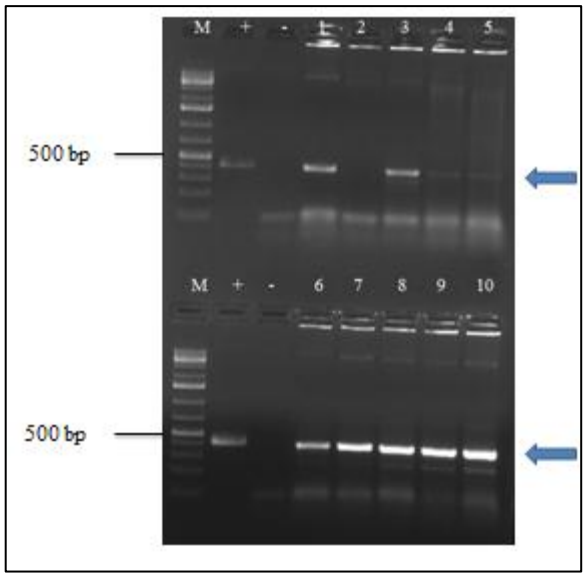


Figure 4.9. Amplification of the CP gene fragments (393 bp) via colony PCR
Lane 1: 1 Kb plus DNA ladder (Thermo scientific); lane 2: positive (Gene fragment); lane 3: negative (No template); lane 3-8 and 6 to 10: CP gene fragment (10 colonies)

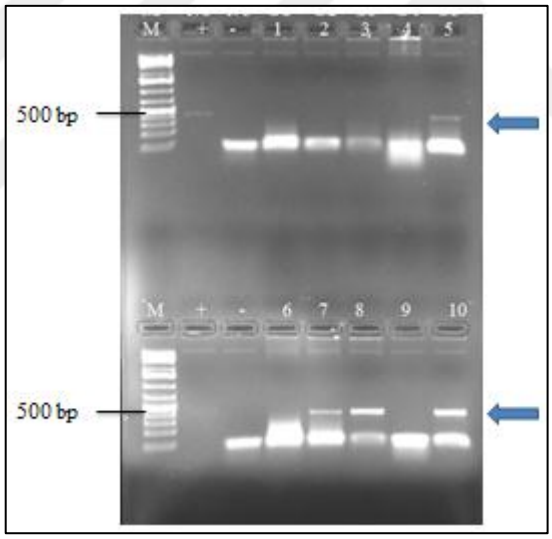


Figure 4.10. Amplification of the GSS fragments (407 bp) via colony PCR
Lane 1: 1 Kb plus DNA ladder (Thermo scientific); lane 2-5 and 6-10: GSS gene fragment (5 colonies); lane 6: positive (Gene fragment); lane 8: Negative (No template DNA)

4.3.4 Confirmation of dsRNA synthesis in bacteria

Positive colonies grown in *DH5α* were used as a source of ligated plasmids. The plasmids with inserts of fragments of desired genes were extracted and they were transformed into competent *HT115* (*DE3*) cells. About 2 µL of extracted plasmid was used for transformation. After transformation, bacteria were kept at 37 °C for 24 hours

and then colony PCR was conducted using *L4440* vector primers to obtain the desired gene fragments (CP: 493 bp; P450: 406 bp and GSS: 507 bp). Successful transformations are shown in Fig. 4.11, 4.12 and 4.13. Positive colonies (2 colonies for CP gene; 2 colonies for GSS from lower half; and 2 colonies for P450) were further used for dsRNA synthesis. The dsRNA of targeted genes was induced by adding IPTG to the bacterial growth medium in order to produce dsRNA. The dsRNAs of all target genes were successfully synthesized in the bacteria.

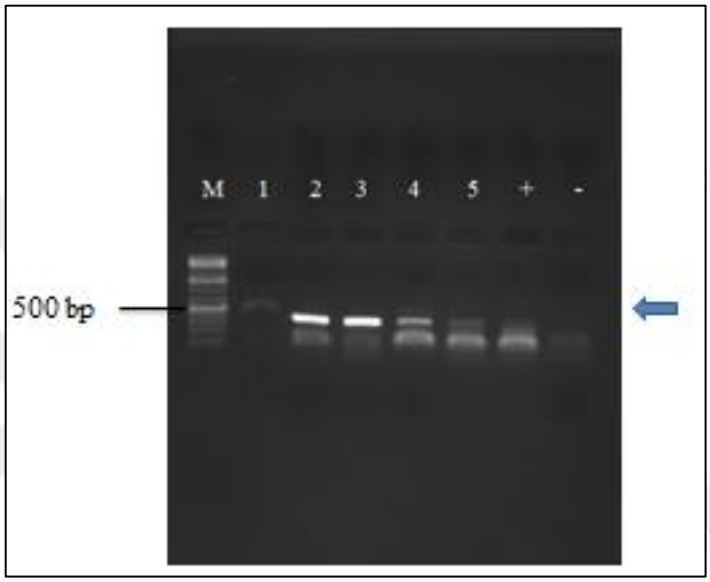


Figure 4.11. Amplification of the GSS fragments (407 bp) via colony PCR
Lane 1: 1 kb plus DNA ladder (Thermo scientific); lane 2-6: GSS gene fragment (10 colonies); lane 7: negative (No colony); lane 8: positive (empty vector)

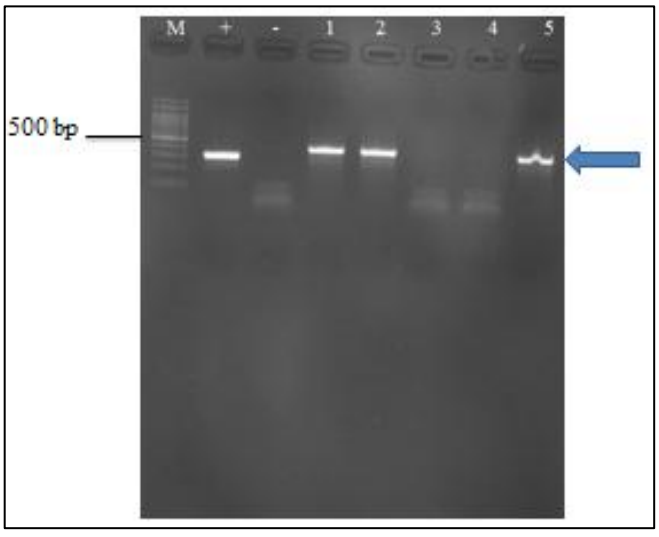


Figure 4.12. Amplification of the P450 gene fragments via colony PCR

Lane 1: 100 bp plus DNA ladder (Thermo scientific); lane 2: positive (empty vector); lane 3: negative (no colony); lane 4-8: P450 gene (5 colonies)

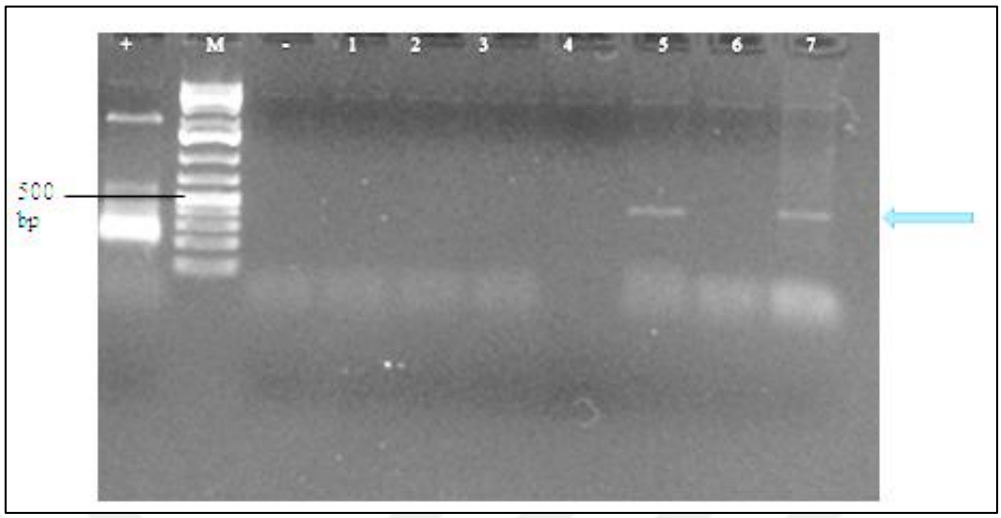


Figure 4.13. Amplification of the cuticular protein gene fragments via colony PCR Lane 3: 1 Kb plus DNA ladder (Thermo scientific); lane 1: Positive (Empty vector); lane 4: negative (No colony); lane 5-11: CP gene fragment (8 colonies)

4.3.5 Restriction analysis of clones

The recombinant *L4440* vectors with CP, P450 and GSS gene fragments were confirmed by restriction analysis for confirmation of clones. Respective recombinant plasmids with CP, P450 and GSS gene fragments were digested using *Kpn*I and *Bgl*II. The expected sizes for gene fragment were 306, 393 and 407 bp for P450, CP and GSS, respectively (Figure 4.14 A, B and C).

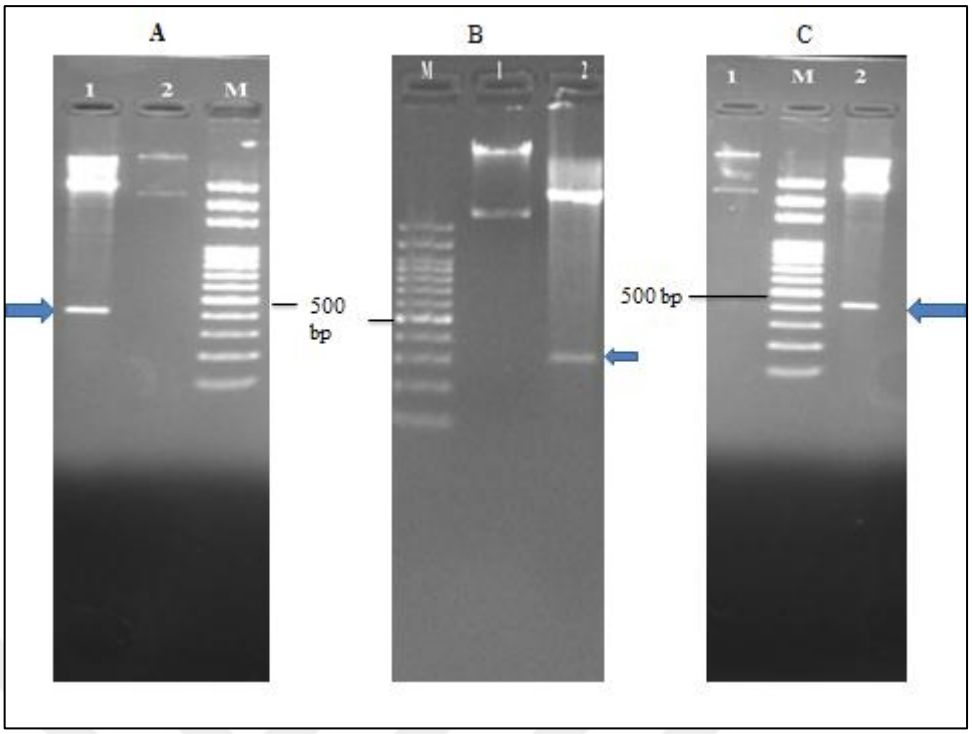


Figure 4.14. Restriction analysis of all three genes
A is showing restriction analysis of CP gene fragment in lane 1, undigested plasmid in lane 2 while 100 bp DNA ladder (Solis BioDyne) in lane 3; B is showing 100 bp plus DNA ladder (Thermo Scientific) in lane 1; undigested plasmid in lane 2 and digested P450 gene fragment in lane 3; C is showing undigested plasmid in lane 1, 100 bp DNA ladder (Solis BioDyne) in lane 2 and digested GSS gene fragment in lane 3

4.3.6 Quantification of dsRNA

Total RNA from bacteria was extracted to analyze the level of synthesis of dsRNA in bacteria. TRI reagent was used for extraction of total RNA. Extracted RNA was treated with DNase-I to degrade the DNA. Prepared pellet was dissolved in double distilled water (ddH_2O) and concentration was checked by loading 4µg of extracted RNA on the 1% agarose TBE gel. Expected size of CP and GSS genes were 421 and 435 bp, while the expected size of P450 gene was 334 bp (Figure 4.15 A and B).

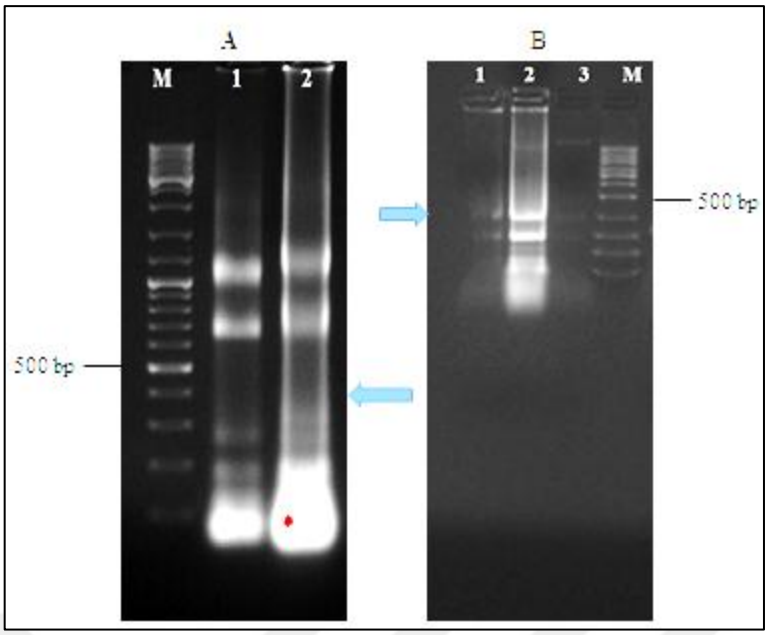


Figure 4.15. (A) shows the dsRNA of P450 gene fragment in lane 1 and 2, while 100 bp plus DNA ladder (Thermo Scientific) while right picture is showing dsRNA of GSS in lane 1, CP in lane 2 and 3 while 500 bp plus DNA ladder (Thermo Scientific) in lane 3

4.4 Percent Identity Matrix

4.4.1 Percent identity matrix of GST family

For percent identity matrix, sequence of our targeted gene (GSS) and members of GST family were retrieved from NCBI. There were no sequences available on GSS but taking in consideration the closeness of GSS and GSTs as they belong to the same superfamily of genes (Munyampundu et al. 2016), similarity matrix and phylogentic tree was constructed on the comparison of our target gene with GST family. In total, 15 sequences were subjected to analysis on Clustal Omega.

