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PhD THESIS

NİĞDE ÖMER HALISDEMIR UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND PPLIED SCIENCES

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

PYRAMIDING OF INSECTICIDAL GENES IN POTATO TO ENCODE RESISTANCE AGAINST COLORADO POTATO BEETLE, *LEPTINOTARSA DECEMLINEATA* (SAY), (CHRYSOMELIDAE: COLEOPTERA)

MUHAMMAD SALIM

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PhD Thesis

Supervisor

Prof. Dr. Ayhan GÖKÇE

January 2019

The study titled "**Pyramiding of Insecticidal Genes in Potato to Encode Resistance against Colorado Potato Beetle, *Leptinotarsa decemlineata* (Say), (Coleoptera: Chrysomelidae)**" and presented by **Muhammad SALIM** 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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THESIS CERTIFICATION

It is certified that I have written this thesis by myself. I further confirmed 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 SALIM

ÖZET

PATATESTE GEN PİRAMİT TEKNİĞİ KULLANARAK PATATES BÖCEĞİNİN, *LEPTINOTARSA DECEMLINEATA* (SAY), (CHRYSOMELIDAE: COLEOPTERA), DİRENÇ YÖNETİMİ

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İnsektisidal gen aktarılmış transgenik patates bitkilerinin *Leptinotarsa decemlineata* (Say) larva ve erginlerinin yaşam tablosu değerleri ile bu bitkilerin toksik ve davranışsal etkileri araştırılmıştır. Çalışmada iki *Bacillus thuringiensis* geni (*cry3A* ve *35S-SN19)* ve bitki proteinaz inhibitörü *Oryza cystatin II* (*OCII*), iki farklı kombinasyon olan *cry3A + 35S-SN19* ve *OCII+ 35S-SN19* şeklinde patates çeşitleri Agria ve Lady Olympia'ya aktarılmıştır. Yaşam tablosu çalışmasında transgenik bitkilerde beslenen larva ve erginler bitkilerden kaynaklanan toksite nedeniyle ölmüş olup yaşam tablosuna ait değerler yalnızca transgenik olmayan bitkilerde yetiştirilen patates böcekleri için hesaplanmıştır. Lady Olympi'da yetiştirilen böcekler için doğal artış oranı (r) 0.15/gün, üreme gücü sınırı (λ) 1.16/gün, net üreme gücü (R_0) 233.81 yavru/dişi ve ortalama döl süresi (T) 37.43 gün bulunmuştur. Agria'da yetiştirilen böcekler için r, λ, R_0 ve T sırasıyla 0.12/gün, 1.13/gün, 120.81döl/dişi ve 39.75 gün olarak hesaplanmıştır. Transgenik bitkilerin larva ve erginlerde yüksek oranda ölümlere neden olduğu, larvalarının erginlere oranla daha hassas olduğu saptanmıştır. Sonuç olarak, gen piramit tekniği ile elde edilen transgenik patates bitkilerinin imidacloprid dirençli patates böceği populasyonlarının kontrolünde kullanma imkânına sahip olduğu düşünülmektedir.

Anahtar Sözcükler: Patates böceği, Imidacloprid, İnsektisit direnci, Gen piramit tekniği, Yaşam tablosu, Patates, Agria ve Lady Olympia

SUMMARY

PYRAMIDING OF INSECTICIDAL GENES IN POTATO TO ENCODE RESISTANCE AGAINST COLORADO POTATO BEETLE, *LEPTINOTARSA DECEMLINEATA* (SAY), (CHRYSOMELIDAE: COLEOPTERA)

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Lethal, behavioural and life table parameters of Leptinotarsa decemlineata were studied on transgenic potato plants under laboratory conditions. Two Bacillus thuringiensis genes (cry3A and 35S-SN19) and one plant proteinases inhibitors Oryza cystatin II (OCII) were transferred with two different combinations of cry3A+ 35S-SN19 and OCII+ 35S-SN19 into cultivars Lady Olympia and Agria. The life table parameters of CPB were studied only commercial potato cultivars due to high mortality on transgenic counterparts. The intrinsic rate of increase (r), the finite rate of increase (λ), the net reproductive rate (R0) and the mean generation time (T) were 0.15/day, 1.16/day, 233.81 offspring/female and 37.43 days on Lady Olympia while r, λ , R0 and T were 0.12/day, 1.13/day, 120.81offsprinf/female and 39.75 days on cultivar Agria, The transgenic plants caused high mortalities and larval stages were more susceptible than the adult stage. The data regarding foliage consumption showed that a very low level of consumption in all transgenic plants as compared to their control plants. All these results indicate that these transgenic potato plants exhibit high level toxicity to CPB and the transgenic potato plants with gene pyramiding technique could provide a useful tool in control of imidacloprid resistant CPB population.

Keywords: Colorado Potato beetle, imidacloprid, insect resistance, gene pyramiding, Two sex life table, Potato, Agria, Lady Olympia

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SYMBOLS AND ABBREVIATIONS

Symbols	Descriptions
BAP	6-Benzylaminopurine
bp	Base Pair
Bt	*Bacillus thuringiensis*
CPB	Colorado potato beetle
cm	Centimeter
Cry	Crystal
DNA	Deoxyribonucleic acid
DS-1	Double stacked 1
DS-2	Double stacked 2
EDTA	Ethylene diamine triacetic acid
ELISA	Enzyme linked ımmunosorbent assay
FAOSTAT	Food and agriculture organization statistical databases (United Nations)
Ti plasmid	Tumor inducing plasmid
MS medium	Murashige and skoog medium
NAA	1-Naphthaleneacetic acid
µM	Micro-molar
mM	Milli-molar
g/ L	Gram per liter
mg/ L	Milligram per liter
ml	Milliliter
µl	Microliter
µmol m-2 s-1	Unit of measuring light (micromoles per meter square per second)
W	Watt
%	Percent
°C	Degree centigrade
LB	Luria-bertani medium

pH	Power of hydrogen
PCR	Polymerase chain reaction
PI	Proteinase Inhibitor
OCI	*Oryza cystatin I*
OCII	*Oryza cystatin II*
ddH2O	Double-distilled water
dNTPs	Dinucleotide triphosphate
RSM	Regeneration selection medium
NaCl	Sodium chloride
U	Units
NaOH	Sodium hydroxide
PBS	Phosphate saline buffer
SDS	Sodium dodecyl sulphate
ng	Nano gram
OD	Optical density
rpm	rounds per minute
RNA	Ribonucleic acid
UV	Ultraviolet
Kb	Kilobase
KCl	Potassium chloride
TE	Tris ethylene diamine tetra acetic acid

CHAPTER I

INTRODUCTION

Potato (*Solanum tuberosum* L.) is one of the most important food crops worldwide which ranks 3rd after rice and wheat in terms of human consumption. Potato is regarded as one of the most important crops with its ability to overcome malnutrition and poverty related issues of farmers in the world due to its high capability to grow well in adverse conditions, high yield potential with a very high harvesting index above 75% and its high nutritional value (Scott et al., 2000; Thiele et al., 2010; Munib et al., 2016). The Potato tubers are highly nutritive as they are rich sources of carbohydrate, vitamins, protein and minerals such as calcium and potassium and antioxidants that are associated with many health benefits that include lower rates of heart disease, immune system improvement, reduce risk of cataracts, macular degeneration and reductions in some types of cancers (Brown, 2005). According to FAO, the total world potato production was 381,682,000 tonnes in 2014 (FAOSTAT, 2017). The literature shows that there are over 4000 edible varieties of potato grown throughout the world and is regarded as food of more than a billion poor people worldwide (Vincent et al., 2013). Major producer countries of potato are China, Russia, India, USA, Ukraine, Poland, Germany, Belarus, Netherlands and France. These countries share approximately 70 per cent of the total potato production (FAOSTAT, 2017). Turkey ranks 13th in terms of potatoes production. Potato contributes 3% to the Turkey national economy and ranks first in Turkey in terms of trade, area and production among tuber crops. It is a highly significant crop, provides raw material for agricultural industry in Turkey. Potato is grown on an area of 128392 hectares in Turkey. Annual production of potato in Turkey is 4166,000 tonnes with an average yield of 324475 hectogram per hectare (FAO STAT, 2017). Central Anatolia including Niğde shares more than 61% potato production in this regard (Anonymous, 2000).

Potato is vegetatively propagated crop which is plagued by numerous biotic and abiotic factors resulting in 40 % crop losses in potato fields and storages (Oerke, 2006). Among biotic factors, disease organisms and insect pests are major biotic constraint in potato production. The most common pests are fungal diseases including Verticillium wilt (*Verticillium dahlia* K.), Fusarium dry rot and wilt, early blight (*Alternaria solani* S.), black scurf (*Rhizoctonia solani* K.), viral diseases including PVA, PVS, PLRV, PVX, and

PVY and bacterial diseases including common scab (*Streptomyces scabies* L.) (Kepenekci et al., 2013).

The literature concerning potato insect pests is one of the greatest one. For example, a search done in 30th September 2018 in the database Scopus with the keywords "potato insect pests" yielded 1937 entries revealed that potato crop is heavily attacked by insect pests. Major insect pests include potato tuber moth (*Phthorimaea operculella* Z.), Colorado potato beetle (*Leptinotarsa decemlineata* (Say), leaf hoppers (*Empoasca fabae* L.) Cutworms (*Agrotis ipsilon* (H)) and disease vectors especially aphids and whiteflies (Vaneva and Dimitrov, 2013).

Among these insects, Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say), (Coleoptera: Chrysomelidae) is the most devastating and damaging pests of potato in Turkey and in the rest of the world (Kivan, 2004; Alyokhin, 2009) and its impact is measured on potato production on a world scale (Kaplanoglu, 2016). The first report on CPB was made in 1811 by Thomas Nuttall but then reported by Thomas Say in 1824 (Jacques, 1988). This oligophagous pest is native to United States and Mexico, but presently distributed throughout Europe, western Asia and United States (Alyokhin et al., 2008). This pest occurs in most areas where the host plants like potatoes or other solanaceous are grown (Alyokhin, 2008). The first major outbreak of CPB infestation was in Nebraska state where it happened during 1859 in potato fields (Jacques, 1988). Over the next few years, CPB population increased rapidly and reported in Europe and the rest of the continent (ca. 1875) and Asia. Currently, CPB population is spread to Asia, Europe and North America and continues to spread (Alyokhin et al., 2008). The pest has a very diverse and complicated life history, high reproduction rate together with high foliage consumption, (one CPB female on average laying 300-600 eggs), dispersal and diapause mechanisms, and its bet-hedging reproductive strategies, its ability to adapt to different climatic conditions make it a very complicated and challenging agricultural pest to control. Both immature stages and adults of CPB are active leaf feeder, feed mostly on the green parts of the plants, making irregular holes in and along leaf margins, but they also attack stems and exposed tubers (Alyokhin, 2009). Most of the damage is done by the fourth larval instar and adult stage. A single 4th instar larva can consume approximately 40 cm^2 leaf area, while at adult stage it is approximately 10 cm^2 leaf area per day (Ferro et al., 1985). Extensive feeding results in reduction in leaf surface area,

decrease in plant vigor to produce and store nutrients, which affects tuber size and number, resulting in reduced yield of potato. If left uncontrolled, the population may increase abruptly and results in decrease of potato yield by 85%. (Roush and Tingey, 1994; Zhou et al., 2012). The pest passes winter in soil (7.6 to 12.7 cm deep in soil as diapausing adults) (Lashomb and NG, 1984). These CPB adults (Overwintered beetles) emerge from soil in early to mid-May depending on the climate of the territory and their physiological conditions. In the central Anatolia and Thrace region, the beetle passed through two generations, the first generation causing yield loss in potatoes whereas the second generation, cause damages to eggplants (Hare, 1990; Has, 1992).

Different techniques are used for the control of *L. decemlineata*. These include cultural control, physical control, screening, sex pheromones, biological control and chemical control. Among these, chemical and biological control methods are most effective in controlling *L. decemlineata* infestation in potato. Potato production on large scale without using insecticides to control CPB, is nearly impossible because use of chemical insecticides against CPB have made the foundation for the control for CPB and approximately 34 % of total insecticide use is used in potato crop is mostly applied for control of CPB, due to this fact CPB has been credited for the creation of modern insecticide industry (Gauthier et al., 1981).

However, extensive use of insecticides in potato against *L. decemlineata* have so many adverse effects like insecticide resistance, effect on non-target organisms, emergence of new pests, destruction of natural enemies, human intoxication, pesticides residue in food chain and environmental pollution (Bakhsh et al., 2014; Gokce et al., 2012; Sohail et al., 2012; Saljoqi et al., 2015). Also, high and continuous selection pressure of different insecticides against CPB has resulted in insecticide-resistant in different CPB populations. Due to this fact, CPB population has develop resistance to every insecticides used against them and present reports suggest that CPB has evolved resistance to more than 56 chemical insecticides belonging to different major insecticides groups (www.pesticideresistance.org). Similarly, conventional breeding programs for developing insect resistant potato cultivars are often laborious and unsuccessful due to the fact that potato plant is tetraploid and hence it is very difficult to produce insect resistant variety. Till now, no single potato cultivar is commercialized that give complete protection against CPB.

To overcome all these problems of pesticides and conventional breeding programs, scientists have started to seek safer alternatives for managing CPB such as the use of plant resistance and *Bacillus thuringiensis* in a transgenic form to replace toxic chemicals in their integrated pest management (IPM) strategies. Usefulness of IPM strategies are to reduced insecticides burden and application costs, increased efficacy against selected target insect pests and to minimize pests scouting needs as compared to calendared based insecticide spray programs (Dhaliwal et al., 2004).

The most successful story in Genetic engineering is to transform genes of economic benefit from other plants into other cash crops such as cotton, tobacco and other crops to develop resistance against insect pests (Dhaliwal et al., 2004). During last 30 years, various methods have been used to develop insect resistant transgenic crops and to reduce the unnecessary pesticides usage (Christou et al., 2006). These transgenic plants are gaining popularity among farmers and considered as one of important components of pest management program in some countries (Kos et al., 2009). There is no doubt that among the transgenic plants, those plants containing *Bacillus thuringiensis* (*Bt*) toxins, have drawn high attention both economically and ecologically and achieved significant success against various insect pests species. The advantages of Bt toxins are that these Bt toxins kill major target pests only and cause little or no harms to other organisms.

Bacillus thuringiensis (*Bt*) is considered as the most imperative source containing resistant genes against various insect orders. *B. thuringiensis* is an aerobic, gram positive, spore-forming, soil-dwelling bacterium that hoards high host specificity with acute crystal insecticidal proteins during sporulation. These crystal proteins of spore-forming soil bacterium are toxic to larvae as well as some adult stages of chewing insects of different orders e.g. Coleoptera, Lepidoptera, Homoptera, Diptera, Hymenoptera, Mallophaga and Orthoptera, and to mites, nematodes and protozoa (Herrnstadt et al., 1986; Andrews et al., 1987; Hofte and Whiteley, 1989; McGaughey and Whalon, 1992; Cohen et al., 2000). Spores of *B. thuringiensis* are already widely used as biopesticide in commercial agriculture to control several key pests. These *Bt* spores are sprayed against insect pests in various crops (Gasser and Fraley, 1992). The introduction of the Bt toxin gene via genetic engineering makes host plants resistant against target insect pests. The crystal protein cry3A was expressed in potato and provided resistance against the most serious defoliating pest *L. decemlineata* (Adang et al., 1993; Perlak et al., 1993).

Many studies have documented different cry genes that produce specific delta-endotoxin proteins effective against different insects' orders in different economically significant crops. For example, cry toxins (cry1Ia and cry1Ba) are found to have very toxic effect against Lepidopteran insects. Similarly, cry toxin (cry1Ia) has high toxicity against coleopterans insects. The hybrid combination of these two-cry genes resulted in the hybrid *B. thuringiensis* delta-endotoxins (*SN19* gene) that gives resistance against both coleopteran and lepidopteran pests in a single potato plant. A Hybrid gene (*cry1B*a/*cry1Ia*) that consist of domains I and III of Cry1Ba and domain II of Cry1Ia, was designed by Naimov et al. (2001, 2003) and has high toxicity against Colorado potato beetles. Transgenic plants produced from this hybrid gene construct were toxic to both immature stages of CPB and adults as well as insects belonging to other groups such as immature stages of European corn borer and potato tuber moth (Naimov et al., 2003).

In addition to Bt toxin, plant proteinase inhibitors (PI) which are a group of plant protective proteins play significant role in protecting commercial crops against destructive insect pests. These PIs are produced as result of different biotic as well as abiotic factors stresses such as pest attack, temperature ups and down, UV radiation and mechanical wounding (Leo et al., 2002). After ingestion due to their inhibition activity, these PIs has the capacity to bind to catalytic sites of insect digestive proteinases resulting in the formation of stable complexes. Insects suffer deficiencies of important and essential nutrients such as amino acids necessary for insect normal growth and development and can lead to high mortality in the target insect pests (Schlüter et al., 2010; Smigocki et al., 2013). These PIs give resistance to many transgenic plants against different families of insects (Tanpure et al., 2017). Plant transformation with a gene coding for a cysteine proteinase inhibitor (cystatin) proposed as a key strategy to hinder with the digestive physiology of Hemipteran and Coleopteran insect pests (Liang et al., 1991). *Oryza cystatins* (OCs) isolated from rice (*Oryza sativa*) are among the well-known proteinase inhibitors of digestive cysteine proteinases. These *OCs* have the ability to provide resistance in crops against insect pests and pathogens that owns cysteine proteinases for digestive protein hydrolysis (Urwin et al., 1995; Ribeiro et al., 2006). Since cysteine proteinases targets are absent in the human gut but are the main proteolytic enzymes in *L. decemlineata* gut, makes it appropriate for human consumption as well as development of CPB resistant potato crops containing *OCs* genes (Arai and Abe, 2000).

First generation *Bt* crops (plants transformed with single gene) efficiently controlled some of the major agricultural insect pests but their selective toxicity appeared as a big hindrance for their continuous use in future. Generally, several insect species attack a single plant at different growth stages, therefore plants having only one gene which is effective against a single target species are not able to shield itself against other insects attacks. Due to various reports of insect resistance against transgenic crops, they have established worries among the farmers communities on the durability of these *Bt* crops (Luttrell et al., 2004; Ali et al., 2007; Bravo et al., 2008; Tabashnik et al., 2008; Gassmann et al., 2011; Zhang et al., 2006).

In insect, field evolved resistance is related to prolonged exposer to toxin in the field. The resultant resistance against the specified toxin in the pest population is related to genetically change that resulted in susceptibility reduction of a pest population to a specific toxin (Tabashnik et al., 2013).

In case of increased insect resistance to transgenic *Bt* plants, it is imperative to develop new strategies for integrated pest management. The most important method for inhibiting process of evolution of pest resistance against transgenic crops is the gene pyramiding technique. Pyramiding involve the use of stacking multiple genes that leads to at a time multiple expressions of target genes in a single transgenic cultivar or variety (Manyangarirwa et al., 2006). Insect pest control with *Bt* crops can be enhanced with the use of pyramid *Bt* crops. These pyramid *Bt* crops consist of different distinct genes that kill insect pests belonging to different families (Carriere et al., 2015). Most of these toxins produced by pyramid *Bt* crops belong to either the Cry protein family or to the vegetative insecticidal protein (Vip) family. These pyramids Bt crops were first commercialized in 2003 and recently have become increasingly widespread in the USA and other countries (Carriere et al., 2015). For example, in 2014, a pyramid cotton producing Bt toxins cry1Ac and cry2Ab accounted for 96% of the 12 million ha of *Bt* cotton in India (Choudhary and Gaur, 2015). The using of pyramiding several *Bt* genes have certain advantages for resistance management as compared to the plants containing single toxins because pyramided *Bt* crops have different toxin combination with different mode of action to circumvent cross resistance (Roush, 1998; Ferré and Van Rie, 2002).

Gene pyramiding studies with different toxins in various plants have been reported with different binding sites in the lepidopteran midguts (Hernández and Ferré, 2005). Computer simulations studies predicted that the possibility of pests evolving instant resistance to two Bt toxins would decrease if the toxins had different binding sites (Roush, 1998). These evidences can be helpful in designing different combinations of Cry toxins and plant Proteinase inhibitors for effective management of resistant development in different insects against *Bt* crops. The development and successful incorporation of gene pyramiding technology in potato against Colorado potato beetle with broad spectrum resistance will be advantageous for the farming community, enabling them to tackle targeted insect pest by reducing insecticide inputs significantly, ultimately leading to high crop productivity and boost in the economy. Therefore, good agronomic practices and sustainability of potato production are very important for agriculture and mankind in the future.