The Table 4.4. shows the percent identity matrix of our gene GSS viz. GEEF01119768.1 to other related genes of GST family viz. KU522334.1, KU522333.1, KU522308.1, KU522307.1, KU522311.1, KU522319.1, EF026111.1, EF026112.1, KU522313.1, KU522315.1, KU522314.1, KU522326.1, KU522331.1 and KU522327.1 gene sequences.

Percent identity of GEEF01119768.1 with GST genes varied from 33.17%-43.04%. Greater percent identity of GEEF01119768.1 was found with KU522327.1 (43.04%), KU522331.1 (40.80%) and KU522334.1 (39.95%).

Lowest percent identity of GEEF01119768.1 was observed with KU522315.1 (33.17%), KU522314.1 (34.38%) and EF026112.1 (34.60%). Less percent identity of our targeted gene (GSS) with other genes confirms that dsRNA was designed only for this gene.

Table 4.4. Percent identity matrix of GST related genes with our targeted gene

Gene	GST1	GST2	GST3	GST4	GST5	GST6	GST7	GST8	GST9	GST 10	GST 11	GSS*	GST 13	GST 14	GST 15
GST 1	100	59.53	36.74	37.15	41.56	32.72	33.85	33.33	39.58	38.12	38.25	39.95	32.79	25	-
GST 2	59.53	100	39.55	38.23	41.94	37.76	37.96	37.52	41.21	40.28	39.04	37.76	32.44	31.76	32.47
GST 3	36.74	39.55	100	54.15	43.43	46.68	47.9	46.38	48.56	46.95	44.34	35.44	34.28	35.37	39.68
GST 4	37.15	38.23	54.15	100	43.81	48.37	48.83	47.63	45.54	47.19	47.38	34.73	32.18	25.53	-
GST 5	41.56	41.94	43.43	43.81	100	41.85	41.9	40.64	48.26	50.47	50.14	38.13	35.44	35.33	36.09
GST 6	32.72	37.76	46.68	48.37	41.85	100	95.98	96.71	49.13	48.06	48.33	35.5	33.33	39.71	-
GST 7	33.85	37.96	47.9	48.83	41.9	95.98	100	98.61	48.97	48.91	50.16	34.86	25	35.42	-
GST 8	33.33	37.52	46.38	47.63	40.64	96.71	98.61	100	47.48	47.57	48.3	34.6	25.56	38	-
GST 9	39.58	41.21	48.56	45.54	48.26	49.13	48.97	47.48	100	65.52	66.58	36.75	35.19	26.Eyl	-
GST 10	38.12	40.28	46.95	47.19	50.47	48.06	48.91	47.57	65.52	100	73.35	33.17	29.97	33.33	29.31
GST 11	38.25	39.04	44.34	47.38	50.14	48.33	50.16	48.3	66.58	73.35	100	34.38	23.91	31.37	-
GSS*	39.95	37.76	35.44	34.73	38.13	35.5	34.86	34.6	36.75	33.17	34.38	100	37.93	40.8	43.04
GST13	32.79	32.44	34.28	32.18	35.44	33.33	25	25.56	35.19	29.97	23.91	37.93	100	50.35	54.78
GST14	25	31.76	35.37	25.53	35.33	39.71	35.42	38	26.Eyl	33.33	31.37	40.8	50.35	100	59.51
GST15	-	32.47	39.68	-	36.09	-	-	-	-	29.31	-	43.04	54.78	59.51	100

GST1: KU522334.1, GST2: KU522333.1, GST3: KU522308.1, GST 4: KU522307.1, GST 5: KU522311.1, GST6: KU522319.1, GST7: EF026111.1, GST8: EF026112.1, GST9: KU522313.1, GST10: KU522315.1, GST11: KU522314.1, GSS*: GEEF01119768.1, GST13: KU522326.1, GST14: KU522331.1, GST15: KU522327.1; Symbol (-) represents no similarity

4.4.2 Percent identity matrix of CP family

Similarity of our targeted gene with other genes of CP family was calculated using Clustal Omega. For this purpose, total of 31 sequences were retrieved from NCBI and analyzed.

The percent identity matrix among our targeted gene viz. GEEF01064138.1 and other related CP genes viz. EU194559.1, EU194558.1, EU194557.1, EU194556.1, EU194555.1, MG601708.1, MG601706.1, MG601705.1, MG601704.1, MG601703.1, MG601702.1, MG601701.1, MG601698.1, MG601697.1, MG601696.1, MG601695.1, MG601694.1, MG601693.1, MG601692.1, MG601691.1, MG601689.1, MG601687.1, MG601686.1, MG601684.1, MG601683.1, MG601680.1, MG601679.1, MG601678.1, MG601675.1, and MG601647.1 was calculated (Table 4.5 and 4.6).

Percent identity matrix of our targeted CP gene viz. GEEF01119768.1 with other members of CP family varied from 0.00%-44.07%. Our targeted gene GEEF01119768.1 shared greater identity of 44.07%, 43.97% and 43.02% with MG601702.1, MG601689.1 and MG601691.1, respectively.

Our targeted gene shared lower percent identity with MG601678.1 (0.00%), MG601686.1 (16.67%) and MG601679.1 (16.67%). Due to less percent identity of our targeted gene (CP) with other members of this gene family has confirmed the specificity of our designed dsRNA.

Table 4.5. Percent identity matrix of CP related genes with our targeted gene

Gene	CP1	CP2	CP*	CP4	CP5	CP6	CP7	CP8	CP9	CP10	CP11	CP12	CP13	CP14	CP15	CP16
CP 1	100	39.08	**31.Mar**	32.23	33.99	35.99	38.03	39.37	35.81	39.85	31.11	37.65	45.16	41.03	36.89	-
CP 2	39.08	100	**37.21**	32.19	37.01	41.78	41.08	39.39	37.45	37.85	23.91	22.41	37.25	34.48	23.53	-
CP*	31.Mar	37.21	**100**	37.06	40.9	43.97	42.3	41.95	43.02	36.88	21.82	16.67	16.67	26.32	28.57	-
CP 4	32.23	32.19	**37.06**	100	45.26	43.99	42.63	43.18	44.14	31.58	-	-	-	-	-	-
CP 5	33.99	37.01	**40.9**	45.26	100	46.37	45.78	47.93	50	31.38	-	-	-	-	-	-
CP 6	35.99	41.78	**43.97**	43.99	46.37	100	82.46	64.44	59.76	34.15	-	-	-	-	-	-
CP 7	38.03	41.08	**42.3**	42.63	45.78	82.46	100	68.28	59.02	36.99	-	-	-	-	-	-
CP 8	39.37	39.39	**41.95**	43.18	47.93	64.44	68.28	100	77.88	36.45	-	-	-	-	-	-
CP 9	35.81	37.45	**43.02**	44.14	50	59.76	59.02	77.88	100	36.88	18.Mar	22.May	25	26.Eyl	20.75	-
CP 10	39.85	37.85	**34.46**	31.58	31.38	34.15	36.99	36.45	36.88	100	41.22	34.21	34.63	35.51	38.72	36.19
CP 11	31.11	23.91	**21.82**	-	-	-	-	-	18.Mar	41.22	100	36.54	38.4	37.6	41.18	37.44
CP 12	37.65	22.41	**16.67**	-	-	-	-	-	22.May	34.21	36.54	100	42.37	41.83	39.01	38.48
CP 13	45.16	37.25	**16.67**	-	-	-	-	-	25	34.63	38.4	42.37	100	59.49	37.96	41.96
CP 14	41.03	34.48	**26.32**	-	-	-	-	-	26.Eyl	35.51	36.6	41.83	59.49	100	41.42	41.05
CP 15	36.89	23.53	**28.57**	-	-	-	-	-	-	39.01	41.18	37.44	37.96	41.42	100	54.41
CP 16	-	-	-	-	-	-	-	-	-	36.19	37.44	38.48	41.96	41.05	54.41	100

CP6: MG601689.1, CP7: MG601647.1, CP8: MG601692.1, CP9: MG601691.1, CP10: MG601695.1, CP11: MG601683.1, CP12: MG601686.1, CP13: MG601679.1, CP14: MG601675.1, CP15: MG601680.1, CP16: MG601678.1; Symbol (-) represents no similarity

Table 4.6. Percent identity matrix of CP related genes with our targeted gene

Gene	CP17	CP*	CP19	CP20	CP21	CP22	CP23	CP24	CP25	CP26	CP27	CP28	CP29	CP30	CP31	CP32
CP17	100	**35.45**	35.48	41.82	38.58	38.43	41.73	36.39	32.95	32.88	30.96	35.31	26.58	35.13	28.49	30.81
CP*	35.45	**100**	33.08	44.07	37.42	37.42	29.48	32.91	31.21	32.83	31.77	34.29	29.23	31.83	24.64	26.14
CP19	35.48	**33.08**	100	44.76	44.78	45.33	29.Eyl	38.03	27.18	32.84	33.19	33.38	22.Eyl	31.25	28.41	26.42
CP20	41.82	**44.07**	44.76	100	42.34	42.66	31.94	36.76	32.53	36.4	33.43	37.34	29.61	41.49	31.Mar	27.May
CP21	38.58	**37.42**	44.78	42.34	100	99.42	35.44	43.01	30.25	34.09	54.55	33.48	33.71	41.49	28.89	25.56
CP22	38.43	**37.42**	45.33	42.66	99.42	100	35.13	43.88	31.17	35.81	54.55	33.8	33.71	41.49	28.89	25.56
CP23	41.73	**29.48**	29.Eyl	31.94	35.44	35.13	100	48.71	32.19	30.77	-	34.08	22.86	39.53	25.64	28.21
CP24	36.39	**32.91**	38.03	36.76	43.01	43.88	48.71	100	30.92	31.91	100	32.63	32.2	38.24	29.69	34.38
CP25	32.95	**31.21**	27.18	32.53	30.25	31.17	32.19	30.92	100	38.81	47.92	40.99	31.18	38.56	31.79	30.16
CP26	32.88	**32.83**	32.84	36.4	34.09	35.81	30.77	31.91	38.81	100	35.6	45.29	33.84	40.1	32.86	32.16
CP27	30.96	**31.77**	33.19	33.43	54.55	54.55	-	100	47.92	35.6	100	53.43	27.Ara	36.33	29.41	19.61
CP28	35.31	**34.29**	33.38	37.34	33.48	33.8	34.08	32.63	40.99	45.29	53.43	100	35.67	44.13	38.94	30.96
CP29	26.58	**29.23**	22.Eyl	29.61	33.71	33.71	22.86	32.2	31.18	33.84	27.Ara	35.67	100	48.04	38.94	34.98
CP30	35.13	**31.83**	31.25	35.99	41.49	41.49	39.53	38.24	38.56	40.1	36.33	44.13	48.04	100	43.41	46.2
CP31	28.49	**24.64**	28.41	31.Mar	28.89	28.89	25.64	29.69	31.79	32.86	29.41	32.55	38.94	43.41	100	52.1
CP32	30.81	**26.14**	26.42	27.May	25.56	25.56	28.21	34.38	30.16	32.16	19.61	30.96	34.98	46.2	52.1	100

CP17: MG601701.1, CP*: GEEF01064138.1, CP19: MG601708.1, CP20: MG601702.1, CP21: EU194556.1, CP22: EU194555.1, CP23: EU194559.1, CP24: EU194557.1, CP25: MG601697.1, CP26: MG601705.1, CP27: MG601706.1, CP28: MG601696.1, CP29: EU194558.1, CP30: MG601703.1, CP31: MG601704.1, CP32: MG601698.1; Symbol (-) represents no similarity

4.4.3 Percent identity matrix of P450 family

Percent identity matrix of our targeted gene (P450) with 17 other members of this family was computed using Clustal Omega. All the relevant sequences were retrieved from NCBI and subjected to analysis.

The percent identity matrix of our targeted gene viz. GEEF01131148.1 with other related genes of P450 family viz. NM_001365727.1, NM_001365732.1, KF044272.1, KF044270.1, KF044266.1, KF044265.1, KF044264.1, KF044259.1, KJ476505.1, KJ476504.1, KJ476503.1, DQ117464.1, DQ117463.1, DQ117462.1, DQ117461.1, and DQ117460.1 is shown in Table 4.7.

Percent identity of GEEF01119768.1 with other members of its family varied from 29.67%-48.61%. Greater identity of GEEF01119768.1 viz. 48.61%, 32.63% and 32.53% was found with KF044259.1, KF044266.1 and KF044272.1.

Lowest percent identity of GEEF01119768.1 was observed with KU522315.1 (33.17%), KU522314.1 (34.38%) and EF026112.1 (34.60%). Less percent identity of our targeted gene (P450) to other genes confirms that dsRNA was designed only for this gene.

Table 4.7. Percent identity matrix of P450 related genes with our targeted gene

Genes	CYP1	CYP*	CYP3	CYP4	CYP5	CYP6	CYP7	CYP8	CYP9	CYP10	CYP11	CYP12	CYP13	CYP14	CYP15	CYP16	CYP17
CYP 1	100	**32.53**	38.79	35.29	36.17	37.17	33.46	33.74	37.72	36.82	36.75	34.55	34.4	34.99	35.65	35.57	35.92
CYP*	32.53	**100**	48.61	30.29	30.77	31.2	30.63	32.63	29.27	29.67	29.67	29.71	29.71	28.13	29.17	27.Şub	27.Şub
CYP 3	38.79	48.61	100	35.6	39.06	35.89	40.19	36.92	36.99	35.09	35.25	37.35	37.27	38.32	35.71	35.97	35.82
CYP 4	35.29	30.29	35.6	100	47.04	46.93	40	37.66	38.79	38.26	38.18	37.59	37.5	37.98	39.2	38.17	38.17
CYP 5	36.17	30.77	39.06	47.04	100	73.53	41.12	40.88	41.19	40.1	39.93	36.43	36.52	40.4	40.37	41.05	41.14
CYP 6	37.17	31.2	35.89	46.93	73.53	100	41.41	40.38	40.57	39.02	39.2	38.36	38.36	40.29	39.75	42.24	42.24
CYP 7	33.46	30.63	40.19	40	41.12	41.41	100	43.83	40.2	37.7	37.7	41.67	41.55	41.04	41.56	41.2	41.2
CYP 8	33.74	32.63	36.92	37.66	40.88	40.38	43.83	100	41.69	40.59	40.68	42.36	42.36	41.44	42.14	42.73	42.82
CYP 9	37.72	29.27	36.99	38.79	41.19	40.57	40.2	41.69	100	63.19	63.25	46.15	46.08	44.68	46.86	46.87	46.54
CYP 10	36.82	29.67	35.09	38.26	40.1	39.02	37.7	40.59	63.19	100	98.93	46	45.86	46.06	48.47	48.53	48.59
CYP 11	36.75	29.67	35.25	38.18	39.93	39.2	37.7	40.68	63.25	98.93	100	46.27	46.13	46.12	48.95	49	49.06
CYP 12	34.55	29.71	37.35	37.59	36.43	38.36	41.67	42.36	46.15	46	46.27	100	99.87	50.29	49.86	52.72	52.97
CYP 13	34.4	29.71	37.27	37.5	36.52	38.36	41.55	42.36	46.08	45.86	46.13	99.87	100	50.16	49.72	52.72	52.98
CYP 14	34.99	28.13	38.32	37.98	40.4	40.29	41.04	41.44	44.68	46.06	46.12	50.29	50.16	100	52.59	52.92	52.42
CYP 15	35.65	29.17	35.71	39.2	40.37	39.75	41.56	42.14	46.86	48.47	48.95	49.86	49.72	52.59	100	55.55	55.48
CYP 16	35.57	27.Şub	35.97	38.17	41.05	42.24	41.2	42.73	46.87	48.53	49.06	49.72	52.72	52.92	55.55	100	99.93
CYP 17	35.92	27.Şub	35.82	38.17	41.14	42.24	41.2	42.82	46.54	48.59	49.06	52.97	52.98	52.42	55.48	99.93	100

CYP1: KF044272.1, CYP*: GEEF01131148.1, CYP3: KF044259.1, CYP4: DQ117464.1, CYP5: KF044265.1, CYP6: KF044264.1, CYP7: KF044270.1, CYP8: KF044266.1, CYP9: DQ117460.1, CYP10: KJ476505.1, CYP11: KJ476503.1, CYP12: DQ117462.1, CYP13: DQ117461.1, CYP14: NM_001365727.1, CYP15: NM_001365732.1, CYP16: KJ476504.1, CYP17: DQ117463.1; Symbol (-) represents no similarity

4.5 Phylogenetic Relationship of Targeted Gene(s)

4.5.1 Phylogenetic relationship of GST gene family

Phylogenetic relationships of our target gene and closely related members of GST gene family were retrieved from NCBI. In total, 15 sequences of CPB were subjected to analysis.

Phylogenetic relationships of GEEF01119768.1 gene which was targeted in our study was found with members of GST family. The figure below shows the neighbor-joining tree showing the relationship between GEEF01119768.1, KU522334.1, KU522333.1, KU522308.1, KU522307.1, KU522311.1, KU522319.1, EF026111.1, EF026112.1, KU522313.1, KU522315.1, KU522314.1, KU522326.1, KU522331.1 and KU522327.1 gene sequences. Bootstrap values (1000 replicates) for all the nodes are shown in neighbor joining tree (Figure 4.16).

Genes EF026111.1 (GST2), EF026112.1 (GST1) and KU522319.1 (GST epsilon 10) were clustered in one main clade. Second clade consists of genes with sequences KU522313.1 (GST epsilon 4), KU522314.1 (GST epsilon 5) and KU522315.1 (GST epsilon 6).

On other side, sequences of gene KU522333.1 (GST Theta 2) and KU522334.1 (GST Theta 3) showed highest similarity. On separate branch KU522326.1 (GST Sigma 1), KU522331.1 (GST Sigma 5b) and KU522327.1 (GST Sigma 2) have showed similarity and grouped in separate branch. While, our targeted sequence GEEF01119768.1 (comp114026_c0_seq1) gene was in a separate branch. No closeness was observed with respect to this gene.

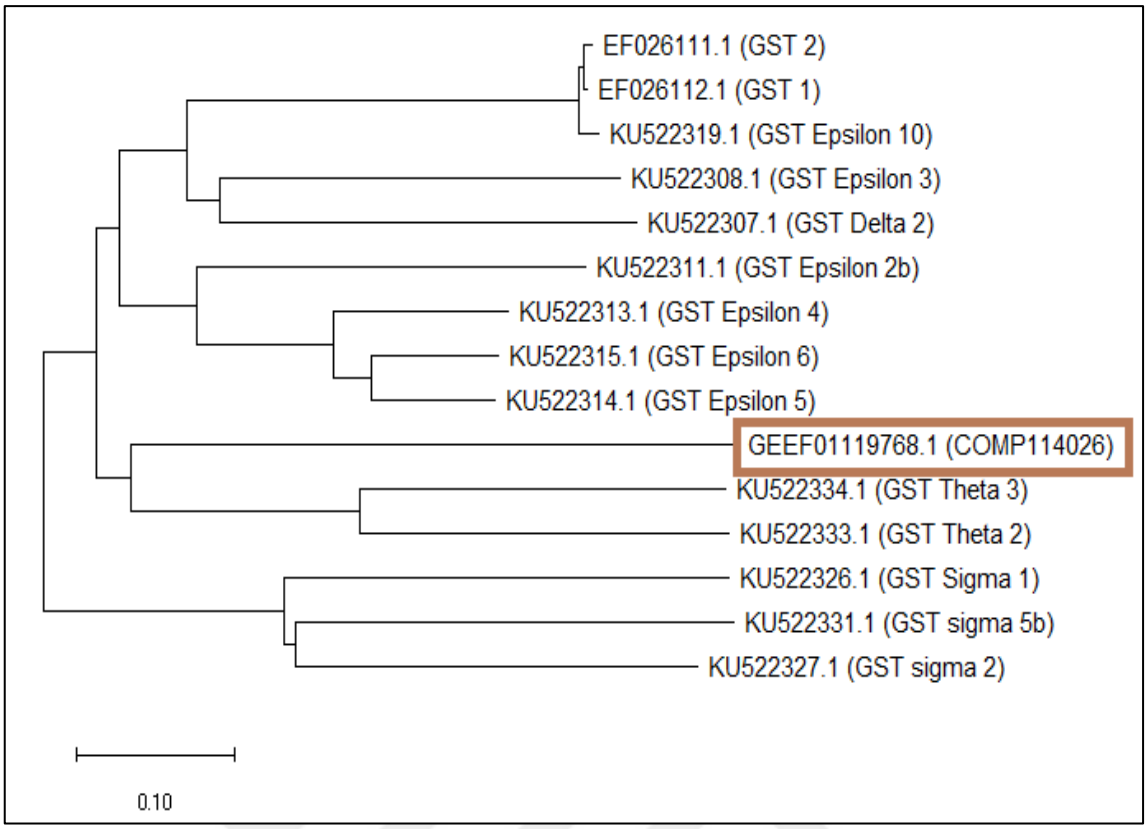


Figure 4.16. Neighbor-joining phylogenetic tree showing the relationship between GST gene family in Colorado potato beetle; box is showing out targeted gene

4.5.2 Phylogenetic relationship of CP gene family

Phylogenetic relationships of gene sequences of CPB related to CP were retrieved from NCBI. In total, 31 CP related sequences of CPB were subjected to analysis.

Phylogenetic relationship of GEEF01064138.1 (Comp105889_c0_seq1) gene which was targeted in our study was found with other members of CP family. The figure below shows the neighbor-joining tree showing the relationship between GEEF01064138.1, EU194559.1, EU194558.1, EU194557.1, EU194556.1, EU194555.1, MG601708.1, MG601706.1, MG601705.1, MG601704.1, MG601703.1, MG601702.1, MG601701.1, MG601698.1, MG601697.1, MG601696.1, MG601695.1, MG601694.1, MG601693.1, MG601692.1, MG601691.1, MG601689.1, MG601687.1, MG601686.1, MG601684.1, MG601683.1, MG601680.1, MG601679.1, MG601678.1, MG601675.1, and MG601647.1 gene sequences. Bootstrap values (1000 replicates) for all the nodes are shown in neighbor joining tree (Figure 4.17).

Phylogenetic tree on CP genes divided 31 genes in three big groups. First group was divided into 3 main branches. First branch in 1st group re-divided in two clads containing EU194559.1 (Ld-CP4), MG601683.1 (CP-141) and MG601684.1 (CP-110). Second branch divided into EU194558.1 (Ld-CP3) and MG601697.1 (CP-74). Similarly 2nd group contains MG601694.1 (CP-59), MG601708.1 (CP-84) and others. In the 2nd clad, MG601705.1 (CP-138), our targeted gene GEEF01064138.1 (Comp105889) and MG601703.1 (CP-64) were categorized in one clad. While 2nd branch was further sub-divided in MG601708.1 and in one clad while MG601679.1 (CP-20) and MG601675.1 (CP-40) in separate clad.

Last group was divided in two main branches. Each branch was further subdivided into MG601704.1 (CP-22), MG601680.1 (CP-37), MG601693.1 (CP-58) and many others.

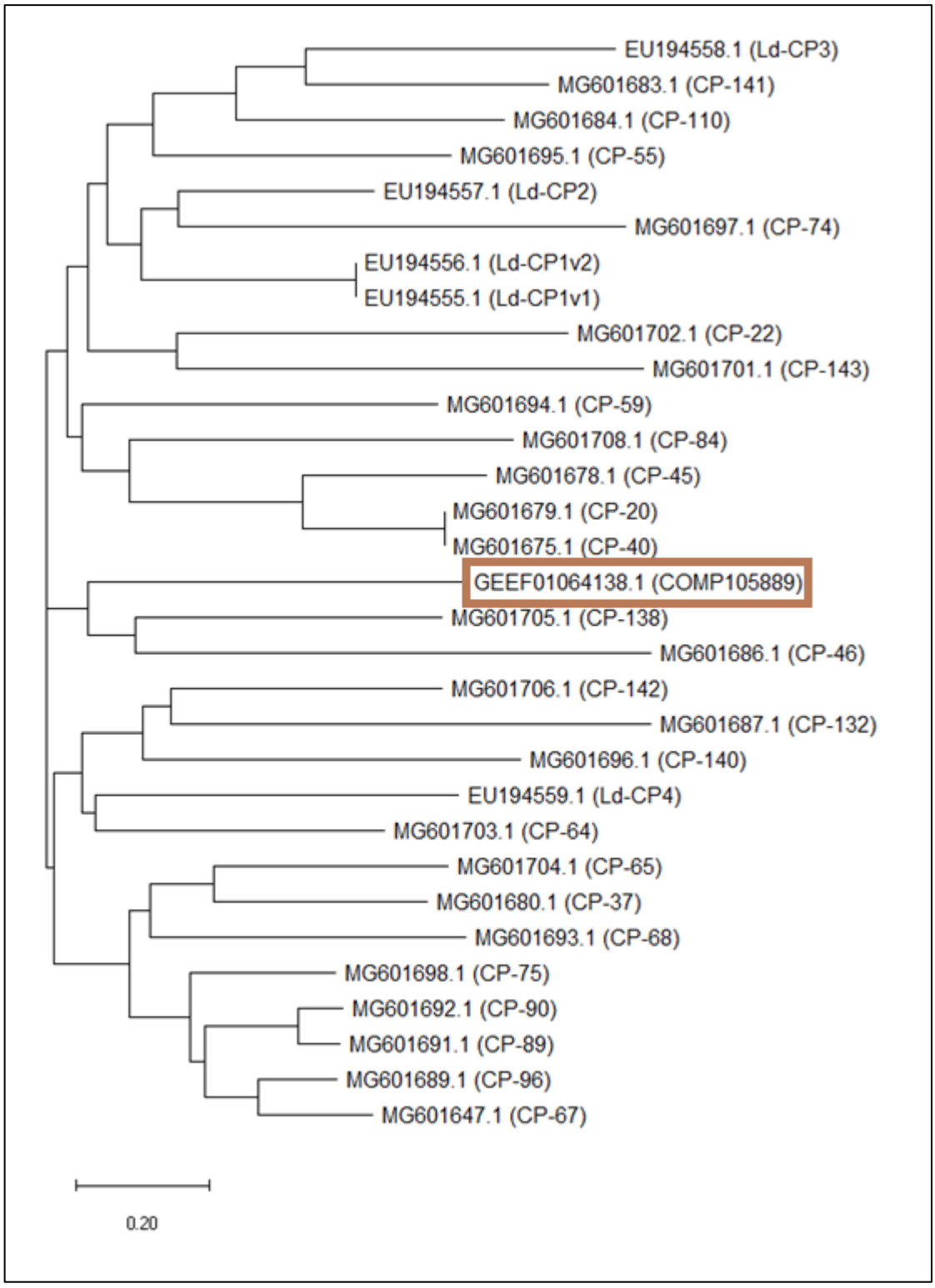


Figure 4.17. Neighbor-joining phylogenetic tree showing the relationship between CP gene family in Colorado potato beetle; box is showing out targeted gene

4.5.3 Phylogenetic relationship of P450

Phylogenetic relationships of gene sequences of CPB related to P450 were retrieved from NCBI. In total, 17 P450 sequences of CPB were subjected to analysis.