Life table studies are very important and a fundamental tool in a study related to population ecology. Many ecologists have used life table parameters in their studies to predict the development and projection of insect population and to compare the fitness cost of insect populations under different conditions. In traditional female-based age-specific life tables, male insect populations are ignored, moreover there is no stage differentiation which is very important while studying life table parameters (Lewis, 1942; Leslie, 1945; Carey, 1993). Due to these limitations, female-based life tables can be erroneous and misguiding due to errors in results (Huang and Chi, 2011). Therefore, one of the main aims of this study is to study the life table parameters of CPB on different transgenic plants of Agria and Lady Olympia and their control plants using the correct age-stage, two-sex life table theory.

1.1 Aims and Objectives

The main aim of this thesis is to study the lethal and sub lethal effects of transgenic potato plants produced by gene pyramiding technique on Colorado potato beetle.

In order to achieve this objective, the following parameters were studied;

1) Isolation and cloning of three insecticidal genes (*cry3A*, *35S-SN19* and *OCII*) from another source.
2) Cloning of insecticidal genes in stacked form in plant expression vector (pCAMBIA 1301).
3) Agrobacterium mediated transformation of potato cultivars with plant expression vector harboring insecticidal genes.
4) Confirmation of gene integration and expression in primary transformants.
5) Leaf biotoxicity assays to determine efficacy of introduced gene(s) against Colorado potato beetle (*Leptinotarsa decemlineata*).

Studying the life table parameters of *L. decemlineata* on transgenic potato and their respective control plants.

CHAPTER II

REVIEW OF LITERATURE

2.1 Importance of Potato Crop

Potato, *Solanum tuberosum* L., is one of the world's most important staple food crops in human history (Vincent et al., 2013). Potato has various nutritional values and are consumed as fresh and processed forms as human food, animal feed and chips etc. in underdeveloped and malnourished countries (Burhans, 2008; Kart et al., 2017). According to The United Nations Food and Agriculture Organization (FAO), potatoes are at the top of the list of products that contributes to the resolution of these problems, faces the inauguration and undernourishment of millions of people and has declared 2008 as " the International Year of the Potato (IYP)" to increase awareness and recognize the impact of potato on human life (Giordanengo et al., 2008).

Turkey ranks 13th in terms of potatoes production. Potato play a jugular vein role in Turkey economy and a good chunk of the population in the country depends directly and indirectly on agriculture. It is known that cultivation of potato is primary source of income for tens of thousands producer's families and the most important staple food item for many families in Turkey (Kart et al., 2017). It was reported that about 56% of the potatoes produced are consumed freshly in Turkey (Kart et al., 2017). Potatoes has a history of about 150 years in Turkey; are grooming as a sector by the production, marketing and consumption phases. In fact, it is the natural agro-ecological resources of Turkey that evidenced it to have a very privileged position in terms of potato production (İşler, 2012). In Turkey, potato is grown in more than 70 provinces, including Niğde, which shares more than 14% potato production in this regard (TÜİK, 2015; Kart et al., 2017). The potato crop experienced both biotic and abiotic stress factors in all stages of its vegetation period, resulting in decrease production of potato in Turkey as compared to other developed countries. The major constrains are biotic factors which primarily includes insect pests and disease outbreaks. The literature reported more than 270 insects and 17 mites species that attack potato in fields and storage conditions worldwide (Alkan et al., 2017). In some cases of sever CPB attack, up-to 100% yield losses in potato have been documented (Oerke, 2006; Alyokhin, 2009).

Major insect pests include potato tuber moth (*Phthorimaea operculella*), Colorado potato beetles (*Leptinotarsa decemlineata*), leaf hoppers (*Empoasca fabae*) and Cutworms (*Agrotis ipsilon*) (Vaneva and Dimitrov, 2013; 1999). Among these insects, Colorado Potato beetle is considered as the most devastating pests of potato worldwide (Alyokhin, 2009). CPB is considered as a one of the major limiting factors to potato production in many countries including Turkey. CPB is also considered as a harmful pest of other solanaceous crops like egg plants, tomatoes and other night shade plants (Kostic et al., 2016).

2.2 Introduction to Pest

2.2.1 History of pest status

The Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Chrysomelidae: Coleoptera), is one of the most economically significant pests of solanaceous crops which include eggplant (*Solanum melongena* L.), potato (*Solanum tuberosum* L.), tomato (*Solanum lycopersicum* L.) and several other species in the Solanaceae family (Grafius and Douches, 2008). Besides, CPB also feeds on a number of weeds which include belladonna, henbane, horse nettle, jimson weed, mullein and thistle (Kuepper, 2000). The pest is thought to have originated in the southern United States and central Mexico and earned its public name after establishing a reputation as a serious pest species in North America (Alyokhin et al., 2008). The pest remains unnoticed till 1859, when the major outbreaks occurred in potato fields about 100 miles west of Omaha, Nebraska (Jacques, 1988). The word “Colorado” was not reported until 1865. Walsh and his colleagues in 1865 found beetles feeding on buffalo-bur in the territory of Colorado. They confirmed that these beetles were native to Colorado. However, it was Riley, who used the word Colorado potato beetle in 1867 (Jacques, 2003).

After the start of potato cultivation in the southern United States, CPB quickly adapted to this new host and became one of the world’s most important destructive insect pest of the plant family Solanaceae (Hare, 1990). Since then, CPB swiftly switched to feeding on the cultivated potato plants (Casagrande, 1987; Jacques, 1988; Weber, 2003).

The succeeding expansion in beetle geographic range was mind-boggling, reached to Canada before 1880 (Radcliffe and Lagnaoui, 2007) and currently established in most provinces, including Prince Edward Island, New Brunswick, Manitoba, Québec, Ontario and Alberta, where most of Canadian potato cultivation occurs (AAFC, 2013). During World War I, CPB was also introduced to Western Europe, where the first population was established in France in 1922. CPB infestation in Turkey (Edirne province) was first observed in 1963 (Has, 1992). Later, the insect started to approach other host and was further reported on bitter sweet (*Solanum dulcamara* L.), deadly nightshade (*Atropa belladonna* L.) and Jimson weed (*Datura stramonium* L.) in Turkey (Has, 1992). By the end of the 20th century, it rapidly spread to Asia and Western China. Currently, the beetle's range covers about 16 million km^2 and continues to expand (Weber, 2003).

2.2.2 Life cycle of CPB

Colorado potato beetle is a multivoltine species with complex and diverse life history which varies greatly from area to area and due to climatic conditions (Jacques, 2003). *L. decemlineata* has a facultative overwintering diapause stage that plays a highly significant role in their adaptation to the surrounding environmental conditions, and greatly confers to its success as a pest of cultivated potatoes (Alyokhin et al., 2015). CPB remains in diapause stage for about four months, though some studies have documented long-term dormancy patterns lasting for one or more years (Senanayake et al., 2000; Tauber and Tauber, 2002). The diapause stage takes place in soil as adult in close vicinity to fields where they have passed the previous summer and is usually induced by short day light duration (Alyokhin, 2009). Survival during the cold weather during the diapause stage is a very tough process, due to which high rate of mortality occur in overwintering CPB adults ranging from approximately 50 to 75% (Alyokhin et al., 2008).

The beetle emergence is more or less synchronized with potato plant phenology. Post-diapause beetles walk or fly from their overwintering sites. In case of non-rotation CPB adults colonize in the same field and then after overwintering, they either emerge in the same filed or move on from their overwintering sites. In case of rotation, the beetles can fly up to 100 kilometers to find most suitable host habitat (Ferro et al., 1991; 1999). After colonization in spring, the beetle continues to feed on newly sprouted host for several days and then start mating (Koštál, 2005; Yocum et al., 2011). CPB adults can also fly

over short distances in colonies, where they lay eggs and find new adults for mating (Tauber, 2002).

CPB is a polygamous insect, both adults (males and females) perform several mattings with different partners during their life time (Alyokhin, 2009). Male CPB protects the female during and after the mattings and display aggressive actions towards other males (Szentesi, 1985). The Female CPB after reaching maturity, secret sex pheromone in air in order to attract males for mattings (Alyokhin, 2008). It is reported that in order to completely fill the female's spermatheca, at least three mattings are required for a female to reach its full reproductive potential (Boiteau,1988a).

Once mated, the females start oviposition within a week or more depending upon the environmental conditions. The eggs are laid in batches, one batch contains 40 to 70 eggs which is laid on the underside of host leaves (Kostic et al., 2016). Colorado potato beetles are very fertile. An average female produces up to 4000 eggs under laboratory conditions and up to 800 eggs over her lifespan under filed conditions (Hare, 1990). Females can recognize their own species eggs and avoid oviposition on the same leaflet. Eggs hatch takes 4-10 days depending upon the environmental conditions (Capinera, 2001).

Larvae after hatching, start feeding directly or move over short distances to find host. Feeding starts within 24 hours of eclosion (Alyokhin, 2008). The larvae have a tendency of maintain body temperature. The larvae are even capable of feeding on the upper surface of host leaves at low ambient temperatures and as ambient temperatures increase, they regulate their body temperature and start moving within plant canopies (May, 1981; Lactin and Holliday, 1994; Alyokhin, 2009). Immature stages consisted of four larval instars that last for a total period of 20-25 days (Capinera, 2001). Pupation takes place in soil near the host plant at an average depth between 5-12 cm, where the last larval development has been completed (Feytaud, 1938). Pupal stage lasts for 5 to 10 days (Capinera, 2001). Depending on environmental conditions, total development from egg to larvae, larvae to pupae and pupae to adult stage takes place from 3-4, 8-11, 9-12 and 45-65 days respectively (de Wilde, 1948; Ferro et al., 1985; Logan et al., 1985). Fast CPB growth occurs between 25-32°C but it seems to vary greatly among different CPB populations having different geographic origins (Boiteau et al., 2008). Shortly after adult's emergence, the adults start developing their reproductive system and flight

muscles (Alyokhin and Ferro, 1999). However, photoperiod has strong impact on CPB adults i.e. those CPB adults that have not well-developed reproductive system and flight muscles are due short-day photoperiod. They feed aggressively for several weeks and then either directly burrow into the soil or walk to the overwintering sites in the fields (Voss, 1989).

There are 3-5 generations per year, depending upon locality and climatic conditions; however, in the central Anatolia and Thrace region of Turkey, two generations have been reported during the season, the first-generation causing yield loss in potatoes whereas the second-generation damages eggplants (Kivan, 2004).

Complex and diverse life history patterns of Colorado potato beetles and their adoptability to harsh and adverse environmental conditions, makes CPB a challenging and difficult pest to control with the existing techniques (Alyokhin, 2008; Alyokhin, 2009). Aggressive feeding, high fecundity rate and flight dispersal connected to diapause stage, allow the CPB to use bet hedging reproductive strategies i.e. during this strategy CPB disperse its offspring's in both within and between fields as well as in time both within and between years, resulting in minimal risk of mortality losses of offspring.

Due to the above-mentioned reasons, the control of this disastrous pest with use of chemical insecticides is wasting of money as well as very difficult task to control CPB populations with the existing techniques.

2.2.3 Colorado potato beetle control

2.2.3.1 Cultural and biological control

CPB populations can be kept under control through combination of several common cultural practices which includes crop rotation, manipulating planting time, planting trap crops and mulching. Crop rotations being widely implemented and effective strategy in controlling CPB infestations. The effect of crop rotation on CPB population was quantified in a study where 95.8% reduction in adult density were observed when potato fields were rotated with a non CPB host crop like wheat or Ry (Weisz et al., 1994). Manipulating time i.e. early planting of potato may help to escape infestation caused by

second generation larvae due to crop harvesting before the emergence of CPB larvae. Similarly planting trap crops that attract beetles and portable field edge trap keeps the overwintering CPBs away from entering the main crops (Boiteau et al., 1994).

Several natural enemies (arthropods species) are capable to control CPB population in fields and some of them have shown potential as biological control agents, but due to heavy pesticide use, their populations are not enough high to control CPB infestation in commercial potato fields. For example, augmentative release of two spotted stink bugs *Perillus bioculatus* (F) and *Podisus maculiventris* (Say) (Hemiptera: Pentatomidae) significantly effect CPB population in potato fields. These bugs feed on the CPB larvae and suppressed their population by 62% and increase potato yield by 65% as compared to control plots (Biever and Chauvin 1992; Cloutier and Bauduin, 1995).

Some beetles e.g. *Coleomegilla maculata* (De Geer) (Coleoptera: Coccinellidae) and *Lebia grandis* Say feed on CPB eggs and larvae. The mortality ranges from 37.8% to 58.1%. Besides these, a large number of CPB parasitic flies e.g. *Doryphorophaga doryphorae* (C) and *D. coberrans* parasitize CPB larvae; and a wasp, *Edovum puttleri*, parasitizes the eggs and have shown significant effect on CPB population. Due to heavy pesticides usages in potato fields and high reproductive output of biological control agents, none of the known natural enemies of CPB have been commercialized (Weber, 1994).

2.2.3.2 Chemical control and insecticides resistance

Insecticides have been the backbone of controlling *L. decemlineata* population on commercial farms. Potato production on large scale without using insecticides to control CPB is nearly impossible because chemical insecticides have taken important place in control of CPB and approximately 34 % of total insecticide sprayed on potato crop is used for control of CPB. Due to this fact, CPB has been credited for the creation of modern insecticide industry (Gauthier et al., 1981). The first broad spectrum insecticide, lead arsenate (Paris green) was dusted on potato leaves by growers in 1865, to protect their crop from CPB foliage damage. Then series of hundreds of chemical compounds with specifically invented application equipment to aid their delivery had been used against this pest during the succeeding decades (Gauthier et al., 1981). DDT and other

organochlorine insecticides were efficiently used against CPB as early as 1939 (Hitchner, 1952; Gauthier et al., 1981). The first report of CPB resistance on DDT in New York dated back to 1952 (Quinton, 1955). Similarly, resistance to other chlorinated hydrocarbons like dieldrin was documented in 1958 (Hofmaster et al., 1967). Since then, failure of insecticides has been reported for wide range of active compounds. Resistance to other organochlorine insecticides like Endrin was reported in 1960 just 3 years after its release. Resistance to Ensulfan was observed in 1991 in the same year of release in the market. Recently organochlorine resistance in CPB population has been reported to increase up to 220X (Sharif et al., 2007). Resistance to organophosphates compounds viz azinphos methyl was reported in 1964, five years after its first release in the market in 1959. Monocrotophos another organophosphates released in 1973 against CPB, resistance was reported in the same year. Similarly, Phorate (organophosphate) released in 1973 for the control of CPB while just one year after its release, resistance was reported in CPB population. Recently, this level of resistance to organophosphates in CPB population has been recorded to enhance up-to 252.1X (MalekMohammadi et al., 2010). The first report of CPB resistance against carbamate (Carbaryl) was documented in 1963, four years after its release in the open market. Resistance to other carbamates viz Carbofuran and Oxamyl were reported in 1976 and 1978 respectively. Recently carbamate resistance in CPB population has been observed to increase up to 18.7 X (Jiang et al., 2010). By the early 1980s, CPB resistance against insecticide had reached an alarming level. In fact, in all major potato growing areas, CPB populations could not be controlled using insecticides. Use of Organochlorines, Organophosphates and carbamates insecticides against CPB had been gradually replaced by pyrethroids during the late 70s and 80. Pyrethroids resistance in CPB was first reported in 1981, just two years after its release in 1979. Currently, CPB resistance to pyrethroids has been reported to increase as much as 2749 times in the field strain (Jiang et al., 2010). Since then, the pyrethroids have been replaced by neonicotinoids and other compounds of novel chemistry during the late 90s. These new chemistry insecticides successfully control CPB infestation in fields where CPB has already developed resistance to every used chemical insecticide used against them. These insecticides in 1995 gave the growers a short period of relief from the devasting pest (Whalon and Ferro, 1998). The first neonicotinoid imidacloprid was used against CPB in USA in 1995. However, CPB resistance to imidacloprid were documented in the same year, reported up-to 100-fold levels of resistance in CPB adult population (Grafius and Bishop, 1996; Zhao et al., 2000). Since

then, after three years of imidacloprid uses, regular monitoring of CPB adult samples from Long Island in USA in 1997 had been observed with more fold increases in resistance to imidacloprid (309-fold) as well as to other active compounds such as dinotefuran, thiamethoxam, acetamiprid, clothianidin, nitenpyram and thiacloprid, despite of the fact that these never having been used in the field at that time (Oslon, et al., 2000; Zhao et al., 2000; Mota-Sanches et al., 2006). In the past six decades, it has been reported that *CPB* has evolved resistance to more than 56 different active compounds belonging to different groups (including imidacloprid and eight other neonicotinoids) (Whalon et al., 2013). Over 50 years, various *Bt* insecticides products have been used in potato crops to control different insect pests (Cannon, 1993), however, due to their high cost and host specificity, environmental sensitivity and non-uniform pest control, they have not gotten enough space among farmers communities in commercial crop production (ILSI, 1998; Castagnola and Jurat-Fuentes, 2012). Practical knowledge regarding how insect develop resistance against *B. thuringiensis* products were also published, indicating that management by single Bt toxin is likely not a sustainable method for control of CPB (Rahardja and Whalon, 1995).

2.3 Mechanisms of Insecticide Resistance in *Leptinotarsa decemlineata*

Resistance in insect herbivore has been referred as "the evolved ability of an organism to survive in response to a selective pressure from exposure to a pesticide by resistance genes". Worldwide more than 4,000 examples of resistance have been reported in about 500 species of insects due to continuous exposure to different insecticides (Georghiou and Lagunes-Tejeda, 1991). Even though IPM alternatives exist, still CPB populations are mostly controlled by insecticides in large scale crop production. The pest has gained notoriety for its tremendous ability to evolve resistance to every insecticide used against it and has evolved insensitivity to more than 56 different active compounds belonging to different insecticide groups with different mode of actions including organochlorines, carbamates, organophosphosphates, pyrethroids, neonicotinoid and Nereis toxin analogues. Different mechanisms have been reported to be associated with the development of insecticide resistance in CPB. These biochemical mechanisms may act individually or concurrently.

Known mechanisms of CPB resistance to various formulations of *B. thuringiensis* and other microbial agents and chemical insecticides include target site mutations or insensitivity, reduce or less insecticide penetration and enhanced insecticide excretion, increase in metabolism by detoxifying enzymes (esterases, carboxylesterases and monooxygenases) and behavioral resistance (Anspaugh et al., 1995; Hoy and Head, 1995; Alyokhin and Ferro, 1999d; Clark et al., 2001). In the Colorado potato beetle, the most studied mechanism of resistance is due to the P450 mediated detoxification system which has been reported across many CPB populations in many countries.

Many studies reported that the piperonyl butoxide (PBO) which is oxygenase inhibitor (P-450 inhibitor) enhances efficacy of imidacloprid, permethrin, abamectin, carbofuran, fenvalerate, azinphosmethyl and carbaryl in high resistant strains of CPB (Soderlund et al., 1983; Rose and Brindley,1985; Ahammad-Sahib et al., 1994; Yoon et al., 2002; Mota-Sanchez et al., 2006).

Another important mechanism of resistance in CPB against insecticides is target site mutation. Resistant to organophosphates and carbamates is mostly associated due to mutation of acetylcholine esterase. Azinphosmethyl resistant in CPB is due to the presence of two mutations in the AChE i.e. S291G and R30K. These two mutated AChEs are responsible for resistance of CPB to azinphosmethyl and carbofuran activities (Kim and Clark, 2002; Stankovic et al., 2004; Kim et al., 2006). However, the target site mutation is not only restricted to AChE, e.g. CPB population resistant to permethrin has a mutation in leucine to phenylalanine, L1014F that leads to change in the sodium channel (Lee et al., 1999; Kim et al., 2005).

Incomplete or reduced penetration and enhanced excretion appear to be less important in reducing CPB sensitivity to insecticides. Yet, they also have their role i.e. these mechanisms may act simultaneously with other mechanisms to enhance metabolism and target site mutation and thus to reduce insecticides toxicities (azinphosmethyl and carbaryl) in CPB (Rose and Brindley, 1985; Argentine et al., 1994). Mota-Sanchez (2003) and Krishnan et al. (2007) also reported that increase excretion contributes to remove imidacloprid and glycoalkaloids form the insect body.

Behavioral resistance in the CPB has been reported against Bt products. When these toxins are ingested by CPB, they increased their flight activity in the resistant strain. In this way physiological resistant in the beetle was reinforced by the behavioral escape from the toxic environment (Alyokhin and Ferro, 1999d).

In another study, Hoy and Head (1995), reported that CPB larvae resistance to *Bt* toxins were more behaviorally responsive and found that CPB populations avoid consumptions of foliage that were treated with higher toxin dose than the susceptible CPB colony larvae. Concluding that CPB has evolved resistance to almost all synthetic and microbial insecticides preparations aimed for its control in potato fields. Due to these resistance alarming situation and difficulties faced by potato growers in controlling this notorious pest, novel approaches are needed to develop effective management strategy for CBP populations in potato fields.

2.4 Genetic Engineering

Genetic engineering offers a variety of ways for manipulating different genes in single plant to get insect resistant plants. Modern approaches have enabled researchers and scientists to develop techniques to transform a gene or multiple genes from different sources into commercially important crop plants to develop resistance against phytophagous insect pests. Insect-resistant genetically modified crops having toxins from *Bacillus thuringiensis* (*Bt*) offers great value in pest management, but they pose high risk due to the evolution of insect resistance against various *Bt* crops containing single toxin. Breeding crop plants for resistance against insects have been tried long time ago, but no commercial variety has ever been shown to express adequate levels of resistance against the Colorado potato beetle and Potato tuber moth (Lagnaoui et al., 2001). However, with the advent of biotechnology, a fast alternative to conventional to improve crop resistance to insects in potatoes is made possible. One of the applications of biotechnology is the use of transgenic plants expressing the plant protection molecules. Plant biotechnology and genetic engineering involve the direct modification of the genetic make-up of an organism to make it with desirable traits. When scientists came to know that plants produce their own insecticide toxins during 1970. They started to developed crop plants by taking genes from wild plants and transfer these genes using genetic engineering

methods (Anderson, 2005). Today, corn, cotton, tobacco and soybeans are commercially grown genetically modified crop plants.

2.4.1 Introduction to *Bt* crops

Bacillus thuringiensis spores had been used as an insecticide many years before the identification of the bacterium. Some researchers suggested that the Bt spores had been used against insect pests by ancient Egyptian. This bacterium was first accidently isolated in Japan in 1901 by the Japanese biologist Shigetane Ishiwatari from an infected silk worm larva. He named this bacterium as *Bacillus sotto*. Approximately ten years later in 1912, a German scientist namely Ernst Berliner isolated the same bacterium from an infected Mediterranean flour moth (*Ephestia kuehniella*) and he changed its name to *Bacillus thuringiensis*, since the infected moth was found in German province of Thuringia (Siegel, 2000). For over 70 years, Bt based insecticides have been used in spray form in crops, containing a mixture of spores and the related protein crystals stay valuable in organic and conventional agriculture to control various insect pests of field crops, vegetables, forests and vector insects. The first Bt based commercial biopesticide formulations such as Dipel and Thuricide was marketed over 50 years ago. Currently numerous sprayable formulations of Bt products with various endotoxins are available for commercial use against different insect pests species. However, the continues use of these Bt based products resulted in evolution of resistance in some major pests. The very first report indicating evolution of insect resistance against sprayed *Bt* spores was published online early 1985, since then many more such cases of field evolved resistance have been documented in various crops pests. This problem was overcome by the development and introduction of transgenic crops. The first genetically modified crops containing endotoxin was grown on large scale in 1996 in the United States of America. Since then, different companies have launched different *Bt* transgenic varieties of different crops including potato, tomato, cotton and corn. In this way, the integrated pest management program gained a new momentum as these *Bt* crops were grown on large scale with the aim of protecting the crops from insect pests damages. Since 1996, transgenic crops have been grown around many countries of the world; they have shown to efficiently control various insect pests including tobacco budworm (*Heliothus armigera* (Hubner)), Colorado potato beetle (*Leptinotarsa decemlineata* (Say)), cotton bollworm (*Helicoverpa armigera* (H)), *Pectinophora gossypiella* (S) (pink bollworm),

Diatraea grandiosella (D), (southwestern corn borer) European corn borer (*Ostrinia nubilalis*) and helped the growers to reduce reliance on the usage of chemical insecticides. These *Bt* crops revolutionized the history of agriculture. With the advancement of genetic engineering and biotechnologies, the insect pests control program took a new dimension that has resulted in the development of different transgenic *B. thuringiensis* varieties in corn, cotton, and potatoes. Currently *Bt* transgenic crops are being extensively grown in many advanced and developing countries (Cohen, 2005; Bravo et al., 2007; Key et al., 2008; Azadi and Ho, 2010; Tester and Langridge, 2010; ISAA, 2017). These Bt transgenic crops have suppressed pest populations, reduced dependency on insecticide sprays, encouraged control via bio-agent, and doubled farmer income (Shelton et al., 2000; Ferry et al., 2004; Tabashnik et al., 2010). These biotech crops were initially grown on 1.7 million hectares land in 1996. Since the start of commercialization, there has been a significant 100-fold increase in cultivated areas of certain *Bt* crops especially in *Bt* cotton and *Bt* corn. This year wise increase is shown in Figure 2.1. It is obvious from Figure 2.1 that a total of 179.7 million hectares of GMO were cultivated in a single year during 2015. Since 1996, a collective total of 2339.5 million hectares of GMO were planted around the entire globe (ISAA, 2017). From the Figure 2.1, it is assumed that these *Bt* based crop technologies are readily accepted worldwide.

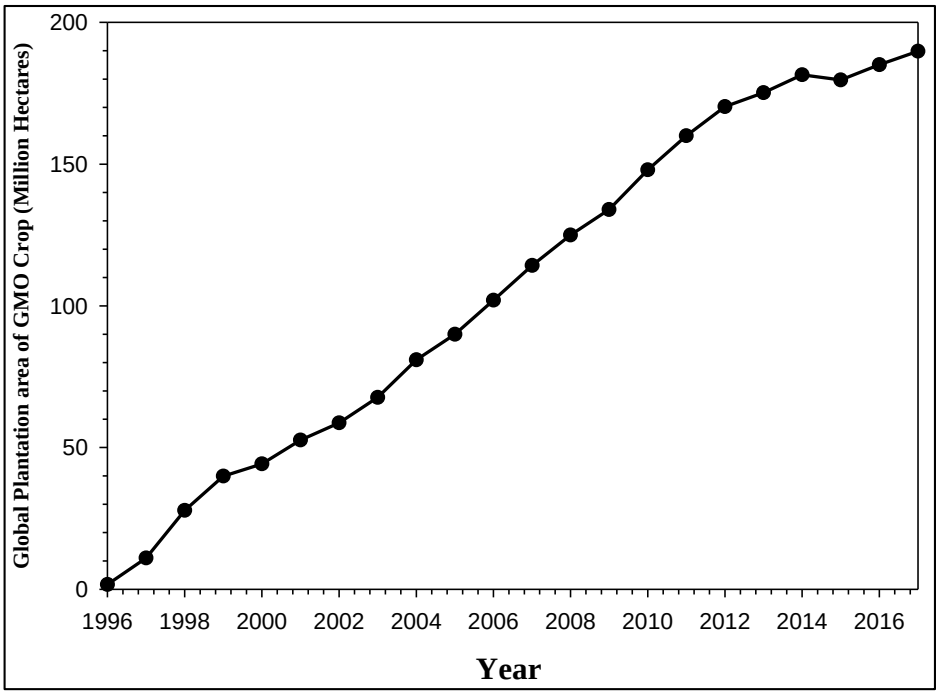


Figure 2.1. ISAAA 2017, Global GMO plantation area (Million hectares) from 1996 to 2017

2.4.2 Mechanism of Bt toxin and insect resistance to *Bt* crops

Agricultural production worldwide is constrained primarily by insect pests. Initially chemical insecticides were found effective in controlling the major agricultural pests, but their continues use has resulted in so many problems like environment pollution, effect on non-target insect and insect resistance (Salim et al., 2016). The transgenic crops produce insecticidal crystal proteins or Bt toxins that are toxic to the insect. The mode of action (MOA) of these Bt endotoxins have been studied in detail by different scientists in various insect orders. From these studies it is evident that the mode of action of these Bt protein relies on three different models. Two of them are based on pore formation activity, while the 3rd one relies on signaling pathway model as shown in Figure 2.2 (Bravo and Mario, 2008; Zhang et al., 2005, 2006). Unlike most other chemical insecticides, these Bt toxin do not acts as a contact poison. To kill insect, these Bt toxins must be ingested. The toxic pathway of these cry toxin undergoes several steps that includes crystal ingestion and solubilization by insect, release of protoxin and conversion into active toxin by midgut proteases (proteolytic activation), cadherin receptor binding (active protein binds to cadherin receptor situated on the brush border membrane of midgut cells) leading to pore formation that causes swelling and leakage of the cells. The swelling continues, resulting in insect midgut cells bursting, allowing the insect mid gut juices to pass into the hemolymph. As a result, insect blood PH arises, that results in insect paralyses and eventually death. (Bravo et al., 2011; Soberon et al., 2009; Vachon et al., 2012). The third model regarding Cyt toxin which constitutes one fifth of the size of the cry protein suggest that they are dissolved and processed like cry toxins, upon releasing they secrete a toxic core which results in cell lyses. They act as a detergent or synergist, attack the lipid portion of the cell membrane resulting cell lysis or increase the activity of other cry toxins.

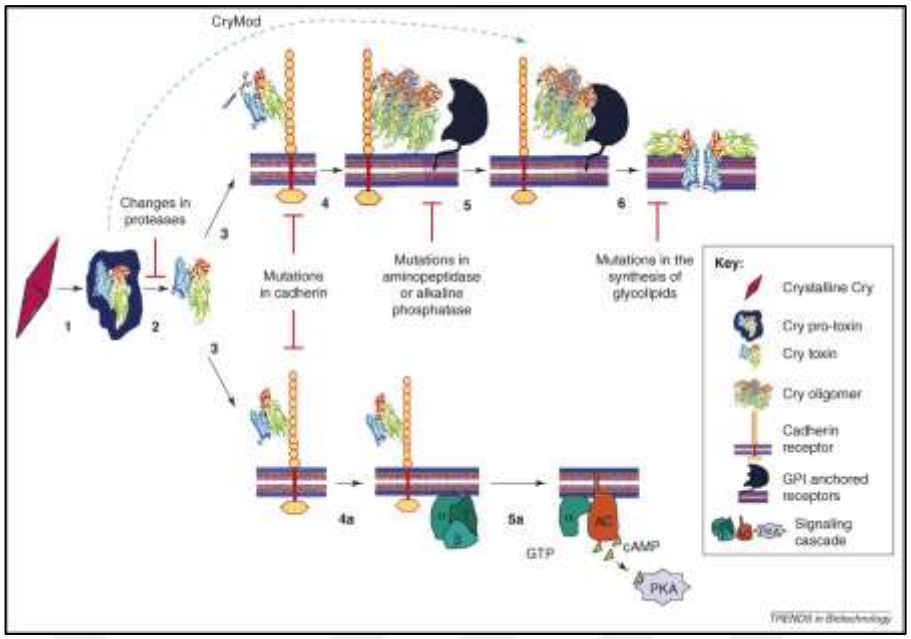


Figure 2.2. Proposed models for cry toxin mode of actions and ultimately resistance mechanism (Bravo and Mario, 2008)

As these first-generation transgenic crops (single toxin) have become more widely cultivated, some of their valuable, environmental and economic benefits have been disappeared, especially due to sudden and rapid evolution of field resistance by insect pests to the first generation Bt crops that produced only a single Bt toxin. These insect resistance reports have made the growers uncomfortable and have raised doubts about the future sustainability of these *Bt* crops. The first insect that has developed resistance to *Bt* cotton is known as bollworm, *Helicoverpa zea* reported between 2003 and 2006 in Mississippi and Arkansas. Since then, many scientists in different parts of the world published different cases of insect resistance to transgenic crops. The insect species that have evolved resistance against different transgenic crops around the entire globe are shown in Table 2.1. Most of the insect resistant species has been reported on large scale cultivated varieties of *Bt* corn and cotton. These reports suggested that the insect pests have evolved resistance to transgenic crops by two different mechanisms. In most cases, the primary mechanism of insect resistance to cry protein is linked to defects in binding specific receptor, that leads to reduce protease activation, higher immune response of the target insect pests or enhanced esterase production mutations in the cadherin receptor in different lepidopterans are known to cause resistance against cry1A toxins (Ferre and Van Rie, 2002; Tabashnik et al., 2013, 2014). For example, resistance to cry1Ac in *H. armigera* conferred by mutation in the promoter region of HaTryR. Lower expression of

cadherin in *Diatraea saccharalis* was linked with resistance to cry1Ab toxin; cadherin silencing with RNAi (RNA interference) was also related with tolerance to this toxin in *D. saccharalis* and *M. sexta* (Soberon et al., 2007; Yang et al., 2011). Similarly, mutation in 3 alleles of a cadherin receptors confers resistance in *P. gossypiella* and *H. virescens* to cry1Ac in *Bt* cotton (Morin et al., 2003; Xu et al., 2005; Fabrick et al., 2011; Ocelotl et al., 2015). Furthermore, resistance in *Plodia interpunctella* to cry1A was due to inactivity of the midgut protease that resulted in decrease activation of cry1A protoxins. Mutations in the aminopeptidase P of *O. nubilalis* were also related with resistance to 3d-Cry toxins (Khajuria et al., 2011). Resistance in *P. gossypiella* to cry1Ac and cry2Ab is due to a cadherin transmembrane mutation disturbing cellular trafficking in pink bollworm (Wang et al., 2018). Mutation in a single amino acid in ABC transporter gene has been linked to confers resistance to Cry1Ab in silkworm (*Bombyx mori*). Resistance in *H. armigera* to cry1Ac toxin has been related with their capability to break down long insecticidal bonds and to exclude these materials, resulted in increased production of gut esterase that bound and sequestered cry1Ac protein (Atsumi et al., 2012).

Figure 2.3. Insect resistance to Bt crops

Insect species	*Bt* Crop	*Bt* gene	Detection year	Country	Reference
Helicoverpa zea	Cotton	*Cry1Ac*	2002	USA	Dennehy et al. (2010)
Helicoverpa zea	Maize	*Cry1Ab*	2004	USA	Dively et al. (2016)
Helicoverpa zea	Maize	*Cry1A.105*	2016	USA	Dively et al. (2016)
Helicoverpa zea	Cotton	*Cry2Ab*	2005	USA	Ali and Luttrell (2007); Tabashnik et al. (2013)
Busseola fusca	Maize	*Cry1Ab*	2006	South Africa	Kruger et al. (2011)
Spodoptera frugiperda	Maize	*Cry1F*	2006	USA	Farias et al. (2014)
Pectinophora gossypiella	Cotton	*Cry2Ab*	2014	India	Tabashnik et al. (2015)
Pectinophora gossypiella	Cotton	*Cry1Ac*	2008	India	Dhurua and Gujar (2011)
Diabrotica v. virgifera	Maize	*Cry3Bb*	2009	USA	Gassmann et al. (2011)
Diabrotica v. virgifera	Maize	*mCry3A*	2011	USA	Gassmann et al. (2014)
Diabrotica v. virgifera	Maize	*Cry34/35Ab*	2013	USA	Gassmann et al. (2016)
Diabrotica v. virgifera	Maize	*eCry3.1Ab*	2014	USA	Zukoff et al. (2016)
Diabrotica v. saccharalis	Maize	*Cry1A.105*	2014	Argentina	Tabashnik and Carrière (2017)
Spodoptera albicosta	Maize	*Cry1Fa*	2013	USA	Smith et al. (2017)
Spodoptera frugiperda	Maize	*Cry1F*	2011	Brazil	Monnerat et al. (2015)
Spodoptera frugiperda	Maize	*Cry1ab*	2010	Brazil	Farias et al. (2014); Tabashnik, et al. (2010)
Spodoptera frugiperda	Maize	*Cry1F*	2007	USA	Storer et al. (2010)

2.4.3 New tools and approaches for breeding of ınsect resistant plants

2.4.3.1 Pyramiding or cry gene stacking strategy (Two genes strategy)

The problem of insect resistance to cry toxin has evolved in single *Bt* crop due to the fact that insects may be able to adopt more rapidly to a single Bt toxin than to multiple ones (Roush, 1998; Zhao et al., 2003). Thus, in order to delay the evolution of insect resistance to *Bt* Crops, several insect resistance management (IRM) strategies have been considered for managing resistance to *Bt*-transgenic crops (Shelton et al., 2000; Bates et al., 2005; Yang et al., 2011; Saljoqi et al., 2015). The primary strategy to overcome insect resistance is the use of high dose &refuge (HDR) (Gould, 1998; Bates et al., 2005; Tabashnik et al., 2008). The HDR strategy requires planting a proportion of non-Bt host plants in close proximity as refuges to maintain an appropriate size of susceptible

population. A drawback of this strategy is the economic loss to the non-Bt-crops in the refuges (Hutchison et al., 2010).

To reduce the percentage of refuges required by the HDR, new generations of transgenic crops with two or more than two Bt-toxins with different mode of actions called pyramid *Bt* crops, plantation have been started in the United States, Australia and elsewhere (Gould, 1998; Ferre´ and Van Rie, 2002; Zhao et al., 2003, 2005). The stacking of more than one useful transgene into plant genomes is called as gene stacking or gene pyramiding (Berger, 2000; Halpin, 2005).

The logic behind the gene pyramid originates from the ancient philosophy when peoples were using insecticide mixtures to control different insect pests with just one spray method. The gene pyramid is considered a permanent insect / disease resistance management strategy (Shelton et al., 2002). However, recently gene pyramiding strategy shows that this method can be used to develop plants resistance against insect pests that were not control by *Bt* plants transformed with single gene.

The main aim of gene pyramiding strategy is to develop transgenic plants with more resistance against pests and to improve crop yield. To obtain transgenic crops with durable and broad-spectrum resistance against insect pests and diseases, the pyramiding of major genes (multigene strategy) implying different mode of action will be a powerful strategy. This strategy has resulted in the so-called 'second generation' of genetically engineered (GE) plants. Gene pyramiding has been principally obtained through crosses between GE plants with different biotech traits (hybrid stacking), such as in Agrisure™ and Viptera™ maize. Other methods involve plant transformation with two or more genes harbored in a single (linked genes or multigene cassette transformation; e.g., Herculex™ maize) or in separate (co-transformation; e.g., Knockout™ maize) gene constructs, or the insertion of one or more genes into an already transgenic plant (retransformation; e.g., Bollgard™ II cotton). The first pyramided transgenic cotton plants expressing two 3D-Cry toxins, cry1Ac and cry2Ab with different mode of actions, were introduced in the US in 2003. These two Cry proteins are known to have different mode of actions and bind to different receptors that results significantly delay in evolution of any insect resistance. The rapid adoption rate of *Bt* pyramided plants has attracted the attention of its superiority over individual transgenic events. Biotech crops with multiple traits were planted on 77.7

million hectares in 2017, representing 41 percent of all biotech hectares planted worldwide and a 29 percent year-over-year increase (ISAAA, 2017). To delay insect resistance to *Bt* crops and to improve efficacy against some pests, and broaden the spectrum of pests controlled, newer *Bt* crops produce two or more Bt toxins have been commercialized in different parts of the world. Most of these products contain multiple *Bt* genes for managing major insects pests. Presently important crops with stacked traits are in maize and in cotton that are resistant to insect pests as well as herbicides application. These crops are mostly stacked with two introduced genes. Many commercial companies such as Syngenta, Bayer Crop Science, Pioneer, Dow Agro Sciences and Monsanto are pursuing to achieve GM crops with stacked traits. These results showcase gene pyramiding as a possibly useful tool that could be successfully applied to the combination pests and diseases for delaying resistance development and in some cases can be successfully applied against the combination of biotic and abiotic stresses.

2.4.3.2 Strategies for gene stacking /pyramiding in plants

Several methods have been used to pyramid (or stack) transgenes into plants that range from using conventional sexual crosses of plants comprising of one single transgene or more of transgenes to be combined into a single germplasm to biotechnologies. The three main strategies are discussed below.

2.4.3.2.1 Iterative procedure / Conventional breeding

Plant harboring one or more transgenes can be successively introduced into another plant by conventional iterative procedures (Figure 2.3). This method is also called as sexual hybridization which result in the development of multi stack hybrid. These techniques have been used to at least combine or enhance existing transgenic traits at the research level. For example, combining herbicide-resistant event with an insect-resistant transgenic event, both being deregulated, can be performed through sexual crossing until all the required genes are present in the progeny. This procedure has been used to produce plants expressing different Bt toxins to interrupt insect resistance development against *Bt* crops. This method has been recently demonstrated in broccoli where *cry1Ac* and *cry1C* (pyramided *Bt* genes) controlled *P. xylostella* resistant to either single protein (Cao et al., 2002), and expressively deferred insects resistance development to *Bt* crops (Zhao et al.,

2003). Iterative rounds of cross-breeding have also been used to introduce novel proteins or biochemical pathways into plants. For example, Arabidopsis plants which were tolerant to one of the environmental toxins (methyl mercury) were formed by crossing plants comprising two different genes for a bacterial organic mercury detoxification pathway (Bizily et al., 2000).