Phylogenetic relationship of our targeted gene GEEF01131148.1 was computed with other sequences of P450. The figure below shows the neighbor-joining tree showing the relationship between NM_001365727.1, NM_001365732.1, KF044272.1, KF044270.1, KF044266.1, KF044265.1, KF044264.1, KF044259.1, KJ476505.1, KJ476504.1, KJ476503.1, DQ117464.1, DQ117463.1, DQ117462.1, DQ117461.1, and DQ117460.1 gene sequences. Bootstrap values (1000 replicates) for all the nodes are shown in neighbor joining tree (Figure 4.18).

Genes DQ117463.1 (CYP6BJ1) and KJ476504.1 (CYP6BJ1v1) were clustered in first clade. On the other hand, sequences of gene DQ117461.1 (CYP6BH1v1) and DQ117462.1 (CYP6BH1v2) showed highest similarity. On separate branch, KF044264.1 (CYP412a1) and KF044265.1 (CYP412a2) were categorized in one clad showing similarity. While, KF044270.1 (CYP302a1) and KF044272.1 (CYP314a1) were grouped in separate branch due to their similarity. Many other genes included targeted sequence in this study, GEEF01119768.1 (Comp114026_c0_seq1) gene were in separate branches.

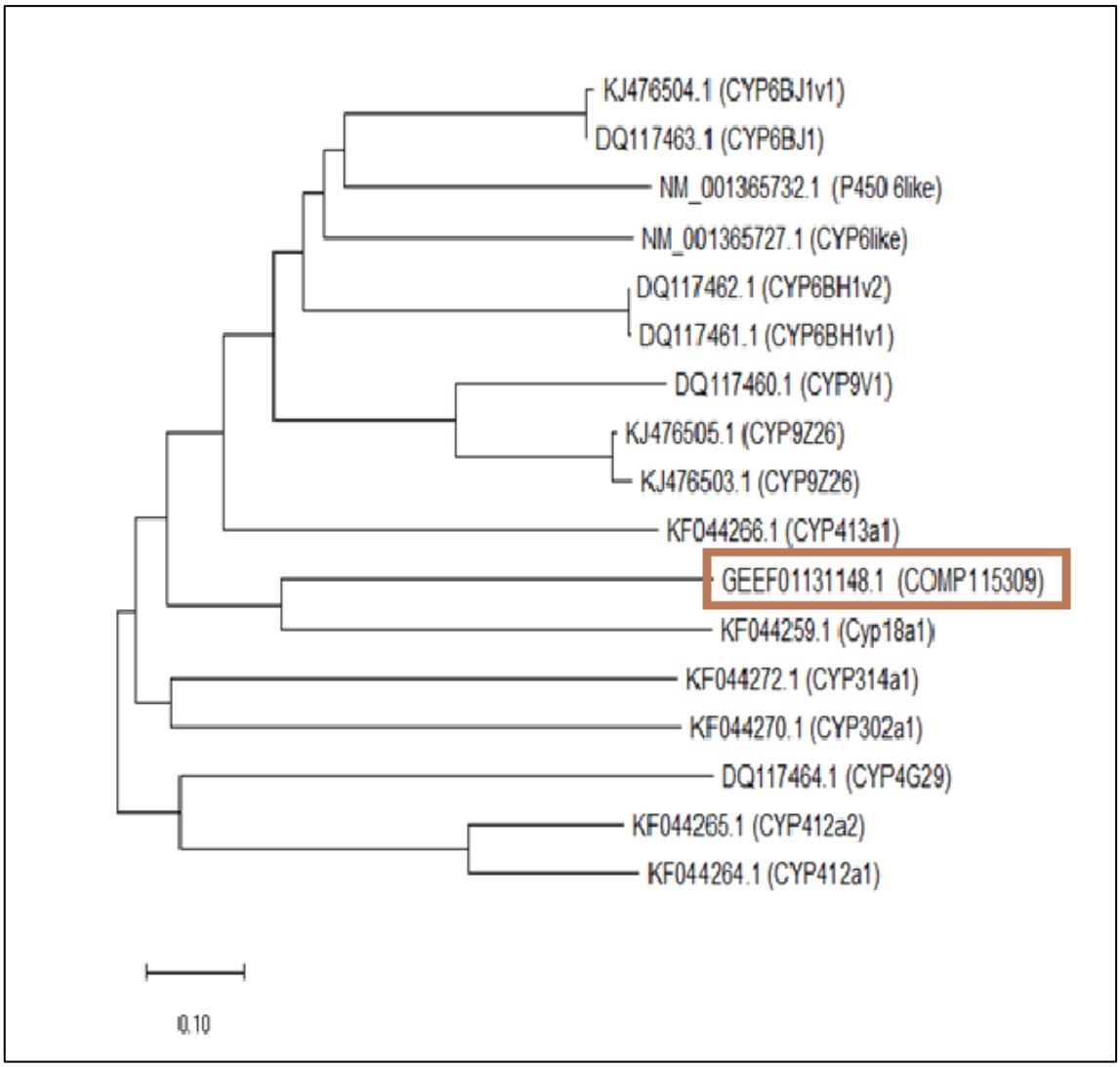


Figure 4.18. Neighbor-joining phylogenetic tree showing the relationship between P450 gene family in Colorado potato beetle; box is showing out targeted gene

4.6 Real-Time Quantitative PCR (qRT-PCR)

Total RNA was extracted from dsRNA fed larvae using TRIzol method. Moreover, cDNA was synthesized from 1µg of RNA according to the protocol described in Fermentas cDNA Synthesis Kit. The qRT-PCR was performed to find relative decrease in gene expression in different CPB larval instars fed on dsRNA and respective control.

4.6.1 Relative expression in CPB 1st instar larvae

Experiment conducted on the 1st instar larvae revealed that the larvae fed on leaves treated with dsRNA, had significantly down-regulated targeted genes than the controls

(untreated) as shown in Figure 4.19. The control group was measured separately for each gene with their respective primers. The expression of control groups were taken as 1.0000 in all the cases. Relative gene expression in case of different dsRNA treatments varied significantly between the treatments. It was found to be 0.0300, 0.0000 and 0.0700 in CP, GSS and P450, respectively ($P \leq 0.05$).

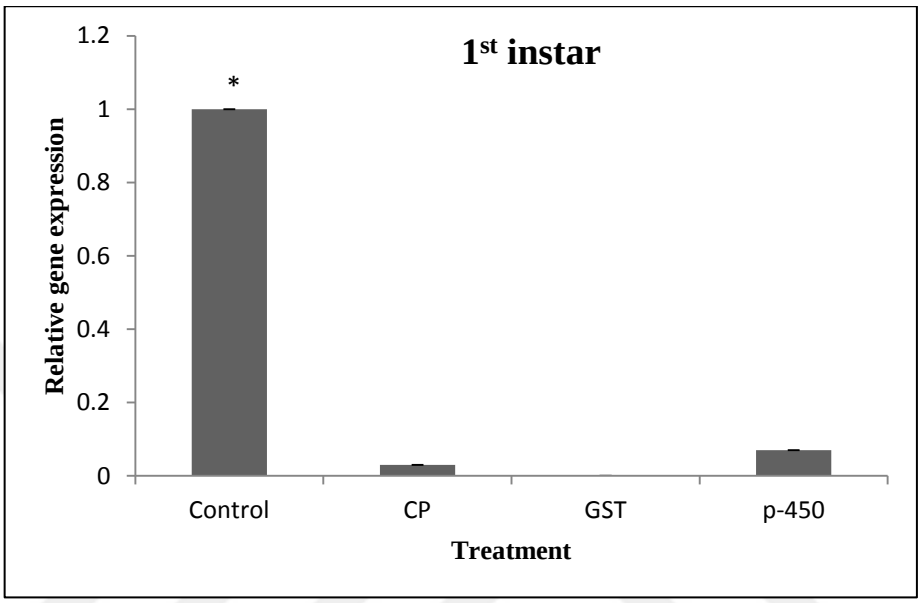


Figure 4.19. Effect of feeding RNAi on target-gene expression (Mean ± SE) in CPB 1st instar larvae after 6 days of feeding assay

4.6.2 Relative expression in CPB 2nd instar larvae

Similarly, the control was measured with primers of all the tested genes and its expression was established as 1.0000 in 2nd instar larvae as shown in Figure 4.20. Experiment on 2nd instar larvae showed that the relative gene expression significantly varied between the treatments than the control. A significant under-expression (0.0100) occurred in larvae exposed to treated leaves with dsRNA synthesized from internal fragment of P450 gene than the control. Similarly, significantly lower expression was also calculated in the dsRNA targeting CP (0.0500) and GSS (0.0900) compared with the control ($P \leq 0.05$).

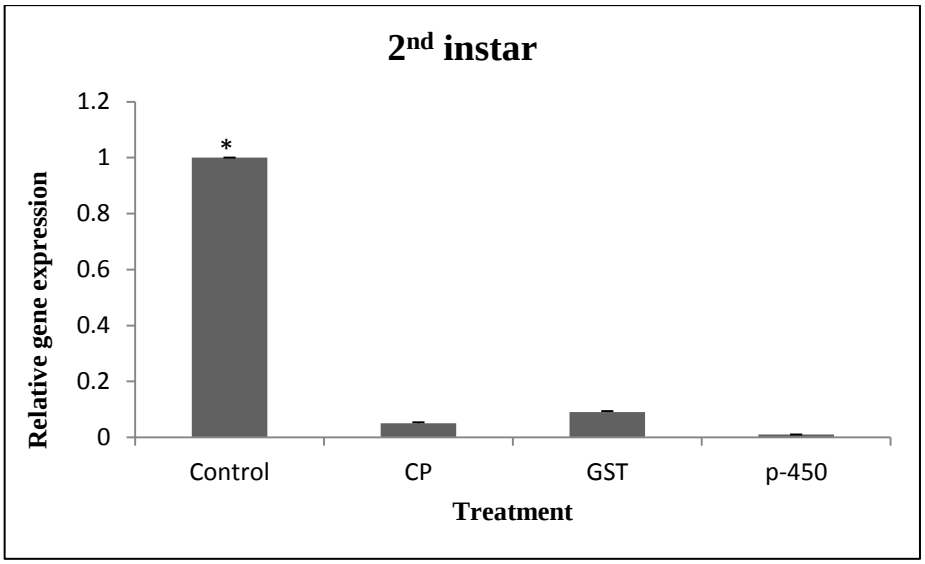


Figure 4.20. Effect of feeding RNAi on target-gene expression (Mean ± SE) in CPB 2nd instar larvae after 6 days of feeding assay

4.6.3 Relative expression in CPB 3rd instar larvae

The control was measured with primers of all the tested genes and its expression was established as 1.0000 in 3rd instar as shown in Figure 4.21. Experiment conducted on 3rd instar showed dramatically lower gene expression in case of all the genes than the control. All the treatments viz. CP, GSS and P450 shown 0.0000, 0.0100 and 0.0300, respectively ($P \leq 0.05$) (Figure 4.21). This could be related with high leaf consumption of this stage as compared with previous two stages.

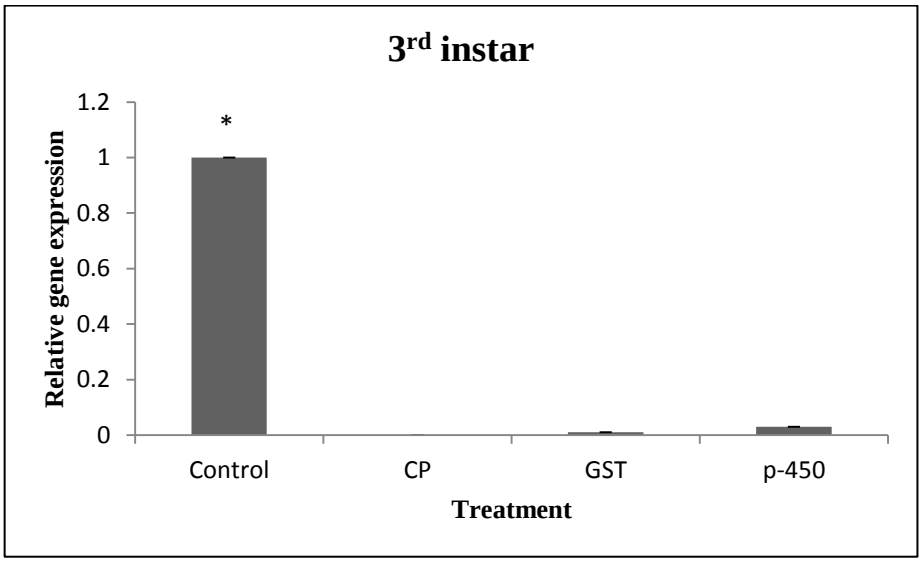


Figure 4.21. Effect of feeding RNAi on target-gene expression (Mean ± SE) in CPB 3rd instar larvae after 3 days of feeding assay

4.6.4 Relative expression in CPB 4th instar larvae

Relative expression of genes targeted by their respective dsRNAs was tested on CPB 4th instar larvae. The control expression was established as 1.0000 to study relative expression of others calculated accordingly in 4th instar. The relative expression of dsRNAs varied significantly between the treatments and the control. Significantly less expression of CP (0.0010), P450 (0.0000) and GSS (0.0004) were observed than the control ($P \leq 0.05$) (Figure 4.22). As it was in the 3rd stage, this stage consumed a large amount of leaf tissue and they probably had taken enough dsRNAs to completely downregulate or silence the targeted genes.

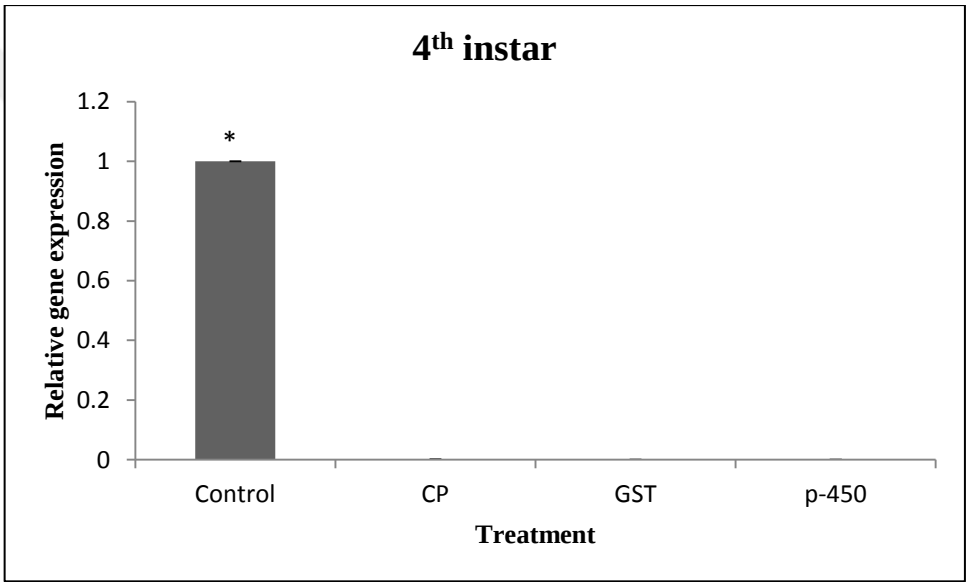


Figure 4.22. Effect of feeding RNAi on target-gene expression (Mean ± SE) in CPB 4th instar larvae after 3 days of feeding assay

4.7 Mortality Effect of dsRNAs on Various Larval Stages of CPB

The effects of feeding with dsRNAs targeting CP, P450 and GSS were tested on various larval instars of the CPB. Percent mortality of the lab resistant 1st instar CPB larvae fed on 3 different dsRNAs is shown in Table 4.8. There was significant difference in mortalities caused by the three genes tested than the control ($P \leq 0.05$). As dsRNA works slowly, based on a preliminary experiment, the data were recorded for 3 and 6 days of continuous feeding. After 3 days of feeding, higher mortality (88.14±2.21%) was observed in the larvae fed on CP-dsRNA than the control. The mortality due to P450 dsRNA was 73.87±0.13% and for GSS-dsRNA it was 67.39±0.37%. However,

there was no mortality in control larvae after 3 days. After 6 days of feeding, the mortality of 1st instar larvae fed on different dsRNAs increased in parallel to increasing time of feeding. Mortalities were 100.00±0.00%, 95.96±2.21% and 90.92±1.72% for CP, P450 and GSS respectively and they were significantly higher than the control after 6 days. There was no mortality in control after 6 days ($P \leq 0.05$).

Table 4.8 Mortalities rates (Mean ± SE) of CPB 1st instar larvae after 3 and 6 days of exposure to 3 different dsRNAs

dsRNA	**Mortality data as % (Mean±SE*) in 1st instar**	
	3 DAT* **	**6 DAT**
CP	88.14±2.21a**	100.00±0.00a
P450	73.87±0.13a	95.96±2.21a
GSS	67.39±0.37a	90.92±1.72a
Control	0.00±0.00b	0.00±0.00b

* SE = Standard Error Degree
**Mean values followed by the different letter in the same column are statistically different ($P \leq 0.05$)
*** DAT= Day after treatment

The mortality of CPB 2nd instar larvae fed on three different dsRNA constructs is shown in Table 4.9. Significant difference was observed in all the treatments both after 3 and 6 days of feeding ($P \leq 0.05$). Higher mortality (57.36±0.32%) was observed in insects fed on CP targeting dsRNA and P450 (37.44±0.32%) than the control. The lowest mortality among dsRNAs was observed in GSS treatment and it was 18.11±0.51%. There was no mortality in control after 3 days. In parallel to extending the feeding period of larvae on the treated leaflets, the mortality increased in all dsRNAs treatments. After 6 days of feeding, a higher mortality was observed in CP treatment with 67.38±0.22%, while, the mortality rates in P450 and GSS treatment were 53.80±0.38% and 37.60±1.65%, respectively. There was no mortality in the control group, after 6 days of incubation.

Table 4.9. Mortalities rates (Mean ± SE) of CPB 2nd instar larvae after 3 and 6 days of exposure to 3 different dsRNAs

dsRNA	Mortality data as % (Mean±SE*) in 2nd instar	
	3 DAT***	6 DAT
CP	57.36±0.32a**	67.38±0.22a
P450	37.44±0.32ab	53.80±0.38ab
GSS	18.11±0.51b	37.60±1.65b
Control	0.00±0.00c	0.00±0.00c

* SE = Standard Error Degree
**Mean values followed by the different letter in the same column are statistically different ($P \leq 0.05$)
*** DAT= Day after treatment

Three days trial was conducted on 3rd larval instar of CPB because 6 days trial was not possible due to initiation of pupal period in exposed insects after 5th day in 3rd instar. There was significant difference between the treatments in feeding bioassay conducted with 3 different dsRNAs as shown in Table 4.10 ($P \leq 0.05$). In the CP treatment the mortality rate was 50.60±0.16% and it was followed by P450 and GSS with 31.29±0.11% and 15.33±0.50% mortality, respectively. There was no mortality in the control group.

Table 4.10. Mortality rates (Mean ± SE) of CPB 3rd instar larvae after 3 days of exposure to 3 different dsRNAs

dsRNA	Mortality data as % (Mean±SE*) in 3rd instar 3 DAT***
CP	50.60±0.16a**
P450	31.29±0.11b
GSS	15.33±0.50c
Control	0.00±0.00d

* SE = Standard Error Degree
**Mean values followed by the different letter in the same column are statistically different ($P \leq 0.05$)
*** DAT= Day after treatment

For 4th instar larvae, 3 days trial was conducted due to the fact that it started pupating right after three days of treatment. Unlike previous stages, the results with CBP 4th instar larvae showed that there was no significant difference in larval mortality among treatments ($P > 0.05$). Feeding of 4th instar larvae on CP targeted dsRNA treated leaflets resulted in 12.19±0.21% mortality. Mortalities of 9.36±0.47% and 1.06±1.06% were

recorded in GSSs and P450 treatments. There was no mortality in the control group (Table 4.11).

Table 4.11. Mortality rates (Mean ± SE) of CPB 4th instar larvae after 3 days of exposure to 3 different dsRNAs

dsRNA	Mortality data as % (Mean±SE*) in 4th instar 3 DAT**
CP	12.19±0.21
P450	1.06±1.06
GSS	9.36±0.47
Control	0.00±0.00

* SE = Standard Error Degree
** DAT= Day after treatment

A preliminary feeding bio-assay was also conducted on adult stage of CPB. However, no mortality was recorded in exposed adults of CPB.

4.8 Effects of dsRNAs on Survival of Various Larval Stages of CPB

Survival rate of 2nd instar larvae fed on dsRNA treated potato leaflets varied significantly between treatments ($P \leq 0.05$). After 3 days, significantly lower survival rate (37.61±0.32%) was observed in case of larvae fed on CP and it was followed by P450 with 67.22±0.30% and GSS with 76.93±0.38% survival rates. As expected, the greatest survival rate among the treatments was observed in the control (100.00±0.00%) and it was significantly higher than the others. Similarly, after 6 days of incubation the survival rate of 2nd instar decreased to 27.91±0.30% in CP-dsRNA treatment while it was lower than the rates in P450 and GSS treatments. Survival rate was 40.92±0.33% and 57.19±0.10% in P450 and GSS treatment, respectively. Before initiation of pupation, significantly higher survival rate (97.56±1.07%) was observed in the control than the other treatments viz. 21.76±0.14% in CP; 31.32±0.11% in P450 and 34.49±0.11% in GSS. After pupation, the survival rate decreased to 4.23±1.07%, 15.33±0.52% and 18.11±0.21% in CP, P450 and GSS treatment and they were significantly lower than the control group (Figure 4.23).

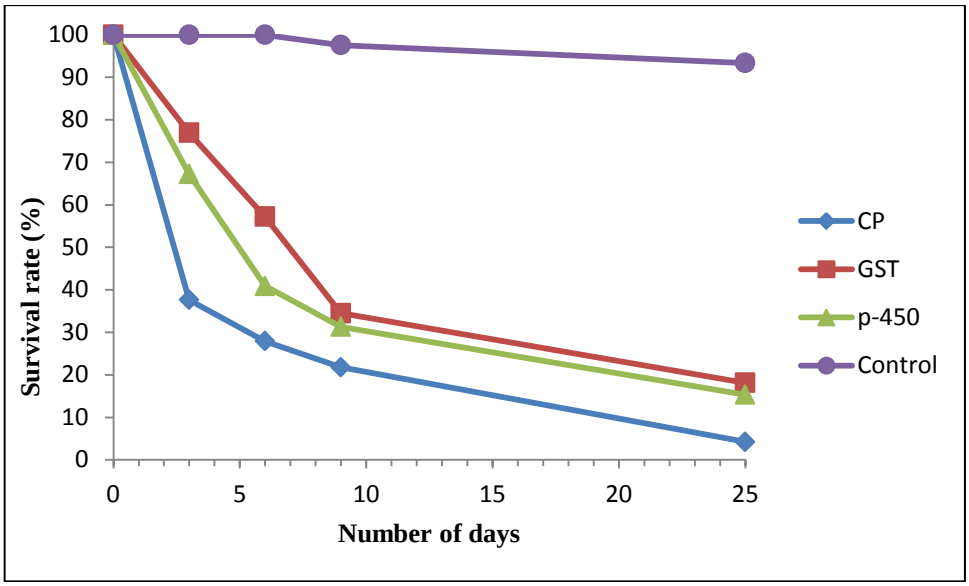


Figure 4.23. Effect of 3 different dsRNAs on survival rate (%) of 2nd instar larvae of CPB

Survival rate of 3rd instars CPB larvae exposed to dsRNAs was calculated up to adult emergence. The survival rate varied significantly after the initial feeding on 3rd instar ($P \leq 0.05$). Survival rate of 3rd instar larvae fed on CP-dsRNA dropped to 42.99±0.75% after 3 days as it decreased to 63.67±0.11% and 70.36±0.51% in P450-dsRNA and GSS-dsRNA treatments, respectively. After 6 days of incubation, the survival rate further decreased to 53.88±0.15% in GSS, 44.11±0.10% in P450 and 34.99±0.11% in CP treatments. After pupation, the survival rate of the insects showed a similar trend and the survival rate due to GSS-dsRNA was 31.30±0.16% and it was 24.92±0.11% for P450. Significantly less number of adults i.e. 15.32±0.21% emerged in case of dsRNA targeting CP. The survival rate in the control was 95.96±1.07% (Figure 4.24).

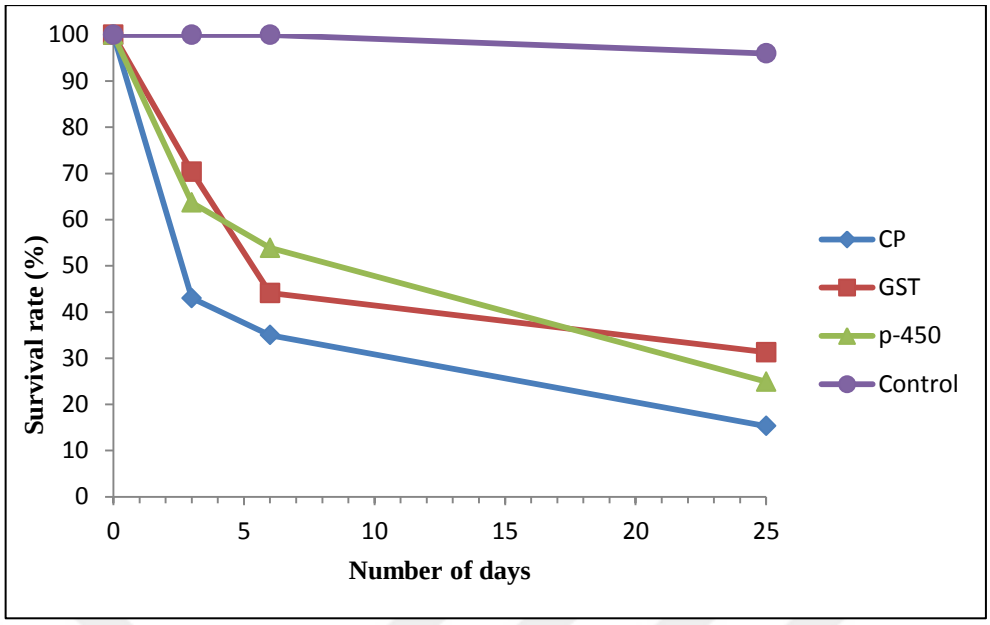


Figure 4.24. Effect of 3 different dsRNAs on survival rate (%) of 3rd larval instar of CPB

There was no significant difference between the survival rates in the dsRNA fed 4th instar larvae during pre-adult stage (P >0.05). After 3 days of feeding, the survival rate of 4th instar larvae fed on CP-dsRNA decreased to 84.13±0.21% and it was followed by GSS-dsRNA feeding with 87.33±1.74% survival rate, while, 97.56±1.06% larvae were alive in P450 treatment. Significantly lower number of adults (47.35±0.31%) emerged from the pupae which were exposed to dsRNA targeting CP when they were 4th stage (P ≤ 0.05). The adult emergence rate from pupae was 50.90±0.51% and 77.63±0.10% in P450 and GSS treatment respectively, and these values were between dsRNA targeting CP and the control group. The survival rate of the 4th instar larvae to adult stage was 97.56±1.06% in the control (Figure 4.25).