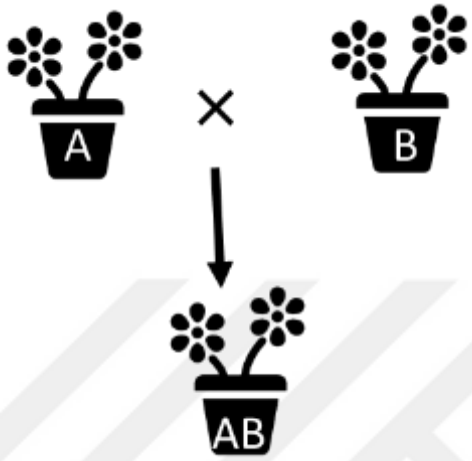


Figure 2.4. Gene pyramiding. Plants with one transgene (A or transgene B) are crossed to each other to yield plants with both characters /transgenes

2.4.3.2.2 Re-transformation

Retransformation is another gene pyramiding method, with which a plant containing one transgene can be transformed with other multiple transgenes with several rounds of transformation (Figure 2.4). For retransformation, every single cycle of transformation, new and different selectable marker are used from the original event. This is very important in this process. This method can be principally useful in trees or woody plants because these are uneasy to propagate by sexual crossing. This method has been proven in various research like modification of flower colors in forsythia by inducing anthocyanin synthesis through sequential transformation with the genes from *Antirrhinum majus* (*AmDFR*) and *Matthiola incana.* This process-initiated anthocyanin synthesis in the double transformant. Due to this process a novel bronze-orange petal color is formed. This method is also useful in trees to combine two genes (involved in lignin biosynthesis) to decrease the environmental impact of papermaking by introducing antisense transgenes.

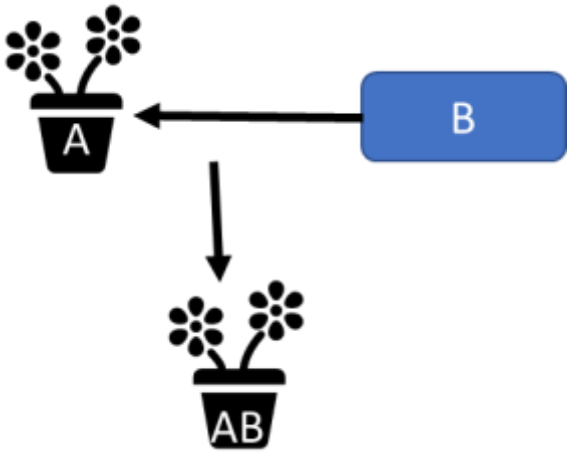


Figure 2.5. Pyramiding genes A and B by retransformation method with which a plant harboring transgene A is retransformed with transgene B

2.4.3.2.3 Co-transformation

Co-transformation is the third method which has been gaining popularity and is considered an important process for the introduction of multi genes into plants. In co-transformation, a single plant is co-transformed with multiple genes simultaneously. This strategy is divided into two categories; (a) single-plasmid co-transformation, in which gene A and B are associated with one piece of DNA. In this method, each gene having its own promoter but transferred together into a plant (Halpin and Ryan, 2004) or (b) multiple-plasmid co-transformation in which transgenes (A and B) are on different pieces of DNA and are transformed together into a plant (François et al., 2002a).

The stacking of multiple gene via co- transformation is fast and can be used in different species via biolistic (direct) and *Agrobacterium*-mediated (indirect) transformation methods. With this method up-to 13 genes are simultaneously co- transformed successfully into plants via direct method and up to 6 gene clusters by single plasmid co-transformation methods (Chen et al., 1998; Goderis et al., 2002; Thomson et al., 2002). Advantage of this process is that the co-introduced genes can settle at high proportion of transgenics and at the same chromosomal position. This approves that (effect) genes are stable to segregate apart in subsequent generations.

Several studies have reported manipulation of transformation conditions to encourage co-transformation and/or co-integration. For example, McCormac et al. (2001) reported

100% co-transformation frequencies in tobacco study using two T-DNAs. Presently, co-transformation methods via particle bombardment has been resulted in successful introduction of three insecticidal genes in *indica* rice. The resultant transgenes showed significant resistance against major rice pests (Maqbool et al., 2001). Similarly, co-transformation of modified cry1Ab and cry1Ac genes of Bt in chickpea has been resulted for improved resistance to *H. armigera* (Mehrotra et al., 2011).

The Proteinase inhibitors *Oryza cystatins I* and *II* genes were stacked successfully through direct co-transformation with both *OCI* and *OCII* genes into the potato genome. Successful expression of both genes in potato resulted in increased resistance against CPB. The resultant transgenic potato plants caused a significant reduction in CPB adult body weight and foliage consumption (Cingel et al., 2014). This method has been used on large scale successfully to produce some of the commercialized GM stacked crop events that are available in the market today.

2.5 Practical Merits of Gene Pyramiding

Problems related with engineering resistance through genetic transformation of single gene is that; there is a possibility that pests may develop resistance to the transgenes used against them, resulting in the susceptibility of the transgenic plants to pest attack. Therefore, gene pyramiding or trestle transfer of multiple genes may be useful in conferring broad spectrum resistance against different biotic and abiotic stresses. Essentially, gene pyramiding allows us to have simultaneous expression of more than one gene(s) associated with resistance in a transgenic plant (Shelton et al., 2002). There is a broad agreement that pyramiding genes for resistance is a useful tool for increasing endurance with many known achievements. Stacked GMOs are an important component of insect resistant management (IRM) strategies to delay development of resistance in pests against the transgenes. Gene staking allows the development of crops with combined protection against multiple pests and diseases as well as adverse environmental conditions such as heat, cold and drought stresses. Second generation dual-*Bt* gene cottons Bollgard II® consisting of *cry 1Ac + cry 2Ab* and WideStrike™ consisting of *cry 1Ac + cry 1F* were introduced in corn and successfully control *H. zea* (Jackson et al., 2003; Gahan et al., 2005). This is because these toxins have different binding sites in larvae midgut. Due to this mechanism, they overcome the normal evolution phenomenon

in insect. This is because a species cannot easily evolve resistance to different toxins with different mode of actions. Since different cry toxins vary in their effectiveness against individual species, these pyramiding of Bt genes in a single crop have effectiveness in controlling different species. Gene pyramiding with different mode of actions may produce more toxic effect and will result in increased efficacy against variety of insect pests (Bates et al., 2005). Currently. Maize verities transformed pyramidically with different genes are resistant against many lepidopteran pests including caterpillars and rootworm. For example, Dow AgroSciences's Herculex XTRA®, Syngenta's Agrisure CB/RW® and Monsanto's Yieldgard Plus®.

2.6 Selected Genes in the Study

2.6.1 *Cry3A* gene

Cry3A gene is extracted from the soil bacterium namely *Bacillus thuringiensis* Berliner strains *San Diego, tenebrionis* and *tolworthii* and is considered as effective in controlling CPB infestation in potato. The protein size of cry3A ranges from 73-75 kDa. The choice of using *Bt* gene to engineer crop plant for resistance against insect pests offers multiple advantages. These Bt proteins are highly specific in their actions against specific insect orders. Further they have not shown any toxicity to birds or mammals including human beings. The first potato transgenic plants expressing the cry3A protein were registered safe for human consumption by the Environmental Protection Agency (EPA) in 1995. This was followed by continuous flow of research in different countries of the world to produce transgenic potato and other plants with more resistant to CPB and other insect pests (Arpaia, 1999). Currently more than 30 events of potato engineered with Bt gene Cry 3A are registered by different companies (ISAA, 2017). These potato events are mostly resistant to infestation caused by CPB in potatoes fields. These events confer resistance against CPB and other coleopteran pests by damaging their mid gut lining.

Sezen et al. (2008) conducted a study with cry3A protein against *Agelastica alni* (L) (Coleoptera: Chrysomelidae) and recorded hundred percent mortality. They also further tested against other coleopteran pests and recorded 90% mortality for *Amphimallon solstitiale* (L) (Coleoptera: Scarabaeidae), and *Melolontha melolontha* (L) (Coleoptera:

Scarabaeidae). Their study suggested that *B. thuringiensis* subsp. *tenebrionis* may be useful as bio agent for other coleopteran pests.

Me et al. (2015) reported in their study that transgenic cry3A potato plants exhibit high toxic activity against Colorado potato beetle. The mortality of the CPB was recoded higher as compared to the controls. Further Insect biomass accumulation and leaf consumption on the foliage of the transgenic plants were significantly lower as compared to non-transgenic plants. They reported that transgenic potato plants could be developed from different wild as well resistant potato varieties for controlling CPB infestation in potato fields and its further spread in potato growing areas in China.

2.6.2 Synthetic hybrid gene (*SN19*)

SN19 gene is a hybrid *Bacillus thuringiensis* delta-endotoxins of cry1Ba/cry1Ia that encode a protein consisting of domains I and III of cry1Ba and domain II of cry1Ia, was first constructed and described by Naimov et al. (2003). This hybrid expression of *Bt* genes has demonstrated a successful strategy for improving insect resistance in transgenic plants. The synthetic version of this hybrid gene was transferred into Red Star potato plants via *Agrobacterium* transformation. After successful transformation in potato plants, the transformed plants resist attack by Colorado potato beetle (Naimov et al., 2003).

Similarly, in another study conducted by Naimov et al. (2006), it is reported that the construction of this hybrid gene has broaden the spectrum of pests control. In this study they transferred the Hybrid delta endotoxin gene into Desiree potato plants via *Agrobacterium* transformation. The resultant transgenic plants resisted attack by Potato tuber moth larvae, European corn borer larvae as well as CPB larvae and adults. These were the first transgenic plants that exhibited resistance against different insect species.

Ahmad et al. (2017) studied effect of insecticidal hybrid SN19 gene in potato against CPB and tomato leaf miner (*Tuta absoluta* Meyrick). They used two different plant expression vectors in four potato genotypes (Tokat 6/24, Tokat 10/1, Innovator and Marabel). After successful transformations, leaf biotoxicity assays were conducted with both CPB and tomato leaf minor. A hundred percent mortality were recorded for both the CPB larvae

and adults as well as tomato leaf minor larvae. The above experimental results confirm the toxicity potential of this hybrid gene and makes it a useful tool for breeding resistance plants against insect herbivores.

2.6.3 Plant proteinase ınhibitors

Currently plant transformation with genes for resistance against CPB and other insect herbivores depend on genes from either *Bt* or plant proteinase inhibitor (PIs). However, plant transformation with proteinase inhibitor (PI) genes are gaining popularity among consumers because of their abundance and stability and effectiveness in controlling insect herbivores. Several authors have reported the adverse effect of Pls on insect growth and development (Rahbe´ et al., 2003; Azzouz et al., 2005). These PIs target mainly chewing insects which use digestive enzymes to digest protein in their food (Michaud, 2000). A well-known and important group of plant proteinase inhibitors are the cystatins, which are inhibitory proteins of cysteine proteinases. As Colorado potato beetles and other Coleopteran generally uses cysteine proteinases for protein digestion. The cystatin super family of proteins is widely distributed among plants and others lower animals which represent potential control proteins for the genetic transformation of several economically important crops against insect herbivory. Recent studies reported that *Oryza cystatin I* (*OCI*) and *Oryza cystatin II* (*OCII*), purified from *Oryza sativa* L. affect the growth and development of several coleopterans including CPB either due to the inhibition of proteinases and result in poor digestion of dietary proteins or reduce the uptake of essential free amino acids which are necessary for growth and development of insect herbivores. They also cause increase production of proteinases in response to the inhibition of digestive proteinases, which leads to loss of appetite and growth reduction in insects including CPB (Atkinson et al., 1995; Womack et al., 2000).

Zhao et al. (1996) investigated wound-inducible cysteine PIs in soybean. He reported that due to this cysteine (PIs), the host plant escapes damage caused by the Western corn rootworm. The beetle larvae have digestive cysteine proteinases in their guts which are inhibited by this PIs. The same pattern of inhibition of proteinases was found in soybean by Hines et al. (1991) in cowpea weevil, *Callosobruchus chinensis* L. and the red flour beetle, *Tribolium castaneum* (H).

Oppert et al. (1993) observed that combinations of cysteine and serine PIs increase toxicity in wheat germ diets against larvae of the red flour beetle, *T. castaneum*. They tested mixture of different cysteine and serine PIs against *T. castaneum*. All PIs inhibit the growth, resulted in reduction in larval weight gain from 82-97% in 17 days after hatching, and later to 40-60% mortality.

Similarly, Bolter and LatoszekGreen, (1997) in another study found that continuing ingestion of cPIs (E-64) has a significant inhibitory effect on CPB larval growth, development and survival, as well as on adult fecundity. They reported increased pupation days in plants treated with PIs as compared to control. Adult fecundity was significantly low (16 ± 2.4 eggs daily) when compared with equal mated pair on control plants (62 ± 5.7 eggs daily).

The proteinase inhibitors *Oryza cystatins* I and II genes were stacked through direct co-transformation method into the potato genome. Successful expression of both genes in potato resulted in increased resistance against CPB that caused a significant reduction in adult body weight. The transgenic potato plants also resulted in less foliage consumption (Cingel et al., 2014).

2.7 Life Tables

Life tables studies are important and useful in assessing various insect population parameters (survival, development and reproduction) under different climatic conditions and on different hosts (Carey, 2001). However, studying life tables using traditional female-based age-specific life tables has some drawbacks, the most important of which is ignoring of the male population as well as the stage differentiation (Lotka ,1907; Lewis, 1942; Leslie, 1945; Birch, 1948; Carey, 1993). Due to these problems, many ecologists had reduced their practical application in studies relating to insect ecology, biological control, mass rearing and pest management program (Huang and Chi, 2012; Huang et al., 2018). Contrary to the traditional female-life tables, the age-stage, two-sex life table (Chi and Liu, 1985, Chi, 1988) takes both sexes into account (male and female) that enables stage differentiation. This approach is used in many studies on insect population ecology, predator-prey relationships, pest management and biological control program.

Besides these, life table studies can also be used to analyze and predict the effect of various abiotic factors on target insect population parameters including survival rate, developmental time and reproductive potential. Life table studies can also be used for comparing different insect population fitness under different climatic conditions. Additionally, age-stage, two-sex life table studies exactly reveal the true-life history parameters of the insect species and has been extensively applied to study relating to various ecological aspects of insect pests and their natural enemies.

Age-stage, two-sex life table studies can also be applied to insects reared in group, because some insect species are gregarious, rearing those insect species individually is not possible due to the fact that it will not survive under natural conditions and will produced wrong results. To overcome these difficulties, age-stage, two-sex life table offers demographic analysis of insects data reared in groups in a very easy and reliable way with accurate results (Huang and Chi, 2011).

CHAPTER III

MATERIALS AND METHODS

The present research work focuses on pyramiding of insecticidal genes in potato to encode resistance against Colorado potato beetle, *Leptinotarsa decemlineata* (Say), (Coleoptera: Chrysomelidae). To develop these transgenic plants, following materials and methods were used.

3.1 Experimental Materials

3.1.1 Plant material (Rice plants)

The rice (*Oryza sativa* L.) cultivar Edirne mature seeds were obtained from the Ministry of Agriculture and Forestry, Directorate of Trakya Agricultural Research Institute (Edirne, TR) for the isolation of *OCII* gene. First the rice seeds were drenched in water for two days and then, the seeds were transferred into 147.85 mL plastic cups (Yöm Plastik Company, Istanbul, TR**)** containing soil consisting of perlite, peat and vermiculate (mixed in the ratio of 1:1:1 v/v/v) (Klasmann- Deilmann GMBH, Germany) and were regularly watered (Figure 3.1). Rice seedlings were kept in growth chamber at 27± 1 ^{o}C with 16:8 h (L: D) photoperiod and at 65% relative humidity.

Figure 3.1. Rice seedling propagation in pots in growth chamber

3.1.2 *In vitro* propagation of plant materials (Potato plants)

Two commercial cultivars of potato (*Solanum tuberosum* L.), namely Lady Olympia and Agria were used for genetic transformation. These are commercially cultivated varieties in the potato growing zone of Turkey with high productivity but are susceptible to insect pests attack in the field condition. To propagate experimental explants, tuber sprouts of both potato cultivars were taken from the Plant Transformation laboratory and were subjected to surface sterilization using NaOCl for 5 minutes followed by three times washing with autoclaved distilled water containing 50 µl of 100% Tween-20. Tuber sprouts were multiplied *in vitro* by monthly subculture of single-node stem explants on basal Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) (Appendix-I). The potato plantlets were then maintained in a growth room with a 16/8 hours light/dark photoperiod and temperature 25 ± 2 °C (Figure3.2).

Figure 3.2. *In vitro* propagation of potato plants for transformation experiments

3.2 Bacterial Strains

During preparation of DS-1 and DS-2 constructs, *E. coli* strains (*DH5α* and *DB301*) and *Agrobacterium* strain (*GV3101*) were used for multiplication and propagation purposes.

3.3 Isolation/Amplification of *Cry3A* Gene for Construct Development

The *cry3A* gene in *pGT-22-b* vector was obtained from Institute of Plant Molecular Biology, Centre of the Academy of Sciences Czech Republic, Ceske Budejovice, Czech Republic. The stock culture of *cry3A* gene in pGT-22-b was grown in Luria- Bertani (LB) medium with selection of antibiotic (100mg/L of ampicillin) overnight. The following day, plasmid isolation was performed using plasmid miniprep kit (Thermo Scientific- Cat #K0503).

3.3.1 Amplification of *cry3A* gene

pGT-22-b plasmid was used as a template to amplify 1832bp fragments of *cry3A* gene using full length gene specific primers (Table 3.1). PCR reaction consisted of 20 µl volume containing DNA template (5ng), forward and reverse primers (50 pM each) specific to the full length of *cry3A* gene, dNTPs (200 µM), 1X PCR Buffer (50 mM KCl, 1.5mM MgCl2 and 10mM Tris-HCl) and Pfu Polymerase (1 unit). The gradient PCR was performed at 95 ºC for 4 minutes for denaturation while annealing temperatures were in the range of 50 ºC and 55 ºC for 1 min to determine the optimum annealing temperature. Extension was carried out at 72 ºC for 2 minutes followed by 40 times. The amplified PCR fragment of *cry3A* gene were resolved on 1% agarose gel and observed under UV light.

3.3.2 Purification of *cry3A* gene fragment from agarose gel

Amplified fragment of *cry3A* gene was purified from agarose gel with GeneJET Gel extraction and DNA cleanup micro Kit (Thermo Scientific- Cat# k0832) in such a way that the required band of *cry3A* gene was cut with the help of sterilized blade from the gel, weighed and incubated with 200 µl of extraction buffer solution in a 1.5 mL tube at 55 ºC till gel slice gets dissolved. After incubation, 200 µl of ethanol (96-100 %) was added and mixed thoroughly. The mixture was then passed through DNA Purification Micro Column and put at room temperature for 10 minutes so that the column may absorb the DNA. The column was then centrifuged for 60 seconds at 14,000 rpm. Supernatant was removed after centrifugation and pellet was washed three times with Pre-wash and wash buffer provided in the kit. Again, supernatant was removed through centrifugation,

the pellet was air dried and warm elution buffer (65 ºC) was added onto the column to resuspend the pellet and kept at room temperature for 30 minutes. After 30 minutes the column was centrifuged and the supernatant was stored in 1.5 mL tube at -20 ^{0}C.

3.4 Confirmation of *pTF101* Plasmid Containing *SN19* Gene

For cloning of *pro35S-SN19-CaMV* terminator fragment in *pCAMBIA1301*, firstly 10 µl glycerol stock of *pTF101* plasmid containing *pro35S-SN19-CaMV* in *E. coli* (*DH5α*) was inoculated in 10 ml of LB broth containing appropriate antibiotics (300 mg/L of Spectinomycin and 100 mg/l of Spectinomycin) and was cultured at 37 ºC overnight in an incubator shaker. The following day, plasmid extraction was performed using plasmid miniprep kit (Thermo Scientific- Cat #K0503). After plasmid extraction, the concentration was checked with spectrophotometer. Then the plasmid was used to confirm the amplification of 480 bp fragment of *SN19* gene using partial gene specific primer (Table 3.1). PCR reaction consisted of 20 µl volume containing DNA template (5 ng), forward and reverse primers (50 pM each) specific to the partial fragment of *SN19* gene, dNTPs (200 µM), 1X PCR Buffer (50 mM KCl, 1.5mM MgCl2 and 10mM Tris-HCl) and Taq Polymerase 1 unit. The PCR machine was set at 95ºC for 5 minutes for denaturation while annealing temperature was 55ºC for 15 seconds. Extension was carried out at 72ºC for 20 seconds followed by 34 times. The amplified PCR fragment of *SN19* gene was resolved on 1% agarose gel and observed under UV light.

3.5 Isolation and Amplification of *OCII* Gene from Rice Plant

3.5.1 Total RNA extraction

The method of Jaakola et al. (2001) was used for extraction of RNA from rice seedlings with some modifications. Young leaves of rice seedlings were cut with the help of a sterilized scissor and flash frozen in liquid nitrogen. The leaf tissues were then ground into a fine powder in a pre-cooled mortar with pestle by the addition of more liquid nitrogen. To each 100 mg of ground sample, 600 µl of pre-warmed (70 ºC) extraction buffer was added and vortexed for 2 minutes. The tubes containing the plant samples were then incubated at 70ºC for 20 minutes. During this time, sample was vortexed after every 5 minutes and were then centrifuged at 14,000 rpm for 12 minutes at 4 ºC. After

centrifugation, the supernatant was transferred to 1.5 mL tubes and again centrifuged at 14,000 rpm for 15 minutes. Again, after centrifugation, the supernatant was transferred to new 1.5 mL tubes and extracted twice with an equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1). Phases were separated at 13,000 rpm for 10 minutes at room temperature. The aqueous phase was transferred to new 1.5 mL tube. Lithium chloride (1/4 volume of 10 M) was added to the aqueous phase and were mixed gently.

RNA samples were then precipitated by incubating for 24 hours at 4 ºC. After incubation, the tubes were then centrifuged at 13000 rpm for 15 minutes at 4 ºC to form pellets. The pellets were then washed with 500 µl of ice-cold ethanol (70%). After washing, the pellets were then air dried in a hood. The tubes containing pellets were removed from the hood after drying. A hundred micro liter of pre-warmed (65 ºC) SSTE buffer (Appendix-1) was added into the tube to dissolve the pellet.

The pellet was then extracted with an equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1). To the supernatant, ice-cold absolute ethanol (100%, twice the volume of supernatant) was added to precipitate RNA at -20ºC overnight. The tubes containing the RNA were centrifuged at 14000 rpm for 15 minutes at 4ºC. After centrifugation, the pellet was washed with 500 µl of ice-cold ethanol (70%). At the end, pellet was air dried in a hood and finally re-suspended in DEPC treated deionized water.

3.5.2 Quantification of total RNA

RNA quantity and quality were determined by using Biospecific-nano Spectrophotometer (Shimadzu Biotech). One microliter of RNA sample was added to the spectrophotometer reader and concentration were recorded.

3.5.3 cDNA synthesis

cDNA was synthesized by using Fermentas cDNA synthesis kit. For cDNA synthesis, reaction mixture of DNase treated RNA (1µg), Anchored oligo dt primers (1µl) and DEPC-treated water (ca. 12 µl) was incubated at 70ºC for five minutes and then quickly placed on ice in order to chill the mixture. After chilling on ice, 5X reaction buffer (4µl), ribonuclease inhibitor (1µl) and 10mM dNTP mix (2µl) was added to the mixture and

again incubated for 5 minutes at 37ºC in incubator shaker. Finally, H minus M-MuLV reverse transcriptase (1µl) was added to the mixture and incubated at 42 ºC for 60 min. Finally, the reaction was stopped by incubating for 10 minutes at 70 ºC in thermocycler machine.

3.5.4 Amplification of *OCII* gene from rice cDNA

cDNA was used to amplify 649bp fragment of *OCII* gene fragment. Primers for the amplification of *OCII* (NCBI Accession No. NC_029260.1) have been given in Table 3.1. PCR was consisted of 20 µl reaction volume containing template (5ng), *OCII* gene specific forward and reverse primers (50 pM each), 200 µM of dNTPs, 1X PCR buffer (50 mM KCl, 1.5mM MgCl2 and 10mM Tris-HCl) and Pfu Polymerase (1 unit). Gradient PCR was done at 95 ºC for 5 minutes for denaturation while annealing temperatures were in the range of 48 ºC and 70 ºC for 1 min to determine the optimum annealing temperature. Extension was carried out at 72 ºC for 2 minutes repeated 40 times. PCR product of *OCII* gene was run on 1% agarose gel for 30 minutes and then observed under UV light.

3.5.5 Purification of *OCII* gene fragment from agarose gel

The amplified PCR product of *OCII* gene was purified from gel with GeneJET Gel extraction and DNA cleanup micro Kit (Cat# k0832) in such a way that the specified band of *OCII* gene was cut with blade from the gel, weighed and incubated with 200 µl of extraction buffer solution in a 1.5 mL tube at 55 ºC temperature till gel slice gets dissolved. The rest of the procedure was the same as discussed in 3.2.1.2. The eluted fragment was then resolved on 1% agarose gel and observed under UV light.

Figure 3.3. Details of various primers with their sequences, annealing temperature and product sizes

Primers	Sequence	Product size (bp)	Annealing Temp (°C)
OCIIF-FL	TAAGATCTATCTATGCGGGCATCATCTC	649	65
OCIIR-FL	ATGGTAACCGCGAAAATGTTGTGTATTGC		
Cry3AF-FL	AC CCATGGGCGCCGATAATAAC	1832	55
Cry3AR-FL	CACAGATCT TTATCAGGATCCAGCGCTAT		
Cry3AF- par	TGAGGGGATACGGGACAACA	509	55
Cry3AR- par	TCCTTGCATCAGGAAGCACAT		
SN19F- par	TGCATTGCTGAGGGTAACAAC	480	55
SN19R- par	AGCATCACGAAGCAAAAGCAA		
hptII-F	TTGGGAATCCCCGAACATCG	597	55
hptII-R	CTCTCGGAGGGCGAAGAATC		
Chv A-F	CGAAACGCTGTTCGGCCTGTGG3	890	65
Chv A-R	GTTCAGCAGGCCGGCATCCTGG3		

3.6 *pCAMBIA 1301* Plasmid Extraction

Three colonies of *E. coli* containing *pCAMBIA 1301* were grown overnight in liquid LB medium with antibiotic (50 µg/mL of kanamycin). The following day, plasmid isolation was performed using plasmid miniprep kit (Thermo Scientific- Cat #K0503). After plasmid extractions, the concentrations were checked on a Spectro photometer. The plasmid (*pCAMBIA 1301*) was then run on 1% agarose gel and observed under UV light. The map of the plant expression vector (*pCAMBIA1301*) is shown in Figure 3.3.

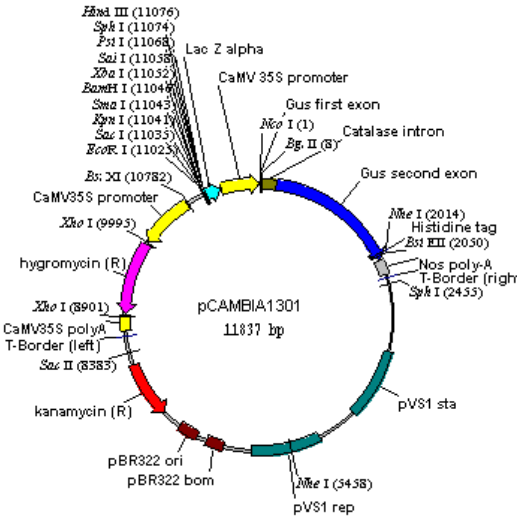


Figure 3.4. Map of plant expression vector pCAMBIA 1301 to be used for cloning (http://www.cambia.org/)

3.7 Construction of DS-1 Plasmid Construct

3.7.1 Construction of *SN19 pCAMBIA 1301* plasmid

To construct *SN19pCAMBIA 1301* plasmid containing, *pro35S-SN19-CAMV* terminator (approximately 2.9 kb) fragment was excised from already available plasmid *pTF101* and further cloned in *pCAMBIA 1301*. Following steps were performed to accomplish the task.

3.7.2 Restriction digestion of *pTF101* plasmid to excise *pro35S-SN19-CAMV* terminator fragment

For this purpose, the *pTF101* vector was digested with *Kpn*I and *Hind*III restriction enzymes to excise desired fragment of *pro35S-SN19-CAMV* terminator from *pTF101*. Following reaction mixture combination was used as given in (Table 3.2). The samples were incubated for 1 hour at 37 ^{o}C for digestion and then for 10 minutes at 65 ^{o}C to deactivate enzyme activities. The digested and undigested vectors were run on 1% agarose gel at 70V for 35 min and analyzed under UV-light. The gel slices containing digested product of desired fragments was cut with sterilized blade and eluted according to the protocol of GeneJET Gel extraction and DNA cleanup micro Kit (Cat# k0832). The eluted fragments of *pro35S-SN19-CAMV* terminator was stored at -20 ^{o}C.

3.7.3 Restriction digestion of *pro35S-SN19-CAMV* terminator fragment and *pCAMBIA 1301* plasmid

For this purpose, the purified *pro35S-SN19-CAMV* terminator fragment and *pCAMBIA 1301* vector were digested with two restriction enzymes (*Kpn*I and *Hind*III) to produce overhangs in desired fragments of *pro35S-SN19-CAMV* terminator and *pCAMBIA 1301* vector. Following reaction mixture combination were used as given in Table 3.2.

Figure 3.5. Reaction mixture of digestion of pro35S-SN19-CAMV terminator and pCAMBIA 1301 with Kpn1 and HindIII

Name	*pTF101* plasmid	*pro35S-SN19-CAMV terminator*	*pCAMBIA 1301* vector
DNA	2µg	1µg	2µg
10x Buffer	2µl	2µl	2µl
*Kpn*I (1U/µL)	2µl	1µl	2µl
*Hind*III (1U/L)	2µl	1µl	2µl
Water	made volume up-to 20µl	made volume up-to 20µl	made volume up-to 20µl

The samples were incubated for 1 hour at 37ºC and then for 10 minutes at 65ºC to deactivate enzyme activities. The digested PCR product and vector was run on 1% agarose gel at 85V for 35 min and analyzed under UV-light. The gel slices containing digested products were cut with blades (sterilized). They were then eluted as per proposed protocol of GeneJET Gel extraction and DNA cleanup micro Kit (Cat# k0832).

3.7.4 Ligation of insert (*pro35S-SN19-CAMV* terminator) into *pCAMBIA 1301* vector

Both insert (*pro35S-SN19-CAMV* terminator) and vector overhangs were ligated by using T4 DNA Ligase (Promega) as per proposed protocol. Vector to insert ratio was 1:5 and ligation temperature for ligation mixture was set 22 ºC for 2 hours and 15 ºC for 3 hours and 4ºC overnight. Following reaction mixture combination was used as given in Table 3.3.

Figure 3.6. Reaction mixture for ligation of 35S-SN19 gene into pCAMBIA 1301 plasmid

Name	Quantity
pCAMBIA1301 Plasmid	50 ng
Insert (*p35S-SN19-CaMV* terminator)	62 ng
10X Ligation buffer (Promega)	1.00 µl
T4 DNA ligase (3U/µl)	1.00 µl
Water	made volume up-to 10µl

3.7.5 Transformation of SN19 pCAMBIA 1301 plasmid in Agrobacterium (LBA4404)

The *Agrobacterium tumefaciens* strain *LBA4404* was used for transformation of *SN19 pCAMBIA 1301* plasmid ligation mixture. Four microliters of ligated product was transformed into competent *Agrobacterium* cells (50µl) and mixed by tapping. These cells were chilled on ice for 30 minutes and were subjected to electroporation. The electroporation device was set at a capacitance of 25µF, voltage 2.4kV with a 200 ohms resistance. After electroporation, 450 µl of LB broth was added and the mixture was incubated at 28ºC for 3 hours at 600 rpm in shaking incubator. Cells were then spread on LB plates containing kanamycin (50 µg/mL) and were then incubated at 28ºC overnight. Following day, colonies were checked and confirmed through colony PCR. Cultures were prepared from the positive colonies in LB broth containing 50 µg/mL of kanamycin and incubated at 28ºC overnight. The following day, the plasmid DNA were extracted from the overnight grown cultures as per protocol using plasmid miniprep kit (Thermo Scientific- Cat #K0503). After extraction, the plasmid were then resolved on 1% agarose gel and observed under UV light and glycerol stocks were prepared and stored at -80ºC.

3.7.6 PCR confirmation of *SN19pCAMBIA1301* plasmid

Colony PCR reaction was conducted to amplify 480 bp fragment of *SN19* gene from plasmid DNA. *pTF101* plasmid was used as positive control. By adding randomly selected colonies, PCR was performed at 95ºC for 4 minutes for denaturation, while annealing temperature was 55ºC for 15 seconds. Extension was carried out at 72ºC for 20 seconds repeated 34 times. The amplified PCR fragments were resolved on 1% agarose gel and observed under UV light.

3.7.7 Transformation of *SN19 pCAMBIA 1301* plasmid into *E. Coli*

For propagation of *SN19 pCAMBIA 1301* plasmid into *E. coli*, 10 µl glycerol stock of *SN19 pCAMBIA1301* plasmid in *Agrobacterium* (*LBA4404*) was grown in 10 ml of LB broth containing 50 µg/mL of kanamycin and incubated at 28ºC overnight. The following day, plasmid isolation was performed using plasmid miniprep kit (Thermo Scientific-Cat#K0503). The plasmid was then transferred in *E. coli* strain (*DB301*) competent cells

(50µl) and mixed by tapping. These cells were then chilled on ice for 30 minutes and were then placed in hot water bath set at 42ºC for 50-60 seconds. LB broth medium (450 µl) was added into the cells and the cells were then incubated at 37ºC for 1 hour in incubator shaker. After 1 hour, cells were spread on LB plates containing 50 µg/mL of kanamycin and incubated at 37ºC overnight. Following day, colonies were checked and confirmed through colony PCR. *pTF101* plasmid was used as positive control. The PCR was performed at 95ºC for 4 minutes for denaturation, while annealing temperature was 55ºC for 15 seconds. Extension was carried out at 72ºC for 20 seconds repeated 34 times. The amplified PCR fragments were then run on 1% agarose gel and detected under UV light. The positive clones were cultured in LB broth containing 50µg/mL of kanamycin and incubated at 37ºC overnight for overnight growth. Following day, glycerol stocks were prepared and stored at -80ºC.

3.8 Cloning of *Cry3A* Gene in *SN19 pCAMBIA 1301* to Develop DS-1 Plasmid

3.8.1 Restriction digestion of gel eluted fragment of *cry3A* and *SN19 pCAMBIA 1301* plasmid

For cloning of *cry3A* gene in the plant expression vector *pCAMBIA 1301*, the purified PCR product of *cry3A* gene and *SN19 pCAMBIA 1301* plasmid were digested with two restriction enzymes (*Nco*I and *Bgl*II) to produce overhangs in desired fragments of *cry3A* gene and *SN19 pCAMBIA 1301* plasmid. Following reaction mixture combination were used as given in Table 3.4.

Figure 3.7. Reaction mixture of digestion of cry3A gene and SN19 pCAMBIA 1301 with Nco1 and BglII

Name	*cry3A* gene	*SN19 pCAMBIA1301*
DNA	1 µg	2 µg
10x Buffer	2µl	2µl
*Nco*I (1U/µl)	1µl	1µl
*Bgl*II (1U/µl)	1µl	1µl
Water	made volume up-to 20 µl	made volume up-to 20 µl

3.8.2 Ligation of insert (c*ry3A* gene) into *SN19 pCAMBIA 1301* vector

Insert (*cry3A* gene) and vector overhangs were ligated by using T4 DNA Ligase (Promega) according to the manufacture's protocol. Vector to insert ratio was 1:1, and ligation mixture was kept at 22 ºC for 2 hours, at 15 ºC for 3 hours and 4 ºC overnight. Detail of the chemicals used for ligation reaction is given in Table 3.5.

Figure 3.8. Reaction mixture for ligation of cry3A gene into SN19 pCAMBIA 1301 vector

Name	Quantity
*SN19 pCAMBIA 1301*Plasmid	50 ng
Insert (*cry3A* gene)	10 ng
10X Ligation buffer (Promega)	1.00 µl
T4 DNA ligase (3U/µl)	1.00 µl
Water	made volume up-to 10 µl

3.8.3 Transformation of DS-1 plasmid construct in *E. coli* Strain (*JM-109*)

The *E. coli* strain *JM-109* was used for ligation mixture transformations. From the ligation mixture, 4µl of ligated product was transformed into chemically competent JM-109 *E. coli* cells (50µl) and mixed by tapping. These cells were then chilled on ice for 30 minutes and were then placed in hot water bath set at 42ºC for 1 minute. LB broth medium (450 µl) was added onto the cells and the cells were then incubated at 37ºC for 1 hour in incubator shaker. After 1 hour, cells were spread on LB plates containing 50µg/mL of kanamycin and incubated overnight at 37 ºC.

Following day, colonies were checked and confirmed through colony PCR. *pGT-22-b* plasmid was used as positive control. The PCR was performed at 95ºC for 4 minutes for denaturation, while annealing temperature was 55ºC for 15 seconds. Extension was carried out at 72ºC for 20 seconds repeated 40 times. The amplified PCR fragments were then run on 1% agarose gel and detected under UV light. The positive clones were grown in LB broth containing 50µg/mL of kanamycin and incubated overnight at 37ºC. The following day, the plasmid DNA was extracted from the overnight grown culture as per protocol using plasmid miniprep kit (Thermo Scientific- Cat #K0503). After extraction, the plasmid was then run on 1% agarose gel and detected under UV light. The plasmid

construct DS-1 carrying *SN19* and *cry3A* genes under *35S CAMV* individually has been shown in Figure 3.4.

The clones confirmed from colony PCR were subjected to restriction digestion analysis. The plasmids were digested with *Nco*1 and *Bgl*II to confirm our clone.

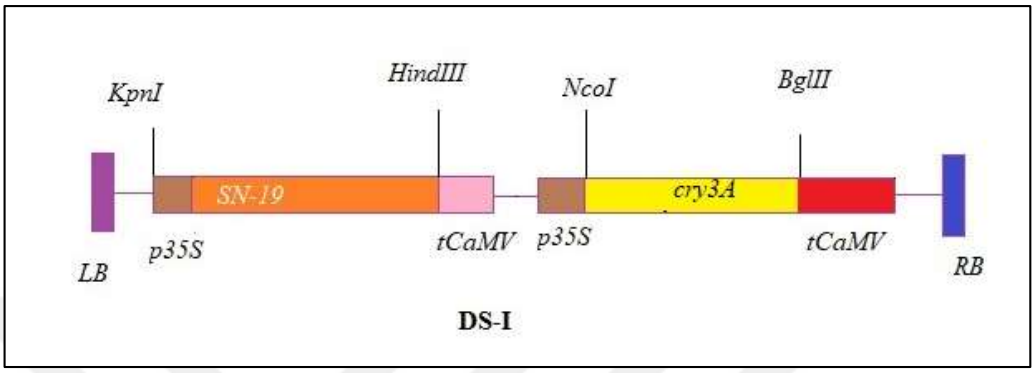


Figure 3.9. DS-1 cassette showing SN19 and cry3A genes fragments under 35S CAMV.
The vector contains nptII gene for bacterial selection whereas as hptII was used for plant selection

3.8.4 Transformation of DS-1 plasmid construct in *Agrobacterium* (*GV3101*)

The *Agrobacterium* strain *GV3101* was used for transformation of DS-1 plasmid. Seven microliters of DS-1 plasmid product was added onto 50µl chemically competent *Agrobacterium* cells and mixed by tapping. These cells were chilled on ice for 30 minutes and were then subjected to electroporation. The electroporation device was set at a capacitance of 25µF, voltage 2.4kV with a 200 ohms resistance. After electroporation, 450 µl of LB broth was added and the mixture was incubated at 28ºC for 3 hours at 600 rpm in shaking incubator. Cells were then spread on LB plates containing 50µg/mL of kanamycin and were incubated at 28ºC overnight. Following day, colonies were checked and confirm through colony PCR. Positive clones were grown in LB broth containing kanamycin 50µg/mL and incubated at 28ºC for 24 hours. The following day, the plasmid DNA was extracted from the overnight grown cultures as per protocol using plasmid miniprep kit (Thermo Scientific- Cat #K0503). After extraction, the plasmid was run on 1% agarose gel and detected under UV light.

3.9 Cloning of *OCII* Gene in *SN19 pCAMBIA 1301* to Develop DS-2 Plasmid

3.9.1 Restriction digestion of gel eluted fragment of *OCII* gene *and SN19 pCAMBIA 1301* plasmid

For cloning of *OCII* gene in plant expression vector *SN19pCAMBIA 1301*, the purified PCR product of *OCII* gene and *SN19 pCAMBIA 1301* plasmid were digested with two restriction enzymes (*Bgl*II and *Bst*EII) to produce overhangs in desired *OCII* gene and *SN19 pCAMBIA 1301* plasmid. Following reaction mixture combination were used as given in Table 3.6.

Figure 3.10. Reaction mixture of digestion with BglII and BstEII for OCII gene and SN19 pCAMBIA 1301

Name	*OCII* gene	*SN19 pCAMBIA 1301*
DNA	1 µg	2 µg
10x Buffer	2.00µl	2µl
*Bgl*II (1U/ µl)	1 µl	3 µl
*Bst*EII (10U/µl)	0.1µl	0.3 µl
Water	made volume up-to 20 µl	made volume up-to 20 µl

The samples were incubated for 45 minutes at 37 ºC for digestion and then for 10 minutes at 65 ºC to deactivate enzyme activities. The digested PCR product and vector was run on 1% agarose gel at 70V for 45 min and detected under UV light. The gel slices containing digested products were cut with sterilized blade and were eluted according to the manufacturer's protocol (GeneJET Gel extraction and DNA cleanup micro Kit, Cat# k0832).