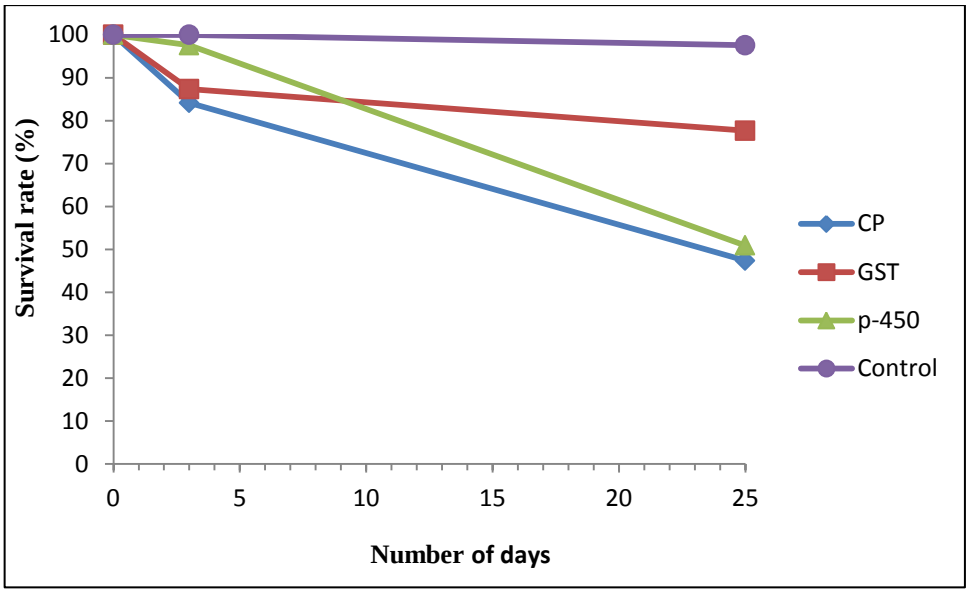


Figure 4.25. Effect of 3 different dsRNAs on survival rate (%) of 4th larval instar of CPB

4.9 Weight Gain in Different Stages of CPB Due to dsRNA Feeding

Weight gain is one of the important parameters of growth in insects and insect growth and damage can be easily assessed by the weight gain especially for the insects that have chewing type mouth parts. Data regarding change in weight depicted significant difference between the treatments. In 3rd and 4th instar larvae, a significant weight change was observed in them after 3 days of exposure ($P \leq 0.05$). Initial weight of tested 3rd instar larvae varied from 26.80 mg to 29.04 mg. Significantly higher final weight was calculated in control (65.65±1.50 mg) and it was followed by the larvae fed on P450 based dsRNA (56.96±1.43 mg). The larvae fed on GSS-dsRNA was weighted 49.87±0.75 mg. Significantly lower final weight was measured in CP-dsRNA fed larvae i.e. 45.10±2.09 mg. Weight gain in the control was significantly higher with 36.65±0.83 mg followed by weight increase in P450 treatment (28.68±0.89 mg). They were followed by the weight change in GSS (20.83±0.54 mg) and CP (18.30±1.14 mg), respectively (Figure 4.26).

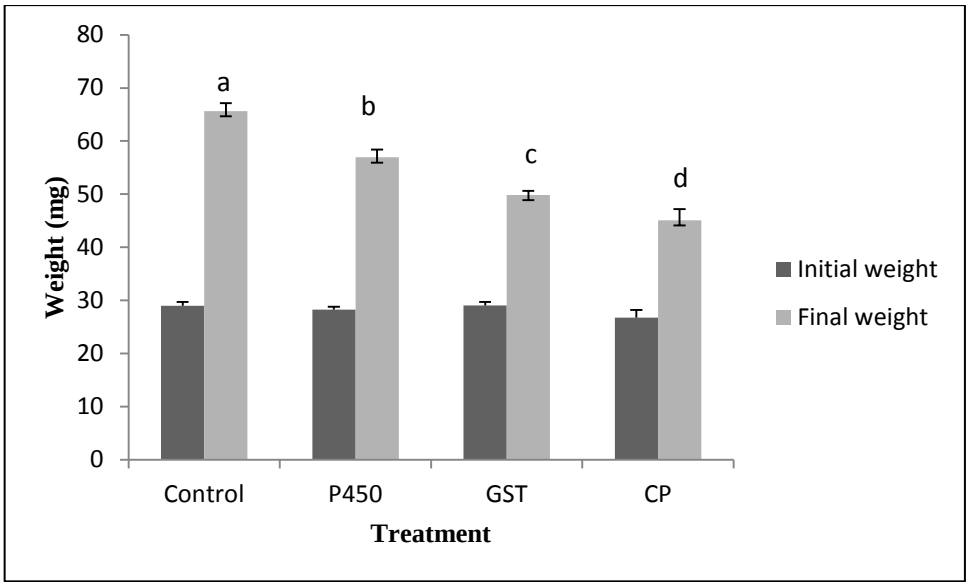


Figure 4.26. Weight gain (mg) in 3rd larval instar of CPB after 3 days of 3 different dsRNAs feeding
*Different letters on error bars represent statistical difference ($P \leq 0.05$)

Varying weight gain in the 4th instar larvae of CPB was observed after 3 days of exposure. Initial weight of tested 4th instar larvae varied from 68.20 to 70.78 mg. The final calculated weight in the control was 158.64±1.87 mg which was statistically similar to the final weight in P450 based dsRNA (154.42±2.21 mg). They were followed by the final weight (121.08±4.22 mg) of larvae fed on GSS-dsRNA. Significantly lower final weight (102.20±3.83 mg) was calculated in CP-dsRNA treatment ($P \leq 0.05$). A higher increase in weight was recorded in control (90.44±2.57 mg) and P450 treatment (85.06±2.27 mg). It was followed by weight gain in the larvae fed on GSS treatment with 52.08±3.88 mg. While, significantly lower increase in weight was observed in larvae with decreased expression of CP (31.42±3.85 mg) (Figure 4.27).

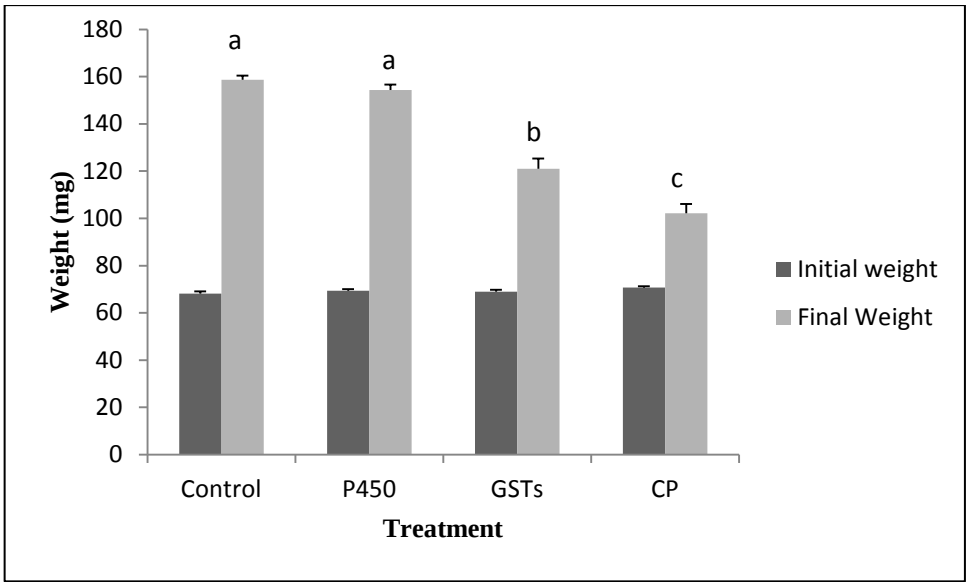


Figure 4.27. Weight gain (mg) in 4th larval instar of CPB when exposed to 3 different dsRNAs for 3 days
*Different letters on error bars represent statistical difference ($P \leq 0.05$)

4.10 Effect of dsRNAs on Pre-adult Duration of Different Stages of CPB

Developmental time for CPB is an important parameter especially regarding assessment of population increase in a growing period of potato plant. Effect of dsRNA on developmental time was assessed on 2nd instar right from the start of bio-assay up to the completion of larval period. There was significant difference between larval developmental time of the 2nd larvae to reach pupae than the control ($P \leq 0.05$). Less time was taken by 2nd instar to reach pupal stage after feeding on CP and GSS based dsRNAs viz. 7.80±0.12 and 8.13±0.37 days, respectively. While, the development time of 2nd instar larvae fed on P450 based dsRNA was 8.23±0.20 days. All of them were statistically less than the control larvae where larval period was 9.10±0.15 (Figure 4.28). Due to higher mortality in pupal stage, it was not possible to calculate pupal duration for 2nd instar.

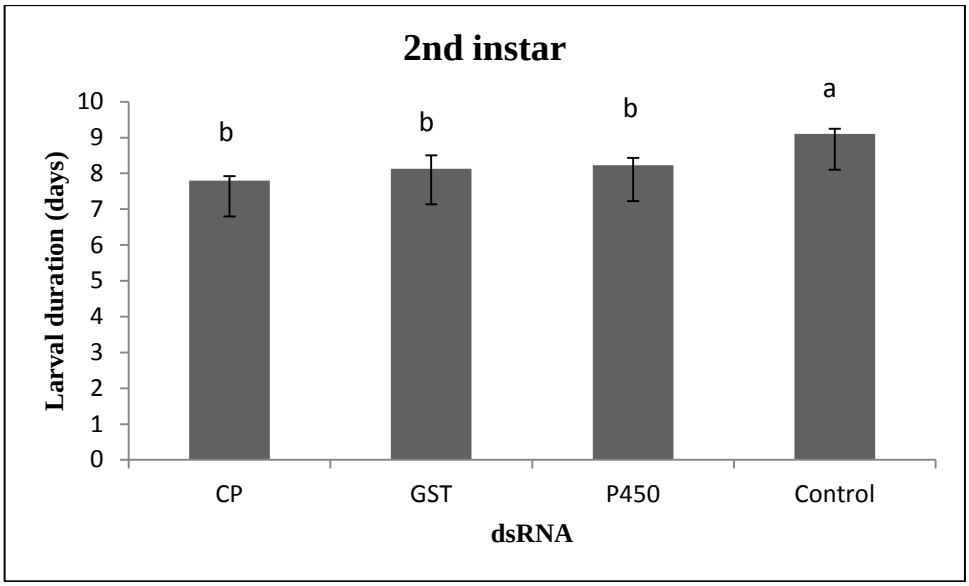


Figure 4.28. Larval duration of CPB 2nd instar larvae after 3 days of feeding on 3 different dsRNAs
* Different letters on error bars represent statistical difference ($P \leq 0.05$)

Developmental time of 3rd instar larvae varied significantly between the dsRNA feeding 3rd instar larvae and control ($P \leq 0.05$). Numbers of days required to reach pupal stage for 3rd instar treated with CP-dsRNA were 5.51±0.20 days. While, in GSS and P450 treatments the calculated periods were 5.85±0.10 and 6.03±0.16 days, respectively. Control larvae took significantly more time (6.96±0.12) to reach pupal stage.

Pupal duration varied significantly among the treatments ($P \leq 0.05$). There was prolonged pupal duration in the larvae which were fed on dsRNA synthesized to target CP i.e. 12.30±0.37 days. The pupal duration in GSS treatment was 11.33±0.60 days. Less pupal duration was observed in P450 with 10.66±0.50 days and control with 10.20±0.37 days (Figure 4.29. A and B).

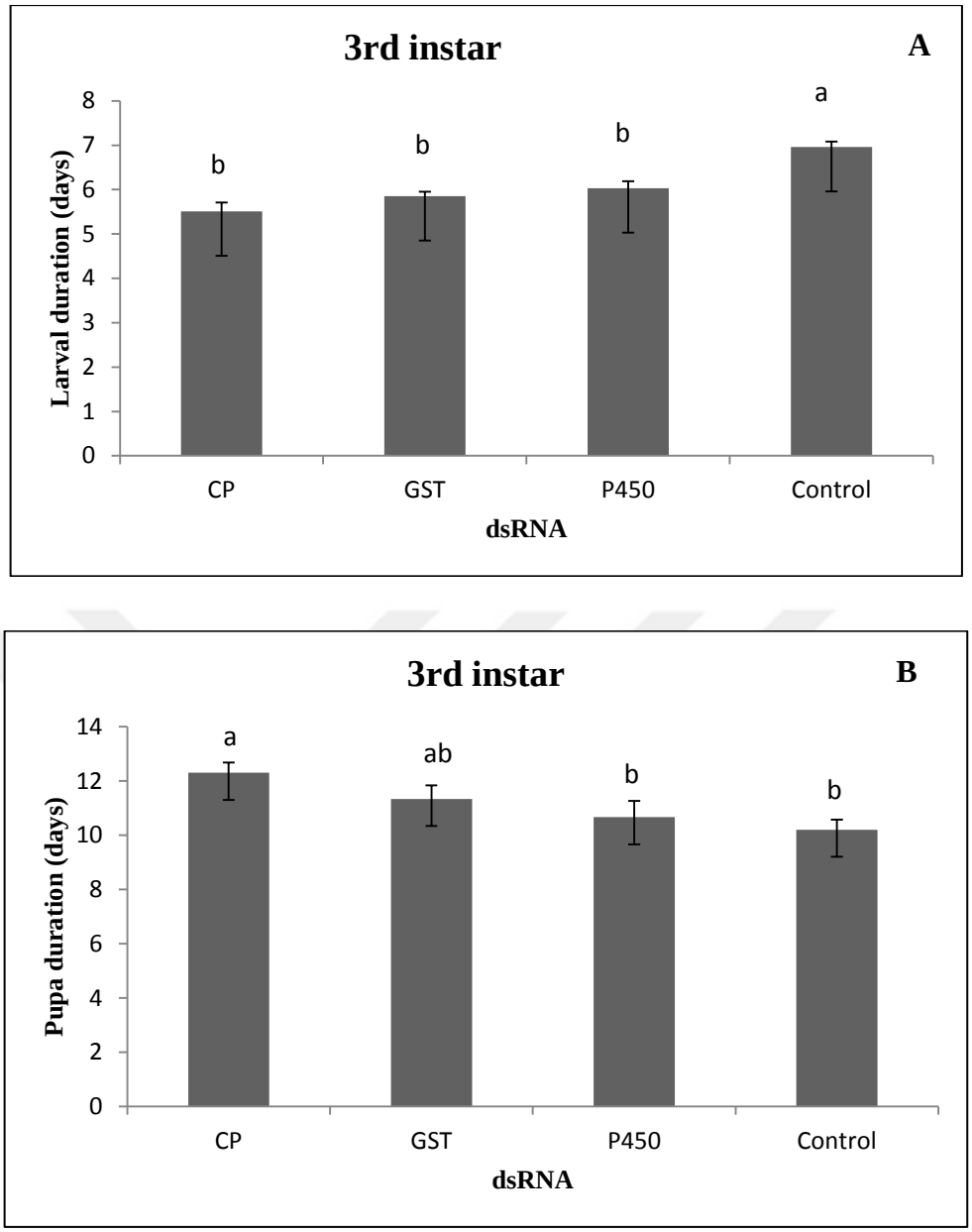


Figure 4.29. Larval and pupal duration of CPB 3rd instar larvae after 3 days of feeding on 3 different dsRNAs (A and B)
* Different letters on error bars represent statistical difference ($P \leq 0.05$)

Larval duration was recorded in the 4th instar larvae after their exposure to dsRNA for 3 days. There was significant difference regarding larval period among the treatments ($P \leq 0.05$). Fewer numbers of days (3.60±0.15 days) were required by 4th instar CPB larvae to reach pupal stage, when fed on dsRNA synthesized to target CP. To reach pupal stage, numbers of days required by 4th instar larvae fed on dsRNA targeting P450 and GSS were 3.90±0.16 and 4.10±0.15 days, respectively. In the control, the duration to reach pupal stage was 4.22±0.21 days.

Pupal duration recorded in the 4th instar larvae after their exposure to dsRNA for 3 days varied between the treatments ($P \leq 0.05$). Pupal duration recorded in CP treatment was 11.20±0.37 days, followed by larvae fed on GSS-dsRNA treatment with 10.40±0.37 days. Pupal duration was 10.20±0.25 days in P450 treatment. The pupal duration in the control was 10.10±0.44 days (Figure 4.30. A and B).

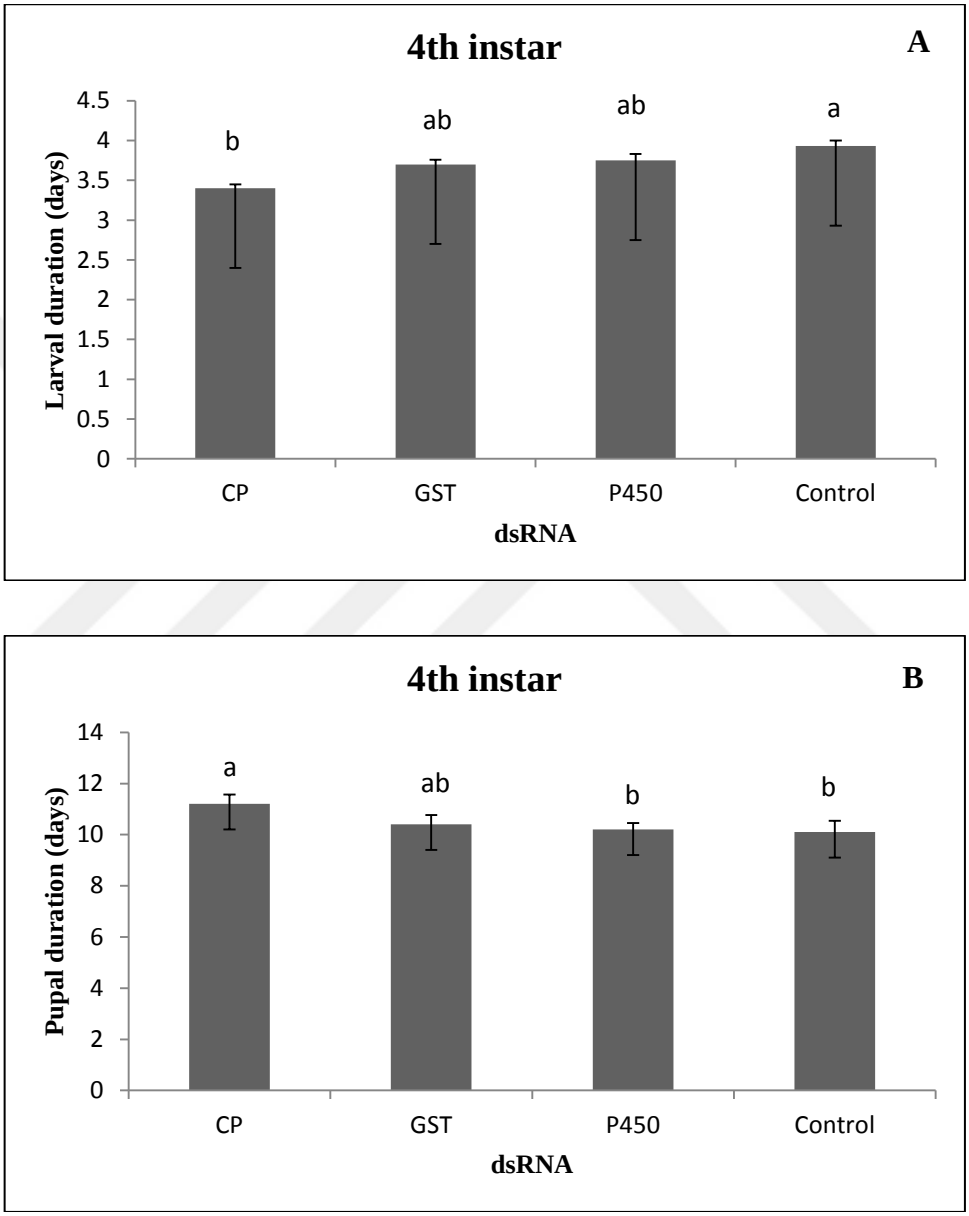


Figure 4.30. Larval and pupal duration of CPB 4rd instar larvae after 3 days of feeding on 3 different dsRNAs (A and B)
*Different letters on error bars represent statistical difference ($P \leq 0.05$)

4.11 Synergism of dsRNA with Imidacloprid

Synergism experiment was conducted on 2nd instar of the lab resistant population, which was 25.64X resistant to imidacloprid. Mortality rate in larvae fed on dsRNAs and the control varied significantly ($P \leq 0.05$). The initial CP feeding caused 34.50±0.11% mortality after 3 days. While, mortality rate recorded in P450 and GSS treatments was 24.92±0.13% and 15.33±0.21%, respectively.

After 3 days of feeding with dsRNAs, reduced dose of imidacloprid was applied on the dsRNA fed larvae and positive control group larvae. After 24 hours of imidacloprid application, the mortality observed in CP fed larvae was 50.59±0.11%. While, the mortality in GSS and P450 mortality enhanced to 47.35±0.21% and 44.12±0.07%, respectively. Mortality in all of the dsRNA fed treatments was significantly higher from the positive and negative control where no mortality was recorded ($P \leq 0.05$).

After 48 hours of imidacloprid treatment, the mortality rate increased to 73.71±0.14%, 63.67±0.11% and 70.36±0.13% in CP, P450 and GSS, respectively. These treatments were significantly different from both control groups ($P \leq 0.05$). While, only 1.07±1.07% mortality rate was recorded in positive control. There was no mortality in the negative control.

After 72 hours of imidacloprid treatment, the mortality rate in CP and GSS further increased to 100.00±0.00% as it was 97.56±1.07% in P450 treatment. Reduced dose imidacloprid caused 4.23±1.07% mortality in the positive control (Table 4.12).

Table 4.12. Synergistic effect of dsRNAs with imidacloprid on CPB 2nd instars larvae

Treatment	Mortality (%) (Mean±SE) before imidacloprid	Mortality data as % (Mean±SE*) after imidacloprid application		
		24 HAT***	48 HAT	72 HAT
CP	34.50±0.11a**	50.59±0.11a	73.71±0.14a	100.00±0.00a
P450	24.92±0.13ab	44.12±0.07a	63.67±0.11a	97.56±1.07a
GSS	15.33±0.21b	47.35±0.21a	70.36±0.13a	100.00±0.00a
Positive control	0.00±0.00c	0.00±0.00b	1.07±1.07b	4.23±1.07b
Negative control	0.00±0.00c	0.00±0.00b	0.00±0.00b	0.00±0.00b

* SEM = Standard Error Degree

**Mean values followed by the different letter in the same column are statistically different ($P \leq 0.05$)

*** HAT= Hours after treatment

CHAPTER V

DISCUSSION

Life table analysis is an effective tool for ecological studies and is used in assessment of growth, survival, reproductive capabilities as well as population projection of insects under varying conditions. Intrinsic rate (r) is assessed by age (x), the age-specific fecundity (m_x) and the age-specific survival rate (l_x) and of insect's population and it helps us to forecast future population especially for insect pests and biocontrol agents (Leslie, 1945; Chi, 1988). Reproductive age of females and the peak value in reproductive age are the determining factors of growth rate of population and are also for forecasting future generations (Lewontin and Felsenstein, 1965). According to life table theory, the population increases only when $r > 0$ and net reproductive rate (R_0) will be greater than 1 and the intrinsic rate of increase (r) is a more useful statistic than R_0, to compare the population growth potential of various insect species (Price, 1997, Southwood and Henderson, 2000; Chen et al., 2017). According to Southwood (1981) and Huffaker et al. (1984), r-strategists possess high intrinsic rate of growth, a large fecundity (large R_0) and short generation time (T) and these are parameters that allow many pest species to find hosts , feed on them and reproduce many offsprings in a growing season of many cash crops. These are the case in our study. The parameters of r, λ and R_0 for the field CPB population were 0.12 day^{-1}, 1.13 day^{-1} and 71.07 offsprings/female, respectively. The projection program estimated that the initial population of 10 insects for the field population could increase to 8036143 individuals only after 120 days. These results show the great growth capacity of CPB and also its threat to the potato plants.

The APOP, TPOP, male longevity, female longevity and total fecundity in the lab susceptible population were 6.58 days, 31.25 days, 49.58 days, 52.94 days and 104.00 eggs per female, respectively. All of these parameters were significantly smaller in the lab susceptible population than those are for the field population. Similarly, other population parameters like r, λ and R_0 which were 0.10 day^{-1}, 1.10 day^{-1} and 38.43 offsprings/female in the lab susceptible population were also significantly lower than the field population. These results are comparable to the previous study conducted by Mansoor et al. (2013) on *Chrysoperla carnea* in which the resistant insect population

had higher survival rate, fecundity, biotic potential, intrinsic rate of increase and other population parameters than the susceptible population. Positive correlations of intrinsic rate of growth with resistant population have been also reported by Abbas et al. (2012) in *Spodoptera litura* and Abbas et al. (2014a) in *C. carnea*. The field CPB population, used in the study, had been exposed to various biotic and abiotic stress factors in the field so these factors could have resulted in survival of the fittest in the insect population while the lab population had been reared without any stress factor so that it was characterized by less fitness parameters. These points were also discussed by Storer et al. (2010) in armyworm.

In our study, dose-response bioassays with three different population revealed that the resistance level in the field population was 7.84 folds. The similar level of the field population resistance to insecticide was reported by Olson et al. (2000) and Clements et al. (2016) who found 10X resistance to imidacloprid in CPB field populations. The field population was collected from the potato field which has been used for potato production since 2014 and application of imidacloprid, thiacloprid, thiamethoxam, cypermethrin, deltamethrin and indoxacarb has been implemented since then. This practice may lead to the development of imidacloprid resistance during these years. It is a well-known fact that the CPB could develop resistance to many active compounds in a very short period of time. For example: after the introduction of imidacloprid in field for the management of CPB, resistance was reported against imidacloprid just in 2 years (Zhao et al., 2000).

Our results showed that the continuous exposure of the lab population to imidacloprid led to 25.64 folds resistance to this active compound. These findings are comparable to the previous findings of Abbas et al (2014b) and Afzal et al. (2015) who have reported enhanced resistance in insects when selection pressure was provided with single insecticide under controlled conditions. This increase in resistance to imidacloprid could be also related with highly diverse resistance mechanisms of CPB (Ioannidis et al., 1991). Metabolic resistance occurring due to increased level of detoxification enzymes viz. esterases, carboxylesterases and/or monooxygenases provides this pest a concrete genomic base to survive in a competitive chemical environment (Kaplanoglu et al. 2017).

We showed that three important genes viz. cuticular protein, cytochrome P450 and glutathione synthetase were overexpressed in the resistant CPB population. These genes were identified by Clements et al. (2016) and Kaplanoglu et al. (2017) among the transcripts in imidacloprid resistant CPB populations. Down-regulation of these genes in feeding bioassays ranging from 0.0000-0.0900 was verified with qRT-PCR. Increased susceptibility to imidacloprid of the 2nd instar larvae of lab resistant population has further confirmed the role of these genes in imidacloprid resistance. The 3rd and 4th larval instars showed more downregulation of the genes and that could be due to more food consumption and thus more intake of dsRNA. Our results are in accordance with the studies by Zhu et al. (2011) on CPB and Zhang et al. (2010) conducted on mosquito, who reported significant downregulation of targeted genes by oral delivery of dsRNA. Amplification of gene silencing effect, favorable pH, dsRNA stability, sytemic spread of dsRNA in coleopterans and RNAi core machinery were the key factors in effectiveness of RNAi-mediated gene silencing in CPB (Cooper et al. 2018).