3.9.2 Ligation of *OCII* gene into *SN19 pCAMBIA 1301* vector

Insert (*OCII* gene) and vector overhangs were ligated by using T4 DNA Ligase (Promega) according to the manufacturer's protocol. Vector to insert ratio was 1:2, and ligation mixture was incubated at 22 ºC for 2 hours at 15 ºC for 4 hours and overnight at 4 ºC. Details of the reaction mixture used for ligation is given in Table 3.7.

Figure 3.11. Reaction mixture for ligation of OCII gene into SN19 pCAMBIA 1301

Name	Quantity
SN19 pCAMBIA 1301 Plasmid	50 ng
Insert (*OCII* gene)	5 ng
10X Ligation buffer (Promega)	1µl
T4 DNA ligase (3U/ µl)	1 µl
Water	made volume up-to 10 µl

3.9.3 Transformation of DS-2 construct in *Agrobacterium* Strain *GV3101*

The A*grobacterium* strain *GV3101* was used for ligation mixture transformations. From the ligation mixture, 4µl of ligated product was transformed into chemically competent *GV3101* cells (50µl) and mixed by tapping. These cells were chilled on ice for 30 minutes. After 30 minutes, they were subjected to electroporation. The electroporation device was set at a capacitance of 25µF, voltage 2.4kV with a 200 ohms resistance. After electroporation, 450 µl of LB broth was added and the mixture was incubated at 28ºC for 3 hours at 600 rpm in shaking incubator. Cells were then spread on LB plates containing 50 µg/mL of kanamycin and were incubated overnight at 28ºC. Following day, few colonies were checked and confirmed through colony PCR. The positive clones were grown in LB broth containing 50µg/mL of kanamycin and incubated overnight at 28ºC. The following day, glycerol stock cultures were prepared and stored at -80 ºC. The plasmid construct DS-2 carrying *SN19* and *OCII* genes under 35S CAMV individually has been shown in Figure 3.5.

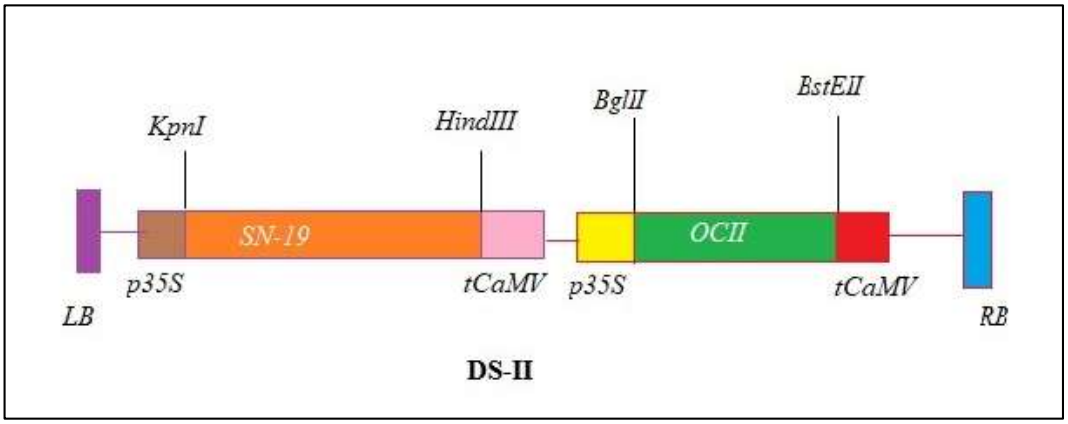


Figure 3.12. DS-2 cassette showing SN19 and OCII gene under 35S promoter. The vector contains nptII gene for bacterial selection whereas as hptII was used for plant selection

3.10 Genetic Transformation of Potato (*Solanum tuberosum* L.) Cultivars with DS-1 and DS-2 Plasmid Constructs

3.10.1 Plant material (potato plants)

Seedlings of potato cultivars (Lady Olympia and Agria) were grown from sprouts developed on sterilized tubers in the growth chambers in Steri Vent boxes (107 x 94 x 96 mm) (Cat. No: S1682.1440) of Duchefa Biochemie B.V. The shoots were sub cultured *in vitro* on basal MS medium (Appendix- II) containing Murashige and Skoog (1962) mineral salts, sucrose and agar under controlled environmental conditions at 27 ± 2ºC with a day length of 16 h light, provided by fluorescent tubes in growth chamber.

3.10.2 Explant propagation

To culture the transformed shoots or leaves cuts, MS (Murashige and Skoog, 1962) broth (Appendix-II) was used. Established shoot cultures of both cultivars were propagated in vitro using single node stem explants on the basal Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). The cultures were incubated under controlled environmental conditions at 27 ± 2ºC with a day length of 16 h light, provided by fluorescent tubes in growth chamber.

3.10.3 Preparation of bacterial inoculum

Agrobacterium tumefaciens strain *GV3101* harboring two different gene construct combinations (DS-1 and DS-2) was used for genetic transformation of potato cultivars. Prior to co-cultivation with potato tissues, the *Agrobacterium* strains harboring their respective genes in individual plasmids were streaked on Luria-Bertani Agar (LB Agar) (Appendix-III) plates containing 50µg/mL of kanamycin and incubated for 24–48h at 28 ºC (Figure 3.6). After incubation period, single colonies from each streaked culture were picked up with the help of small tips and inoculated in 10 mL of Luria-Bertani broth (LB broth) containing 50mg/mL of kanamycin in a 50 mL falcon tube (Figure 3.7). The bacteria were cultured overnight on a rotary shaker at 28 ºC with shaking at 180 rpm.

Figure 3.13. Streaking of Agrobacterium plasmid suspension overnight growth on LB agar plate with antibiotic

Figure 3.14. Overnight grown culture of DS-1 and DS-2 gene plasmids suspensions

3.10.4 Inoculation of explants with *Agrobacterium* Culture

Both leaf discs and internodes were used as ex plants. The explants of both the cultivars were excised with the help of sharp sterilized scissors in LB broth medium. The explants were then incubated for 30 minutes in the *A. tumefaciens* suspensions (O. D= 0.4) in LB broth medium (Figure 3.8). So, for a total 6500 explants were used in the transformation experiment.

After incubation, explants were blotted dry on filter paper. After drying, they were shifted on co-cultivation medium plates (Appendix-IV) for 2 days. The cultures were kept in incubator chamber at 25 °C± 2°C under 16: 8 hours of light: dark photoperiod. After 2 days explants were washed with sterile distilled water and then with sterile distilled water

containing Sulcid (1 mL/ 20mL water) for 20 minutes. After 20 minutes, the explants were dried on filter paper and transferred to regeneration selection media.

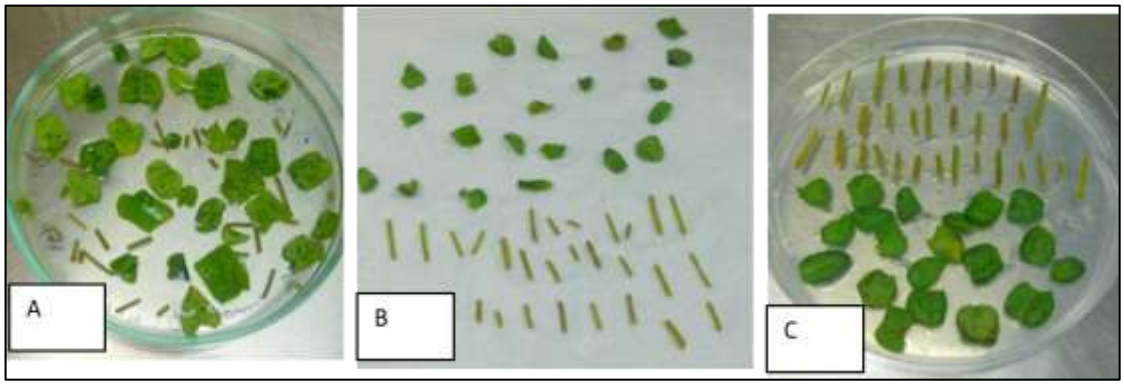


Figure 3.15. Inoculation of explants with Agrobacterium suspension (a), Drying of explants on blotting paper (b). Explants on co-cultivation medium after inoculation (c)

3.10.5 Regeneration selection medium (RSM)

After two days (48hrs) of cocultivation, explants were transferred to regeneration selection medium (Appendix-V) i.e. MS medium containing hygromycin (4mg/L) and sulcid (500mg/L).

3.10.6 Callus and shoot induction percentage

The total number of ex-plants that induce callus formation and shoot regeneration was calculated as per given formulae;

$$\%\ \text{age of callus induction} = \frac{\text{Number of calli formed}}{\text{Total number of explants}} \times 100$$

$$\%\ \text{age of shoot induction} = \frac{\text{Number of shoots obtained}}{\text{Total number of calli}} \times 100$$

3.10.7 Transfer and selection on shoot ınduction medium

Six weeks after growing explants on regeneration selection medium, developed calli were transferred to selective shoot-induction medium (Appendix-VI).

3.10.8 Transfer and selection on root medium

After 5-7 weeks, plantlets of both Agria and Lady Olympia with well-developed shoots were sub cultured and transferred to larger boxes containing root inducing medium (MS supplemented with vitamins, 30 g/L of sucrose, 8g/L of agar, 200 mg/L of Sulcid and 4 mg/ L of hygromycin).

3.10.9 Acclimatization

After root formation, the putative transgenic plants of both Agria and Lady Olympia were transferred into plastic pots containing soil media consisted of mixture of perlite, peat and vermiculite (1:1:1 v/v/v) and were first shifted to growth chamber for acclimatization (27 ± 2 °C, 16:8 hours light/dark photoperiod) and then were transferred in a green house.

3.11 Molecular Analysis of Primary Transformants

3.11.1 Genomic DNA extraction from putative transgenic plants

The plant genomic DNA was isolated from young leaves of both the Lady Olympia and Agria according to the method describe by Saha et al. (1997) and Zhang et al. (2000) with some modifications. In order to isolate the genomic DNA, about 0.5 g fresh leaves were cut from transgenic potato plants as well as from their non transgenic control plants and were ground into a fine powder in a pre-cooled mortar and pestle by the addition of liquid nitrogen. The ground powder of each plant was then put in eppendorf tubes (2 ml). To each 100 mg of grinded sample, 500 µl of DNA Extraction Buffer (Appendix-VII) was added and vortexed for two minutes. The samples in the eppendorf tubes were then incubated at 65 ^{0}C for 1 hour with 12,000 rpm in a thermo cycler machine. The samples were inverted after every 10-15 minutes. The samples were then centrifuged at 4 ^{0}C for 10 minutes at 14,800 rpm in centrifuge machine. The supernatant was transferred into tubes and 0.5 mL of DNA Lysis Buffer was added. The tubes were then again incubated at 65°C for 30 minutes. After incubation, equal volume (500 µl) of Chloroform: Isoamyl Alcohol (24:1) was added and the mixture was centrifuged at 14,000 rpm for 10 minutes at 4 °C. The Supernatant (aqueous phase) was transferred to new 1.5 mL tubes. The above step was repeated. An equal volume of chilled Isopropanol was added and the tubes were

incubated at -20 °C for 1 hour. After incubation, the samples were then again centrifuged at 14,000 rpm for 10 minutes at 4 °C. After centrifugation, the supernatants were discarded, and the tubes were slightly dried on sterile tissue paper without disturbing the pellet. The pellet was then washed with 70 % cold ethanol (500 µL) and then again centrifuged at 14,000 rpm for 10 minutes at 4°C.

After centrifugation, again the supernatants were discarded; the pellets were air-dried and resuspended in 100 µL of TE Buffer (Appendix- VIII). Two µL RNAase (10mg mL^{-1} stock) was added into each tube. The tubes were then incubated at 37 °C for 45 minutes and then at 65 °C for 5 minutes to deactivate enzyme with slight shaking. After incubation, samples were then again centrifuged at 14,000 rpm for 10 minutes at 4°C. After centrifuge, the supernatants were transferred into new 1.5 ml tubes and stored at -20 °C.

3.11.2 PCR assays

After extraction of genomic DNA, the DNA samples were then resolved on 0.8 % agarose gel and observed under UV light. After measuring the quality and quantity of the DNA samples, they were diluted to desirable concentrations. The plant genomic DNA was then subjected to different PCR analyses using the gene specific partial primers of *cry3A*, *OCII*, *SN19* and *hygromycin Phosphotransferase II* (*hpt*II gene). The extracted genomic DNA were also checked with PCR using *Chv A* gene-specific primers 5'CGAAACGCTGTTCGGCCTGTGG3' and 5'GTTCAGCAGGCCGGCATCCTGG3' to amplify 890-bp of the gene for checking any *Agrobacterium* contaminations. The reactions were performed in a 20 µl volume containing 50 pM of each primers, 1X of Thermo Scientific™ DreamTaq Green PCR Master Mix (Cat. No. K1081), 8.5 µl of Thermo Fisher sterile water and 50 ng DNA as template. The PCR reaction conditions for the *cry3A* and *SN19* gene were follows: at 95 ºC for 5 minutes for denaturation followed by annealing temperatures at 55 ºC for 15 seconds. Extension was carried out at 72 ºC for 30 seconds repeated 40 times. Final extension was carried out at 72 ºC for 7 minutes.

Similarly, PCR reaction condition for *hptII* was performed at 95ºC for 4 minutes for denaturation followed by annealing temperatures at 55 ºC for 20 seconds. Extension was

carried out at 72 ºC for 30 seconds followed by 34 times. Final extension was carried out at 72 ºC for 7 minutes.

PCR reaction condition for C*hv A* gene specific primer set was performed at 95 ºC for 5 minutes for denaturation followed by annealing temperatures at 64 ºC for 15 seconds. Extension was carried out at 72 ºC for 20 seconds followed by 34 times. Final extension was carried out at 72 ºC for 8 minutes.

3.11.3 Calculation of transformation efficiency

Transformation efficiency calculations for transgenic plants of both potato cultivars (Agria and Lady Olympia) was performed after confirming different analyses results. Mean Percent Transformation Efficiency (%MTE) was calculated as given below;

$$\%\ \mathrm{MTE} = \frac{\mathrm{nTPR}}{\mathrm{tnEU}} \times 100$$

where, nTPR= Number of transgenic plants regenerated,
tnEU= Total number of all ex-plant used

3.11.4 Enzyme-linked immunosorbent assay (ELISA) of the putative transgenic plants

The transgenic potato plants were analyzed through ELISA for Cry3A protein accumulation. For this purpose, fresh leaves from each transgenic plant along with their respective control were grinded with liquid nitrogen and added (0.5 mg) to eppendorf tubes (2ml). Shortly after it, Protein extraction buffer (Appendix- IX) was added (500 µl) to each tube. The tubes were then centrifuged at 14,000 rpm at 4°C for 15 minutes. After centrifugation, the supernatant was transferred into new 1.5 ml tubes. After protein extraction, ELISA was performed using EnviroLogix Qualiplate^{TM} Kit (Cat. No. AP050NWV10) for CRY3A. The protein in each tube was diluted 10 times with EB2 extraction buffer from the above kit. After dilution, 50 µl of the plant protein and 50 µl of conjugate enzyme were put in each well with slight shake for 30 seconds to mix them properly. Last two samples were used as an internal positive and negative. After mixing, they were covered with parafilm and placed at ambient temperature (37 °C) for two hours.

After incubation, the protein samples were washed three times with PBS buffer (1X buffer, Appendix- X). After washing, 100 µl of substrate from kit were added to each well and covered with parafilm and incubated for 15-30 minutes. Lastly the reaction was stopped by adding 100 µl of stop solution to each well. Then the wells were placed in ELISA machine for reading and data was recorded (Figure 3.9).

Figure 3.16. Bio-Tek FLx800 universal Micro-plate reader for ELISA

3.11.5 Southern blot

Southern blot analyses were performed as per method of Southern (1975) with some modifications. Genomic DNA were extracted from PCR and ELISA positive plant leaves of both Agria and Lady Olympia (transformed with DS-1 plasmid construct) and their non-transgenic control plants using Thermo Scientific plant genomic DNA extraction kit (Cat. No. 0722). Approximately 10 µg DNA of transgenic plant samples were digested with *Kpn*1 to release the fragment from DS-1 plasmid. The digested DNA samples were then run in 1% agarose gel at 30V for 4 hours and then at 12 V overnight. After running, gel depurination was done using 0.25M HCl for 10 minutes with slight shaking at 50 rpm on Digital rocker. The gel was then denatured with a mixture of 1.5M NaCl and 0.5M NaOH for about 30 minutes. Gel was then neutralized with 0.5M Tris-HCl and 1.5M NaCl at pH 7.5. Digested samples of DNA were then transferred to the Hybond^{TM}-N^{+} Nylon Membrane (Amersham; Cat. No. RPN119B) with solution of 10X SSC (Appendix-XI) by the capillary action. DNA was fixed on the membrane by placing the membrane under UV light for about 6 minutes. After fixing DNA, membrane was then transferred

to hybridization tube along with pre-hybridization buffer solution and was treated for 2 hours at 55 ºC. Hybridization with the probe was done for 24 hours. User guide of Fermentas Biotin Chromogenic Detection Kit (Cat. No. K0661) was used for the detection. 1X blocking/washing buffer (10 ml) was used to wash membrane with moderate shaking at about 50 rpm. After washing step, the membrane was dipped in 10 ml blocking solution with moderate shaking, for 30 minutes. After removing the blocking solution properly, membrane was saturated with 10 ml of diluted streptavidin AP conjugate followed by washing with 1X blocking/washing buffer. Afterwards, washing buffer was removed and 10 ml of detection buffer was added and incubated for 30 minutes. Finally, 1X NBT/BCIP was prepared and dissolved in detection buffer to carry out enzymatic reaction which needs incubation of membrane in 20 ml of 1X NBT/BCIP for 30 minutes.

3.11.6 Probe labeling

Labeling of probe for detection of *SN19* gene fragment was done using Thermo scientific Biotin Deca Label DNA labeling kit (Cat. No. K0651). As per manufacturer instructions, 13 µl of template (100 ng), 5 µl of decanucleotides in the 5X reaction buffer along with nuclease free water up to 44 ul were added in the eppendorf tube. The tube was then vortexed for 3-5 seconds to spun down. The tube was placed in boiling water bath for 5-10 minutes and then chilled down quickly on the ice. After spinning the mixture was transferred to eppendorf tube. To the eppendorf tube, 5 µl of Biotin Labeling mix and 1ul Klenew (5U) fragment was added. Tube was then spun down with all added contents, and was incubated for 20 hours at 37 °C. After finishing incubation time, 1µl of 0.5M EDTA at pH 8.0 was added to stop the reaction. The labeled probe (DNA) was estimated by spotting that on the nitrocellulose membrane along with the control DNA labelled as control (provided in the kit) in consecutive (Serial) dilutions and was then finally detected using Thermos Scientific Biotin Chromogenic Detection Kit (Cat. No. K0661).

3.12 Lab Bio-toxicity Assays

3.12.1 Insect culture (Lab Imi-resistant CPB Colony

More than 100 adults of Colorado potato beetle (*L. decemlineata*) (approximately 1:1 sex ratio) were collected from potato research fields in Nigde Omer Halisdemir University in July 2014. Stock cultures of Colorado potato beetle was maintained at 25 ± 1°C under a 16:8 h light–dark photoperiod and 60-65% relative humidity on non-transgenic potato seedlings in the growth chamber as described by Gökçe et al. (2006). Insecticide free seedlings of potato cultivars (Lady Olympia and Agria) were developed in small pots from sterilized tubers in the growth chambers (Figure 3.10). These seedlings were used for beetle rearing and oviposition. To provide selection pressure, a dose of imidacloprid (375 µg/mL) was applied to CPB 2nd instar larvae during each generation. This selection pressure with the above dose was tried for six consecutive generations. After six generations, topical bioassays were performed with CPB 2nd instar larvae to assess level of resistance. Five serial dilutions were made based on preliminary data viz 187.5 µg/mL, 468.75 µg/mL, 937.5 µg/mL, 1875 µg/mL and 3750 µg/mL with the imidacloprid (Confidor®, Bayer, Turkey) in water. Each larva was treated with 1 µl of insecticide suspension with the help of a 50 µl Hamilton syringe (Hamilton Company, Romania) coupled with micro applicator (Burkard Scientific, UK). The treated larvae were transferred into 90 mm Petri dishes and left to dry for 30 min. After that, they were transferred into new 90 mm Petri dishes and provided with pesticide free potato foliage. In the control group, larvae were treated with 1µl distilled water. The mortality rate was recorded 24h intervals for 3 days. The larva was counted as dead if it was not responding when puked with a fine brush (Mota-Sanchez et al., 2006). The experiment was set up in Randomized Block Design and each treatment day represented a block. Each block contained all doses and a control group. The whole experiment was repeated three different days. For each concentration, total 30 uniform CPB 2nd instar larvae (3 replication, each containing 10 larvae) were treated. The data was analyzed with a probit program (PoloPlus, Leora Software Company, Parma, USA) and LD_{10}, LD_{50}, LD_{90}, slope and heterogenicity values were calculated.

From the comparison of LD_{50} values, it was observed that Lab resistant population was 26.09X more resistant than lab susceptible CPB colony (Unpublished Data, Muhammed

Nadir Naqqash PhD Thesis). From time to time they were mixed breed with the imidacloprid resistant field population to avoid inbreeding depression. Moist soil media consisted of mixture of perlite, peat and vermiculite (1:1:1 v/v/v) (Klasmann- Deilmann GMBH, Germany) were used in the growth chamber bottom as a site for pupation.

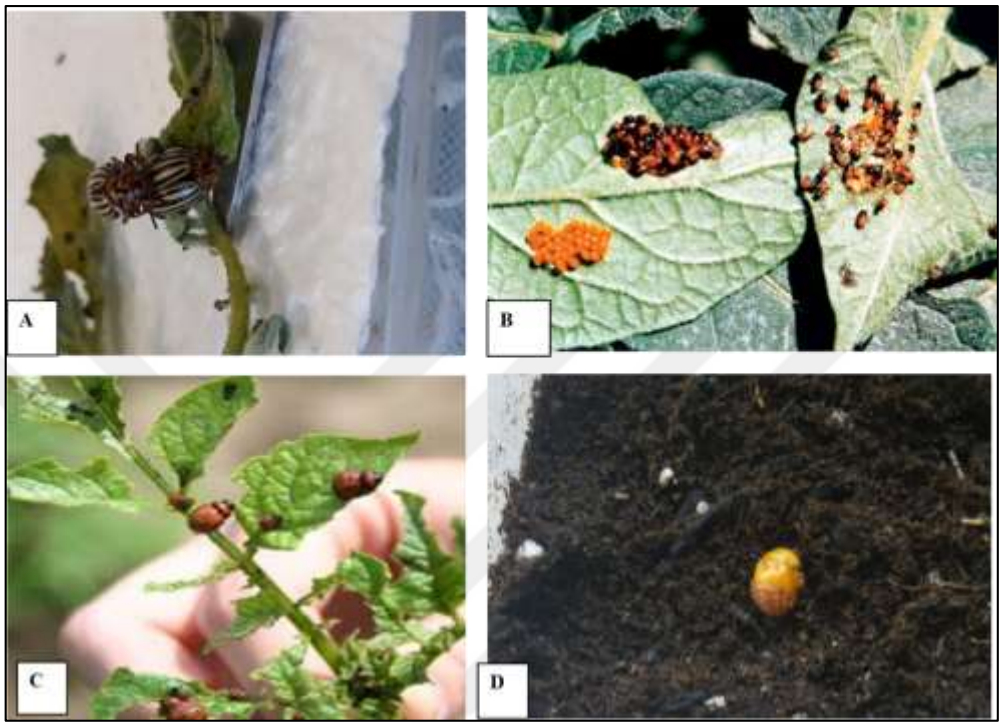


Figure 3.17. Insect culture rearing in lab for leaf biotoxicity assays. CPB adult mating (a), egg and larval instars of CPB (b&c), CPB pupal stage (d)

3.12.2 Life table studies

Prior to beginning the life table study, imi-resistant CPB colony was reared for one generation on each potato cultivar (Lady Olympia and Agria) in the growth chamber at 25 ± 1°C under a 16:8 h light–dark photoperiod and 60-65% relative humidity for eliminating any factor. After completing one generation, 30 mated pairs were shifted to the fresh sprouts of each cultivars.

3.12.2.1 Individually rearing method

The adult CPB were allowed to lay eggs for 24h on 3-4 leaflets stage of pesticide free potato plants. After oviposition on potato plants, fresh egg batches along with leaves (79 and 75 eggs) were randomly collected from Agria and Lady Olympia seedlings

respectively in glass Petri dishes (100x 20mm dimension) (Cat. No: 081.01.100, ISOLAB) on top of a moist WHATMAN® No filter paper (SIGMA-ALDRICH (MeRck), Turkey) until hatching. Each egg was considered as a replicate. Egg hatching data was recorded daily. After hatching, each neonate larva was then transferred into a new glass petri dish containing a potato leaflet (Figure 3.11). Each larva was also considered as a replicate. A fresh potato leaflet of each cultivar was supplied to individual larva every 24 h. The larvae were kept in growth chamber at 25± 1°C under a 16:8 h light–dark photoperiod and 60-65% relative humidity. Data regarding larval development and survival rate were recorded at 24 hours intervals on each potato cultivar. Different larval stages were confirmed by the presence of exuvium. When the 4th instar larvae reached to prepupal stage, they were shifted to individual rearing hard plastic cups (147,85 ml, Yöm Plastik Company, Istanbul, TR) containing sterile soil. The cups containing pupae were placed in the same incubators as those used for larval development and were checked daily for adults emergence. They were kept moist to avoid pupal desiccation. Freshly emerged adults were collected and sexed by examining the ventral tip of the abdomen as proposed by Rivnay (1928) and Gelman et al. (2001) as "males have a slight depression on the posterior ventral tip of the abdomen just anterior to the anal opening". After sexing, one male plus one female (1♂ + 1♀) was transferred into an egg laying apparatus (two 147,85 ml plastic cups combined with a parafilm band). There was a small hole at one of the bottoms of cup where the stem of potato plant was dipped into water solution. There were four holes on each cup for aeriation containing four fresh leaves of each cultivar for oviposition. The base of the leaves was dipped in a small glass bottle (20 ml, SIGMA-ALDRICH (MeRck), Turkey) containing water to keep the leaves moist. The leaves were replaced with two days intervals. Eggs laid by each female per day were counted and recorded daily. Newly hatched larvae were counted to determine the egg hatching rate by dividing of the emerged larvae to the total number of eggs [Hatching Rate= (Number of hatched eggs/Total laid eggs) x100]. Egg hatching, oviposition period and adult longevity at 24 hours intervals were recorded on each cultivar until the death of all adults.

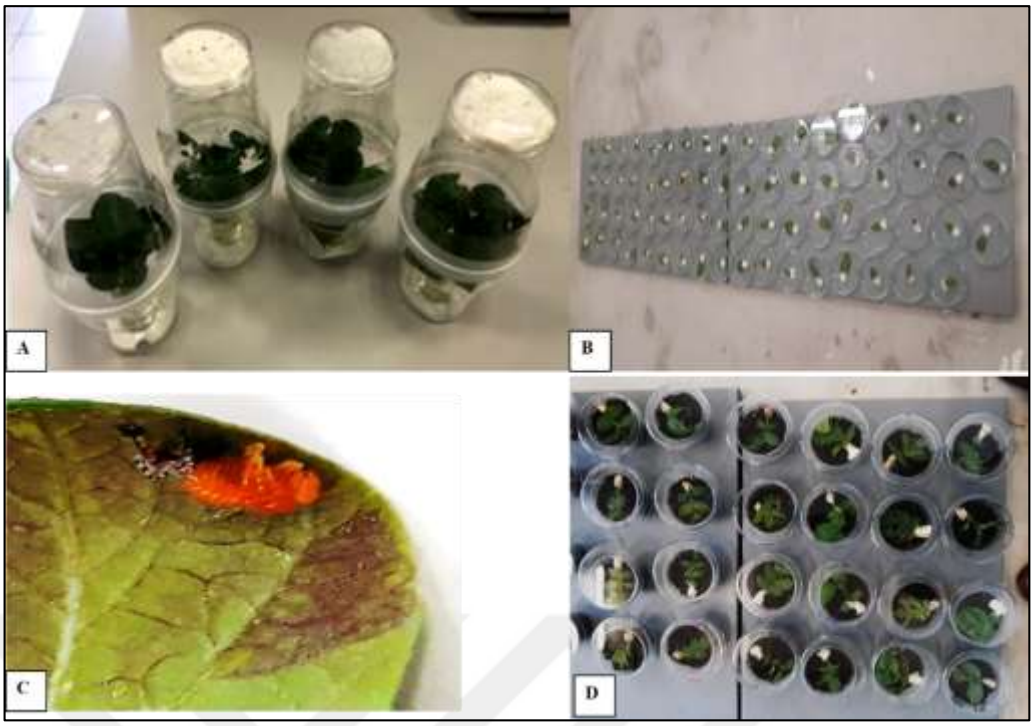


Figure 3.18. Rearing of mated pair of CPB in plastic cups for oviposition (a), individual rearing of CPB larval stages in glass petri plates (b), larval molting and presence of exuvium (c) and individually reared 4th instars larvae shifted to plastic cups with soil for pupation (d)

3.12.2.2 Group rearing method

For group rearing method, seventy eggs were collected in groups from each variety (Agria and Lady Olympia). Due to cannibalism in CPB larvae (the practice of eating the flesh of one's own species), these eggs were first divided into 4 parts. Same procedure was done for the other cultivar. Daily observation was made regarding the number of eggs hatched. After hatching, the larvae were reared in the same groups in larval rearing Steri Vent boxes (107 x 94 x 96 mm (Cat. No: S1682.1440, Duchefa Biochemie B.V. Netherlands) at 25± 1 °C under a 16:8 h light–dark photoperiod and 60-65% relative humidity. Fresh leaflets of each variety were daily provided to the respective larva groups and larval stages were recorded daily until pupation. Full grown larvae in groups were then transferred to pupation cages (107 x 94 x 96 mm), where moist soil was placed in the bottom to facilitate pupation and were kept in the same growth chamber as for larval rearing (Figure 3.12). These cages were checked daily for adult emergence. Newly emerged adults were sexed as method described in 3.6.2.1 and were transferred in groups in two mating cages (107 x 94 x 96 mm (Cat. No: S1682.1440, Duchefa Biochemie B.V.) for each variety and the

number of eggs laid collectively, and dead adults of both sexes were recorded daily until the death of all adults.

Figure 3.19. Rearing of late larval stages of CPB in groups in plastic cage with sterilized soil for pupation

3.12.2.3 Life table study of CPB with transgenic potato plants

Life table study of CPB with transgenic plants of both Agria and lady Olympia from low to high expression levels were tried first with freshly emerged adults and then directly with egg clusters collected from their respective non-transgenic plants. Same experimental conditions were used for studying life table parameters of CPB on transgenic plants of both Agria and Lady Olympia as described in 3.12.2.1 for individual reared CPB life table and grouped reared CPB life table in 3.12.2.2.

3.12.3 Bioassays with transgenic potatoes

The toxicity potential of introduced insecticidal genes in T_0 progeny transgenic potato plants were evaluated against Colorado potato beetle larvae and adults. Larval Mortality, leaf consumption area, adult longevity and reproduction rate were evaluated on both transgenic plants and commercial varieties of potato cultivars.

3.12.3.1 Mortality rates of various life stages of lab resistant CPB fed on transgenic potato plants of Agria and Lady Olympia

The toxicity potential of introduced insecticidal genes in T_0 progeny transgenic potato plants were evaluated against CBP larvae and adults. A single fresh potato leaflet of each putative transgenic potato plants was placed in a petri dishes (100x 20mm dimensions) (Cat. No: 081.01.100, ISOLAB) on top of moist filter paper and pre-starved 1st instar larvae (10 number) were shifted into the petri dish. Each treatment was repeated three time at different occasions. A total of 30 larvae were used in each treatment. The larvae were reared at 25± 1°C under a 16:8 h light–dark photoperiod and 60-65% relative humidity in the growth chamber. A fresh potato leaf was provided at 24 hours intervals. The mortality rate and other parameters were recorded every 24 hours. The same experimental treatments were used for the 2nd, 3rd and 4th larval instars and adults. The experiments were repeated at three different occasions for each stage and each cultivar.

3.12.3.2 Reproduction rate of CPB Lab resistant colony on transgenic potato plants of Agria and Lady Olympia

The reproduction rate of CPB lab resistant colony on transgenic potato plants of Agria and Lady Olympia were calculated. Newly emerged adults were collected from the stock culture. These newly emerged beetles were sexed as method describe in 3.6.2. 1. After sexing, one male and one female were released on three leaflets of each transgenic plants in an egg laying apparatus (two 147,85 ml plastic cups combined with a parafilm band). There was a small hole at one of the bottoms of cup where the stem of potato plant was dipped into water solution. There were four holes on each cup for aeriation containing four fresh leaves of each cultivar for oviposition. The base of the leaves was dipped in a small glass bottle (20 ml, SIGMA-ALDRICH (MeRck), Turkey) containing water to keep the leaves moist.

A total of 30 adult insects (15 pairs) were used in each treatment. The insects were reared on potato leaflets at 25± 1°C under a 16:8 h light–dark photoperiod and 60-65% relative humidity. In the control treatment, non-transgenic control plants were used for oviposition. Eggs laid by each female per day were counted and recorded daily. Newly hatched larvae was counted to determine the egg hatching rate by dividing of the emerged

larvae to the total number of eggs [Hatching Rate= (Number of hatched eggs/Total laid eggs) x100]. Egg hatching, oviposition period and adult longevity at 24 hours intervals were recorded on each cultivar until the death of all adults.

3.12.3.3 Leaf consumption area of various life stages of lab resistant CPB fed on transgenic potato plants of Agria and Lady Olympia

The leaf consumption area of various stages of lab resistant CPB fed on transgenic plants of Agria and Lady Olympia was measured. The experimental set up was similar to procedure described in 3.12.2.1. A single fresh potato leaflet of each transgenic potato plants was placed in petri dishes on top of moist filter paper and pre-starved 1st instar larva was shifted individually into each petri dish. The larvae were reared at 25± 1°C under a 16:8 h light–dark photoperiod and 60-65% relative humidity. Leaf consumption area of each 1st instar larvae was measured individually after 24 hours. The same experimental conditions were used for the 2nd, 3rd and 4th larval instars and adults. For measuring leaf consumption area, a computer program for measuring leaf area software was used (Huang and Chi, 2017). The experiments were repeated at three different occasions for each stage and each cultivar.

3.13 Statistical Analysis

3.13.1 Life table analysis

3.13.1.1 Individually rearing method

All raw life history data of CPB i.e. developmental time of eggs, larvae, pupae, those dying before adult stages, male and female adults and female daily fecundity were analyzed using age-stage, two-sex life table method (Chi and Liu, 1985; Chi, 1988). The life history data were pooled and analyzed by the computer program TWOSEX-MSChart (Chi, 2018). The age-stage specific survival rate (s_{xj}) (where x = age and j = stage), is the probability that a newly oviposit egg will survive to age x and stage j, the age-specific survival rate (l_x) is the probability that a newly laid egg survives to age x, the age-stage specific fecundity (f_{xj}), the age-specific fecundity (m_x; the mean fecundity of individuals

at age *x*), and other parameters, the intrinsic rate of increase (*r*), the net reproductive rate (R_0), the finite rate of increase (λ), and the mean generation time (*T*) were calculated.

According to the age-stage, two-sex life table theory (Chi and Liu, 1985), the age-specific survival rate (l_x) and the age specific fecundity of the population (m_x) are calculated as follows;

$$l_x = \sum_{j=1}^{m} s_{xj} \tag{1}$$

$$m_x = \frac{\sum_{j=1}^{m} s_{xj} f_{xj}}{\sum_{j=1}^{m} s_{xj}} \tag{2}$$

where, *m* is the number of stages of the study cohort.

The net reproductive rate (R_0) represents the mean number of off-spring that an individual can produce during its lifetime i.e. R_0 is the cumulative summation of $l_x m_x$ from birth to death and is calculated as (Chi and Liu, 1985).

$$R_0 = \sum_{x=0}^{\infty} l_x m_x \tag{3}$$

The intrinsic rate of increase (*r*) which is defined as "the rate of increase per head under specified physical conditions, in an unlimited environment where the effects of increasing density do not need to be considered" was calculated by using the iterative bisection method from the Euler–Lotka formula (Goodman, 1982) with age indexed from 0 (age of the newly laid eggs) as follows;

$$\sum_{x=0}^{\infty} e^{-r(x+1)} l_x \, m_x = 1 \tag{4}$$

Similarly, the "*GRR"* (Grass reproductive rate) was calculated as follows;

$$GRR = \sum m_x \tag{5}$$

The finite rate of increase (λ*;* the rate of increase per individual per unit time.) is calculated as follows (Goodman, 1982);

$$\lambda = e^{r} \tag{6}$$

The mean generation time (T; the length of time that a population requires to increase to the R_0-fold of its size at the stable age-stage distribution) was calculated as follows (Goodman, 1982);

$$T = \frac{\ln R_0}{r} \tag{7}$$

Life expectancy another parameter which is defined by Shryock et al. 1973 as "the average time that a member of a population is predictive to live". According to the age-stage, two-sex life table theory, individuals of the same age may have different life expectancies; hence, the age-stage specific life expectancy (e_{xj} is the length of time that an individual of age x and stage j is expected to live) and is calculated according to Chi and Su (2006) by assuming $s'_{xj} = 1$

$$e_{xj} = \sum_{i=x}^{\infty} \sum_{y=j}^{k} s'_{iy} \tag{8}$$

where s'_{iy} is the probability that an individual of age x and stage j can survive to age i and stage y.

The concept of reproductive value was introduced by Fisher (1930) that represents the role of an individual of age x and stage j to the upcoming population commonly use in demography and population genetics studies. According to Tuan et al. (2014) and Huang and Chi (2011), the reproductive value (v_{xj}) in terms of age stage two-sex lifetable theory can be defined as "the contribution of an individual of age x and stage j to the future population" and is calculated as

$$v_{xj} = \frac{e^{(x+1)}}{s_{xj}} \sum_{i=x}^{\infty} e^{-r(x+1)} \sum_{y=j}^{k} s'_{iy}.f_{iy} \tag{9}$$

The bootstrap technique described by Efron and Tibshirani (1993) was used to ensure precise estimates in the variances and standard errors of population parameters. To ensure precise and stable estimates, 100,000 bootstraps was used in this study. The analyses of the life table parameters were simplified by using the computer program TWOSEX-

MSChart (Chi, 2018) available for download at http://140.120.197.173/Ecology /prod02.htm. The paired bootstrap test (Akköprü et al., 2015; Efron and Tibshirani, 1993) was used to compare the differences in developmental time, adult longevity, adult preoviposition period define as "the period between the emergence of an adult female and her first oviposition" (APOP), total preoviposition period define as " the time interval from birth to the beginning of oviposition" (TPOP), oviposition days, and fecundity between treatments. The population parameters between the two treatments were also compared by using the paired bootstrap test based on the confidence interval of difference (Akköprü et al., 2015; Efron and Tibshirani, 1993), where the *P* value of paired bootstrap test was defined as: *P*, Agria to Lady Olympia was also calculated using the experimental data.

3.13.1.2 Group rearing method

All group reared data of CPB i.e. developmental time of eggs, larvae, pupae, those dying before adult stages, sexes (male and female), adults and female daily fecundity were collected according to the age-stage two-sex life table theory (Chi and Liu, 1985; Chi, 1988) and analyzed using TWOSEX-MSChart (Chi, 2018). As the insects were reared in groups, the number of individuals that survived to age "*x"* for each stage were recorded. The survival rate (s_{xj}) to each age-stage unit was calculated as

$$s_{xj} = \frac{n_{xj}}{n_{01}} \tag{10}$$

where "n_{01}*"* (the number of eggs used at the beginning of life table study) and "n_{xj}*"* (the number of insects that survived to age *x* and stage *j*). Since we observed and collected the total number of eggs (E_x) laid by all female adults together (the seventh life stage) at age *x*, female age-specific fecundity "f_{x7}*"* and the net reproductive rate (R_0) was calculated as (Chang et al., 2016).

$$f_{x7} = \frac{E_x}{n_{x7}} \tag{11}$$

$$R_0 = \sum_{x=0}^{\infty} \sum_{j=1}^{k} s_{xj} f_{xj} \tag{12}$$

where "*k*" is the number of life stages. The same procedure as described previously for individually reared method was used for calculating the age-specific survival rate (l_x), the age-specific fecundity (m_x), the finite rate of increase (λ), the intrinsic rate of increase (r), and the mean generation time (T).

The age-specific life expectancy (e_x*;* the time that individuals of age *x* are predictable to live) for group rearing method 20 is calculated as;

$$e_x = \frac{\sum_{i=x}^{n} l_i}{l_x} \tag{13}$$

l_i =the probability that an individual of age *0* will survive to age *i.*
The reproductive value (v_x) in terms of age stage two-sex lifetable theory is calculated as;

$$v_x = \frac{e^{-r(x+1)}}{l_x} \sum_{i=x}^{\infty} e^{-r(i+1)}\, l_i m_i \tag{14}$$

The bootstrap technique as described previously for individual reared method was used to estimate the standard errors for population parameters. All data calculated for each group was subjected to the bootstrap method with 10,000 resampling for estimating the standard errors of population parameters. Difference between treatments were then compared by using the paired bootstrap method (Efron and Tibshirani, 1993; Akköprü et al., 2015).