Phylogenetic analysis of targeted genes viz. CP, P450 and GSS was carried out using the available sequences at NCBI where a total of 15 GST sequences, 31 CP related sequences and 17 P450 sequences are available. Our phylogenetic analyses depicted that imidacloprid resistant CP, GSS and P450 lied in a place after many other relevant genes which show their evolution of CP, P450 and GSS after many natural and other resistance related genes of the same family. This happened may be due to the fact that imidacloprid was introduced after many years of introduction of insecticides belonging to other classes (Hovda and Hooser, 2002). Similarity matrix of this group verifies the results that GSS has 40.80% and 43.04% resemblance with KU522331.1 and KU522327.1, respectively. These genes are also insecticide inducible as reported by Han et al. (2016), so they have quite close resemblance with our targeted gene. Similarity matrix of P450 genes shows the resemblance of our targeted gene to KF044259.1 and KF044272.1 genes with 32.53% and 48.61% similarity. These genes are associated with pyrethroid resistance in CPB as reported by Wan et al. (2013) thus occupy a close relation to our targeted gene which is imidacloprid inducible. The CP gene has shown close relation with MG601647.1 and MG601691.1 with similarity matrix of 40.12% and 39.81%, respectively. Closely related sequences are categorized

near to each other according to the similarity matrix in phylogenetic trees (Hunt et al. 2007).

Our study showed that among 3 different dsRNAs, larvae fed on CP-dsRNA underwent higher mortality i.e. 100.00%, 67.38% and 50.60% in 1st instar, 2nd instar and 3rd instar. These findings are in accordance with the findings of Mun et al. (2015) and Jasrapuria et al. (2012) who have reported significant mortality in red flour beetle due to downregulation of CP. The CP plays a vital role in insect growth, development of penetration resistance to various insecticides and tolerance of environmental factors so that its downregulation could result in higher mortality in insect species (Jasrapuria et al. 2012). Mortality rates were 95.96%, 53.80% and 31.29% in 1st, 2nd and 3rd instar CPB larvae fed on P450 dsRNA. Similar results were also reported by Mao et al. (2007) and Jin et al (2015) who reported significantly higher mortality in *H. armigera* due to downregulation of a P450 gene. Feeding of CPB larvae with dsRNA targeting the GSS produced mortality i.e. 37.60% and 15.33% in 2nd and 3rd instar larvae, respectively. GSTs role in detoxification of xenobiotics during phase II reactions is well documented (Panini et al., 2016; Zhang et al., 2016; Dang et al., 2017). Mortality in case of GSS may occur due to enhanced bio-activity of phytochemicals especially phenolics in potato against CPB (Im et al., 2008).

Our findings showed that mortality rate caused by dsRNAs varied between CPB larval stages, while, a higher mortality (90.92-100.00% and 37.60-67.38%) was observed in the 1st and 2nd instar it was 15.33-50.60% in the 3rd and 1.06-12.91% in the 4th instar larvae. These results are comparable to the findings of Amiri and Bakhsh (2019) and Hussain et al. (2019) who have reported that earlier instars of CPB are more susceptible than the later instars. Despite of the fact that the later instars showed more downregulation of the targeted genes ranging from 0.0000-0.0300, the functions of these genes can be replaced by other closely related genes at later stages by some stage specific gene (s). For example, after 3rd instar, mortality due to downregulation of P450 started decreasing and this could be related with the production of various types of P450 in the insect as reported by Clements et al., (2016) and Kaplanoglu et al. (2017). As discussed above, percent identity matrix of P450 genes shows the resemblance of our targeted gene, which could replace our targeted gene thus decreased mortality was observed at the later instars. Similarly, the decrease in mortality of tested larvae in GSS

treatment could be explained with the percent identity matrix of this group. The targeted GSS has resemblance with many other members of GST genes family. These closely related genes probably replaced the function of our target gene at the 3rd and 4th instar larvae, which may lead to the lower mortalities seen in these stages. Co-regulation of some closely related genes is also reported by Cornman et al. (2008) and Togawa et al., (2008) who reported that some genes are stage specific. Therefore, in the current study the targeted genes could be replaced by some closely related stage-specific gene (s) at 3rd and 4th instar larvae. Additionally, the larvae at later stages also prepare themselves for pupal formation so set of other genes may have activated which decreased the need of our targeted genes in 3rd and 4th instar CPB larvae. As pointed out above many genes in insect body are stage-specific, so role of those substitute genes can be a major factor in the limited effects of dsRNAs especially 4th instar larvae.

Feeding of CPB larvae with dsRNA treated potato leaflets decreased survival and also fitness of CPB larvae. Similar decrease of CPB larvae survival and fitness fed on dsRNA was also reported by Jin et al. (2011). Down-regulation of CP resulted in significant decrease in survival rate to 4.23%, 15.32% and 47.35% in 2nd, 3rd and 4th instar CPB larvae. These findings are comparable to the findings of Jasrapuria et al. (2012) who reported a decreased survival of red flour beetle due to down-regulation of CP genes. In the current study most of the pupae died before adult emergence and this decrease in adult emergence from pupae is also in accordance with the findings of Mun et al. (2015), who reported a decrease in adult eclosion due to downregulation of cuticular protein in red flour beetle. The knock down of CP in test larvae could make them prone to various stresses like increased loss of insect moisture and increased susceptibility to the temperature thus decreasing the survival of insects (Xiong et al., 2018). As the pupal stage is most vulnerable stage among all the stages of insects so downregulation of CP may lead to the higher mortalities in this stage (Arakane et al. 2008). Similarly, the survival rate of 2nd, 3rd and 4th instar CPB larvae fed on P450-dsRNA decreased to 15.33%, 24.92% and 50.90% while reaching the adult stage. These results are in accord with the previous reports of Mao et al. (2007) and Jin et al (2015) who reported a decrease in survival of tested insect species due to downregulation of P450. According to Rewitz and Gilbert (2008) and Gilbert (2004), the P450 genes mediate various growth hormones in insects so it can affect the survival of insects at various growth stages. However, more survival was observed in the GSS treatment than

they were in CP and P450 treatments owing to the fact that the GSSs do not have any vital role in insect growth and/or development.

Less weight gain was recorded in larvae fed on CP-dsRNA viz. 18.30 and 31.42 mg in 3rd and 4th instar larvae of CPB. Decrease in weight might be due to dsRNA feeding is also reported by Jin et al. (2015) in *H. armigera* and Zhu et al. (2011) in CPB, who reported that the weight gain was less in larvae fed with dsRNA. Less weight gain can be attributed to the fact that growth and development of insects are highly reliant on the ability of insect to remodel their exocuticle (Medina et al., 2002; Ahmad et al., 2006). Weight gain in P450 based dsRNA treatment was significantly higher than other dsRNA treatments primarily due to activation of diverse set of P450 enzymes at bigger instars which were able to mediate the insect growth. Weight gain in 3rd instar larvae due to down-regulation of P450 was measured as 28.68 mg. Decrease in weight due to downregulation of P450 in insect species is also reported by Jin et al (2015). However, the weight of 4th instar larvae was similar to the control group and this could be result of not having enough time to show full effects of dsRNA on larval weight at this stage.

Time required to reach pupal stage by 2nd, 3rd and 4th instar larvae fed with CP-dsRNA was 7.80, 5.51 and 3.60 days. These results are comparable to the findings of Qiao et al., (2014) who reported that larval growth in silkworm can be affected by the change in cuticle protein. Similarly, pupal duration viz. 12.3 and 11.2 days in 3rd and 4th instar treated CPB larvae was significantly affected due to CP-dsRNA feeding. It may happen due to the fact that CP related transcripts are more active right after pupation in normal insects. These set of genes are associated with first ecdysone pulse, with variation in their time of expression (Arakane et al. 2008). The P450 based dsRNA altered the duration of larvae during 2nd and 3rd stage which was 8.23 and 6.03 days respectively. Rewitz and Gilbert (2008) reported that the P450 mediates the growth hormones in insects and any interference in this enzyme may lead to change in larval development. Although there is no report on the role of GSSs in insect growth and development, variation in insect development could be due to bioactivity of xenobiotics in potato plants that affect the development period (Im et al., 2008). Beninger et al. (2004) have reported the role of phenolic compounds in growth and development of cabbage looper, so effect of GSS on larval and pupal duration in our study can be attributed to the effect of phenolics in potato.

Synergism experiment showed a remarkable potential of our targeted genes as synergists with imidacloprid. Only one larva survived in P450 dsRNA treatment while all the tested insects died in CP and GSS treatments. These results are in accord with the findings of Clements et al. (2016) who have reported that CP and GSS are more effective synergists than the P450. The CP has important role in growth and also penetration resistance to various insecticides so downregulation of CP resulted in complete decline of the exposed population (Hadley, 1982; Clements et al., 2017). While, role of GST in phase-II reactions is well established and the phase II reactions are quite important in detoxification of neonicotinoids. Yu and Killiny (2018) reported the increase in susceptibility of Asian citrus psyllid to thiamethoxam due to decreased expression of GSTs. Resistant insects can undergo mortality if phase II reactions are hindered by any source like gene silencing. This finding confirms our results (synergist effect of GSS with imidacloprid). Synergistic effect of P450-dsRNA with imidacloprid produced 97.56% mortality of exposed larvae. Use of P450 as synergist is also reported by Bautista et al. (2009) who found decrease in resistance of *P. xylostella* to permethrin due to downregulation of a P450 gene. This study is further supported by the study of Kaplanoglu et al. (2017) conducted on imidacloprid resistant CPB populations.

CHAPTER VI

CONCLUSION

The main objective of the current study was to evaluate of RNAi technology in the control of imidacloprid resistant CPB population by knocking-down the transcriptome of 3 important imidacloprid resistance associated genes; cuticular protein, glutathione synthetase and cytochrome P450 monoxygenase. This is the first study reporting the effects of dsRNA on mortality, growth and survival of different larval instars of CPB. The life table study of lab susceptible and field collected population showed that the lab susceptible population had significantly less intrinsic rate, fecundity, survival and other population parameters than the field collected population. Comparison of lab susceptible and field collected population indicates that some resistant related enzymes like CP and P450 mediating various growth hormones may lead to increase the fitness of field collected population. So, suppressing of resistant genes to produce a susceptible population of CPB by gene silencing can be useful in devising novel control strategies. Bacterially expressed dsRNA was used to conduct oral feeding bioassays on different larval instars of CPB. The mortality rates were greater at the earlier stages. Decreased survival rate of exposed CPB larvae was observed in all the dsRNAs. Similarly, body weight and pre-adult duration were also affected due to dsRNAs. Synergistic effect of all the dsRNAs with imidacloprid on 2nd instar CPB larvae produced high mortality with reduced dose of the both treatments.

Heavy reliance on insecticides for management of CPB has made this pest resistant to 52 insecticides Additionally, input cost of insecticide development/discovery is markedly increasing because each year thousands of chemicals are produced and tested for their insecticidal properties in pesticide industries which require billions of dollars but only small number of active compound are registered. For management of resistance in CPB, eco-friendly synergists like dsRNAs used in this study could play an important role for reducing application dose of many insecticides as imidacloprid was used in the current study.

Although very promising results were obtained with these tested dsRNA in the lab, further studies should be conducted to validate their effects in greenhouse and field

conditions against various CPB populations. Different formulation methods should also be explored to enhance dsRNAs stability in field conditions. Additionally, dose-response bioassays are needed to calculate Lethal Dose 50 (LD_{50}) for utilization these dsRNAs in an effective way. Synergist effects of dsRNAs on various stages especially the 4^{th} larvae and adult should be also studied to understand full potential of dsRNAs in controlling CPB. Studying life table parameters with reduced dose of dsRNAs can help to understand the sub lethal effects of these dsRNAs on population parameters of CPB. Moreover, this technique (RNAi) should be tested against other notorious pests like American bollworm, armyworm, diamondback moth etc. especially as synergist to control these pest species. Different active compounds with different dsRNAs should be sought to discover their full synergist potential. Additionally, effects of these dsRNAs on enzymatic level should be studied to see their possible effects on enzyme activity.

This study revealed that the use of this gene silencing technique can be utilized against imidacloprid resistant CPB and it may also be used against insect pests of different crops. Additionally, these genes can be used in combination with various control methods like gene pyramiding, biocontrol agents, other biopesticides and/or chemical insecticides to devise integrated pest management programs. As a matter of fact, resistance involves diverse genes and metabolic enzyme systems associated with them. Instead of consuming time and money on discovery and development of new chemicals, the chemicals already in the market can be used with RNAi-based synergists against resistant insect-pests. Further research on the use of dsRNA in field and its implementation can significantly decrease the cost of development of new insecticides. It can be a milestone in resistance management of CPB and various other notorious insect pests.

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Appendix-A

LB Broth (1L)

Yeast extract 5g

Bactotrypton 10g

NaCl 10g

Dissolved in 1 liter of distilled water, adjusted pH 7.5 and autoclaved.

Appendix-B

LB AGAR (1L)

LB containing 15g L-1 of Bacto Agar

Appendix-C

10x TBE Buffer

Trimza base 108g

Boric acid 55g

0.5M EDTA 40mL

Adjust volume to 1 liter with distilled water

CURRICULUME VITAE

Muhammad Nadir Naqqash was born on Novermber 9, 1990 in Multan, Pakistan. He completed his higher secondary education in 2008 from Punjab College of Commerce, Multan. He joined Bahauddin Zakariya University, Multan in 2008 for his undergraduate studies. He completed his B.Sc. (Hons) in Agricultural Sciences with specialization in Entomology from Faculty of Agricultural Sciences and Technology, Bahauddin Zakariya University Multan Pakistan in 2012. He continued his Master's degree from Department of Entomology, Faculty of Agricultural Sciences and Technology, Bahauddin Zakariya University Multan Pakistan. He completed his degree of Masters in Agricultural Entomology during August 2014. He enrolled in Graduate School of Natural and Applied Sciences, Department of Production and Technologies at Niğde Ömer Halisdemir University, Niğde, Turkey to pursue his PhD education under the guidance of Prof. Dr. Ayhan GÖKÇE. During his PhD thesis research, he worked on knock down of imidacloprid resistance associated genes in Colorado potato beetle (CPB) using RNAi-based approach. He knows English, Urdu, Saraiki and Punjabi languages.