3.13.1.3 Population projection

Survival rate and fecundity data of CPB population reared individually and in group reared methods on both Agria and Lady Olympia were used to project the population growth of *L. decemlineata* using the TIMING-MSChart program (Chi and Liu, 1985; Chi, 1990; Chi, 2018). For initial population, ten eggs were used to project the population growth of CPB for 60 days duration.

3.14 Data Analysis

All the experiments for genetic transformation and the evaluation of transgenes were analyzed statistically using Statistix 8.1 program (McGraw-Hill, 2008). The mortality

data recorded after different incubation hours for different stages of CPB were corrected using Abbott's formula (Abbott, 1925). The mortality data were then transformed into arcsine to ensure normality and variance homogeneity (Zar, 1999) and submitted to a one-way analysis of variance (Annova), with significance being tested at the 5% level ($P \leq 0.05$) using Statistix 8.1 (McGraw-Hill, 2008). The difference between treatments were tested with multiple comparison test of Tukey with significance at the 5% level ($P \leq 0.05$) using Statistix 8.1 (McGraw-Hill, 2008).

The data regarding leaf consumption area was calculated using computer-based software imaging program, developed by Huang and Chi (2017), was used. They were analyzed with one-way analysis of variance (Annova) and then followed by the Tukey comparison test with the significance level at 5% ($P \leq 0.05$) using Statistix 8.1 (McGraw-Hill, 2008).

Various life table studies data were subjected to analyses using age-stage TWOSEX-MS Chart program (Chi, 2018). The estimation of the standard errors for population parameters was done with the bootstrap method. To estimate the standard error of populations parameters, all data calculated for each group was subjected to 10,000 resampling using the bootstrap method. Difference between treatments were also compared by using the paired bootstrap method based on the confidence interval of difference (Efron and Tibshirani, 1993; Akköprü et al., 2015).

CHAPTER IV

RESULTS

4.1 Amplification and Cloning of *SN19, Cry3A* and *OCII* Genes

For amplification and cloning of above mentioned genes in the *pCAMBIA 1301* vector, following steps were performed.

4.1.1 Amplification of *cry3A* gene

pGT-22-b plasmid was used as a template to amplify 1832bp of *cry3A* gene. The gradient revealed best results at both 50 ºC and 55 ºC (Figure 4.1 A). PCR amplified product was purified from agarose gel with Thermo Scientific GeneJET Gel extraction and DNA cleanup micro Kit (Cat# k0832) (Figure 4.1 B). Eluted fragment was further used for cloning purpose.

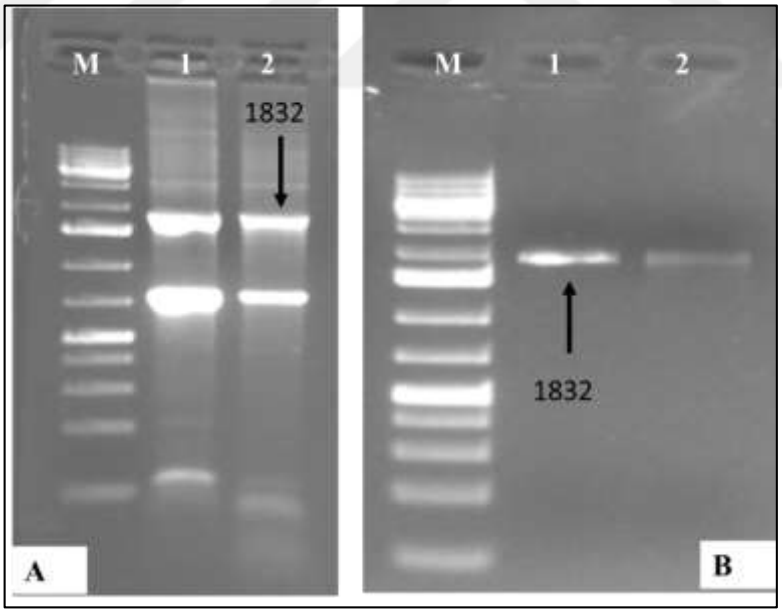


Figure 4.1. Amplification of cry3A gene from pGT-22-b plasmid
PCR assay showed amplification of correct size of cry3A gene, Lane M: 1kb plus ladder (Thermo Scientific), Lane 1-2: cry3 A gene amplified at 50ºC and 55ºC, respectively (A), Gel eluted fragment of cry3A gene (B)

4.1.2 Confirmation of *SN19* in *pTF101* plasmid

The plasmid *pTF101* extracted from overnight culture of *Agrobacterium* were subjected to PCR assay. The revealed the presence of *SN19* partial fragment (480 bp) (Figure 4.2). After PCR confirmation, the isolated *pTF101* plasmid was used further to excise *pro35S-SN19-CaMV* terminator fragment.

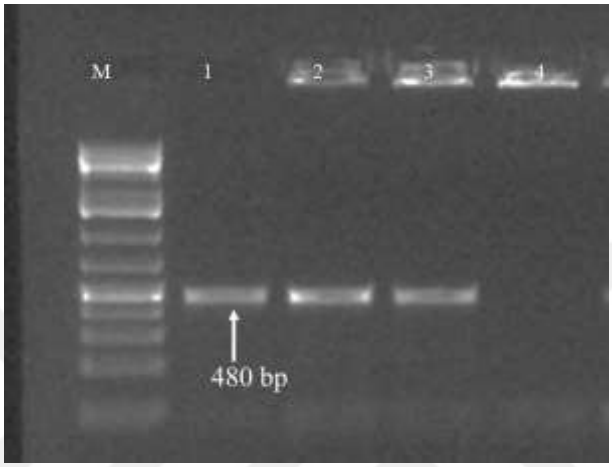


Figure 4.2. Amplification of SN19 gene using internal primers specific SN19 gene M: 1kb plus ladder thermo scientific, Lane 1: positive control (pTF101 plasmid) 2-3: Positive clones of 35S-SN19 gene. 4: negative control with no DNA

4.1.3 Amplification of *OCII* gene

The amplified PCR fragments of *OCII* gene were run on 1% agarose gel for 25 min at 100 V and then observed under UV light (Figure 4.3). PCR product was then purified from agarose gel as per protocol. The size and quality of the eluted fragments were confirmed (Figure 4.4). Gel eluted fragments were further used for cloning purpose.

Figure 4.3. Amplification of OCII gene from rice cDNA M: 1kb plus ladder thermo scientific, Lane 2: PCR amplification of OCII gene at 65ºC

4.1.4 *pCAMBIA 1301* plasmid extraction

Three colonies of *E. coli* containing *pCAMBIA 1301* plasmid were grown overnight in 10 mL LB medium. The following day, plasmid isolation was performed using Thermo Scientific plasmid miniprep kit (#K0503).

4.2 Construction of *SN19 pCAMBIA 1301* Plasmid

To construct *SN19 pCAMBIA 1301*, the pro35S-*SN19-CAMV* terminator (approximately 2.9 kb) fragment was excised from already available plasmid *pTF101* and was further cloned in *pCAMBIA 1301*. Following steps were performed to accomplish the task.

4.2.1 Restriction digestion of *pTF101* plasmid to excise *35S-SN19-CAMV* terminator fragment

The *pTF101* plasmid was digested with *Kpn*1 and *Hindl*II restriction enzymes to excise *35S-SN19-CAMV* terminator fragment. Result revealed proper excision of 2.9 kb desired fragment (Figure 4.4). The digested fragment was cut with sterilized blade and eluted according to the manufacturer protocol. Insert was ready to be cloned in *pCAMBIA 1301* vector.

To produce overhangs of *Kpn*1 and *Hind*III, *pCAMBIA 1301* vector was also digested as described in Table 3.2. The digested fragment was cut with sterilized blade and were eluted according to the protocol. Vector was ready to be used for ligation purpose.

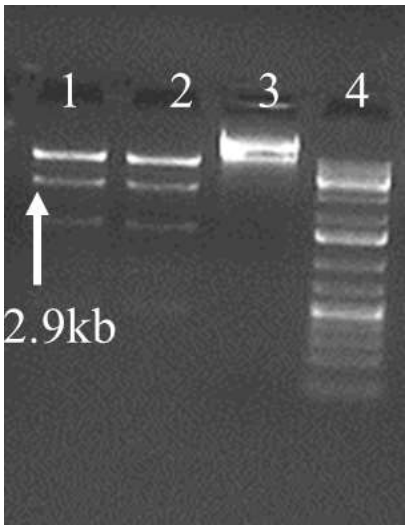


Figure 4.4. Digestion of pTF101 plasmid with Kpn1 and HindIII to excise 35S-SN19-CAMV terminator fragment
Lane 1-2: Digested plasmid, Lane 3: undigested plasmid, Lane 4: 1kb plus ladder (Thermo Scientific)

4.2.2 Transformation of *SN19 pCAMBIA 1301* plasmid in *E. coli* (*DB301*)

After ligation reaction, the ligated product was then transformed to *E. coli* competent cells as per standard protocol of transformation. The positive clones were confirmed through colony PCR using gene specific primers (*SN-19* gene, 480bp). Gel eluted fragments of *pTF101* plasmid was used as positive control (Figure 4.5).

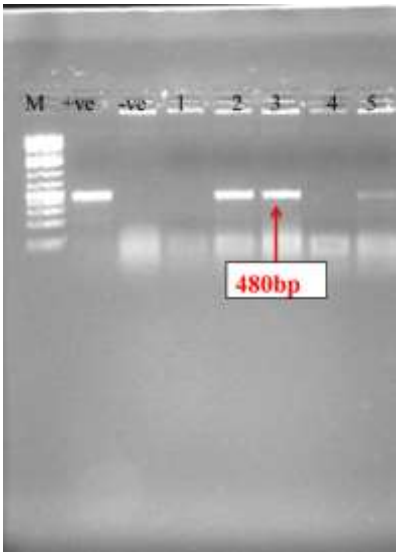


Figure 4.5. Colony PCR assay for confirmation of SN19 pCAMBIA plasmid
M: 1kb plus ladder (Thermo Scientific), Lane +: positive control pTF101 plasmid, Lane -ve: negative control without DNA, Lane 1-5 plasmid DNA results with specific primers, Lane 2,3 and 5 were marked as positive

4.2.3 Restriction digestion confirmation of *SN19 pCAMBIA 1301* plasmid

The positive clones of *SN19* plasmids were further confirmed by restriction digestion analysis with *Kpn*1 and *Hind*III restriction enzymes to excise *35S-SN19-CAMV* terminator fragment. Result revealed proper excision of ~ 2.9 kb desired fragment (Figure 4.6).

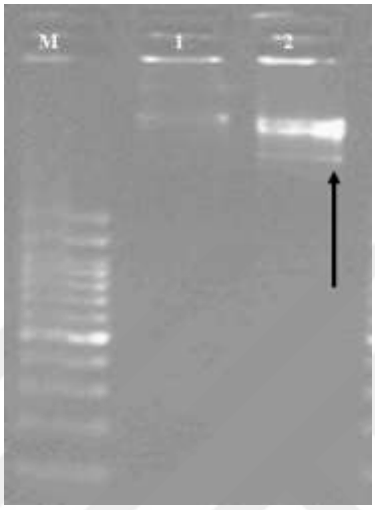


Figure 4.6. Restriction Analyses of SN19 pCAMBIA 1301 plasmid
Lane M: 100 bp DNA ladder (Solis BioDyne), Lane1: Undigested plasmid, Lane 2: Kpn1 and HindIII digested SN19 plasmid

4.2.4 Transformation of SN19 pCAMBIA1301 plasmid in *Agrobacterium tumefaciens* (LBA4404)

For confirmation of *SN19pCAMBIA 1301* plasmid in *Agrobacterium tumefaciens* (*LBA4404*), colony PCR were performed with primers specific to the internal fragment of *SN19* gene. Gel eluted fragment of *pTF101* plasmid was used a positive control (Figure 4.7). The positive clones were grown in LB broth medium overnight with appropriate antibiotic.

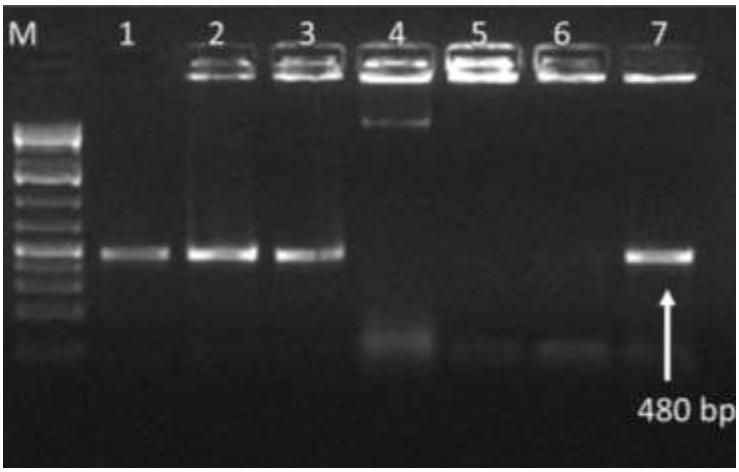


Figure 4.7. Confirmation of SN19 gene internal fragment in SN19 pCAMBIA 1301 in Agrobacterium tumefaciens LBA4404
M: 1kb plus ladder (Thermo Scientific), Lane 1-5: tested clones, Lane 6: negative control without DNA, Lane 7: positive control (pTF101 plasmid)

4.3 Cloning of *Cry3A* Gene in *SN19 pCAMBIA 1301* to Develop DS-1 Plasmid

For cloning of *cry3A* gene in the plant expression vector *SN19pCAMBIA 1301*, the purified PCR product of *cry3A* gene with overhangs of *Nco*I and *Bgl*II and *SN19 pCAMBIA 1301* plasmid were ligated by using T4 DNA Ligase (Promega) according to the manufacturer's protocol.

4.3.1 Transformation in *E. coli* strain *JM-109*

The *E. coli* strain *JM-109* was used for ligation mixture transformations as per standard protocol of transformation. The positive clones of DS-1 plasmid were confirmed by colony PCR by primers specific to the partial length of *cry3A* gene (509 bp). PCR eluted fragment of *cry3A* gene was used as a positive control (Figure 4.8).

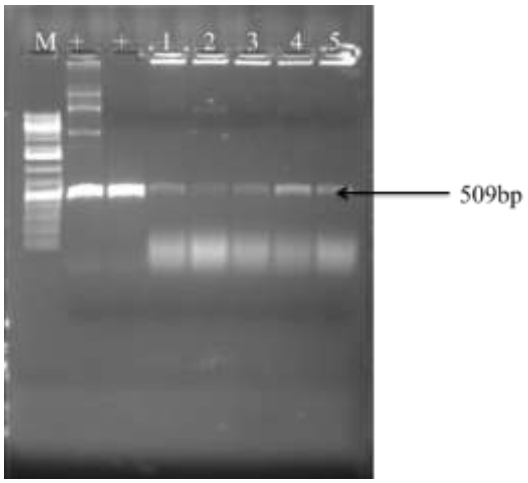


Figure 4.8. Colony PCR to confirm DS-1gene construct using cry3A gene specific primer in SN19 pCAMBIA 1301 plasmid
M: 1kb plus ladder (Thermo Scientific), Lane += pGT-22-b plasmid: Lane 1-5 positive by primers specific to the partial length of cry3A gene

4.3.2 Restriction digestion confirmation of DS-1 plasmid construct

The positive clones of DS-1 plasmids were further confirmed by restriction digestion analysis with two restriction enzymes (*Nco*1 and *Bgl*II) to produce desired fragments of *cry3A* gene in DS-1 plasmid. Result revealed proper excision of ~ 1832 bp desired fragment (Figure 4.9).

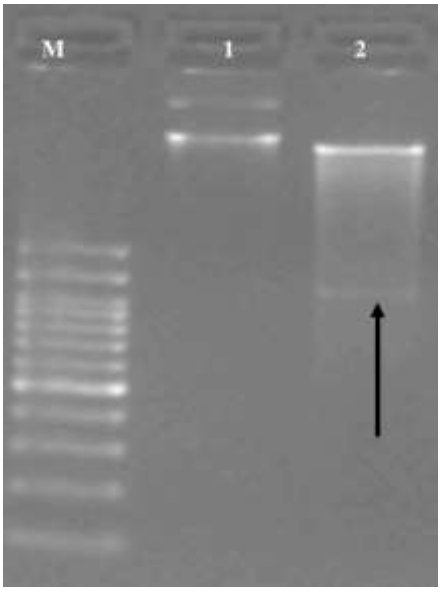


Figure 4.9. Restriction Analyses of DS-1 plasmid
Lane M: 100 bp DNA ladder (Solis BioDyne), Lane1: Undigested DS-1 plasmid, Lane 2: Nco1 and BglII digested DS-1 plasmid

4.3.3 Transformation of DS-1 plasmid in *Agrobacterium* (*GV3101*)

The *Agrobacterium* strain *GV3101* was used for transformation of DS-1 plasmid ligation mixture transformations as per standard protocol of transformation (electroporation method). The positive clones of DS-1 plasmid construct were confirmed by colony PCR using primers specific to the partial length of c*ry3A* gene (509 bp). Gel eluted fragment of *cry3A* gene was used as a positive control (Figure 4.10).

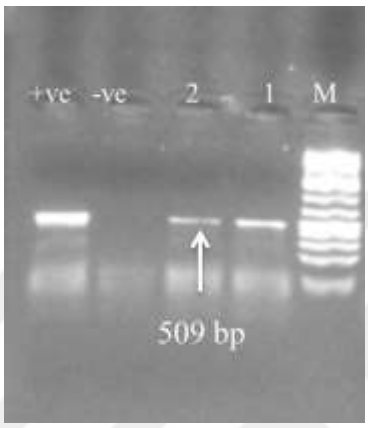


Figure 4.10. Colony PCR to confirm DS-1 plasmid construct using cry3A gene specific primer in SN19 pCAMBIA 1301 plasmid
M: 1kb plus ladder (Thermo Scientific), Lane: pGT-22-b plasmid, Lane 1-2 positive cry3A gene clone results primers specific to the partial length of cry3A gene

4.4 Cloning of *OCII* Gene in *SN19 pCAMBIA 1301* Plasmid to Develop DS-2 Construct

For cloning of *OCII* gene in the *SN19pCAMBIA 1301* plasmid, the purified PCR product of *OCII* gene was digested with two restriction enzymes (*Bgl*II and *Bst*EII) to produce overhangs in desired fragments of *OCII* gene and *SN19 pCAMBIA 1301* plasmid was also digested with the same restriction enzymes. After digestion, both *OCII* gene and vector overhangs were ligated by using T4 DNA Ligase (Promega) according to the manufacturer's protocol.

4.4.1 Transformation in *Agrobacterium* strain *GV3101*

The *Agrobacterium* strain *GV3101* was used for ligation mixture transformations as per standard protocol of transformation (electroporation method). The positive clones of

OCII gene in DS-2 construct were confirmed (649 bp) by colony PCR using gene specific primer. PCR eluted fragment of *OCII* gene was used as a positive control (Figure 4.11).

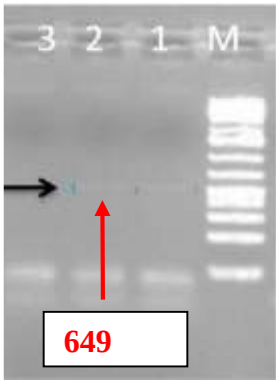


Figure 4.11. PCR result for confirmation of OCII gene in DS-2 plasmid construct clone in in Agrobacterium strain GV3101
M: 1kb plus ladder (Thermo Scientific), Lane 1-3 positive OCII gene clone results with primers specific to the full length of OCII gene

4.4.2 Restriction digestion of DS-2 construct

The positive clones of DS-2 plasmid was further confirmed by restriction digestion analysis with two restriction enzymes (*Bgl*II and *Bst*EII) to produce desired fragments of *OCII* gene Result revealed proper excision of ~ 649 bp desired fragment (Figure 4.12).

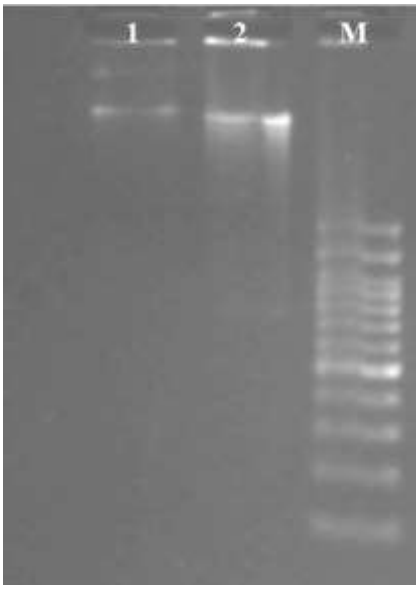


Figure 4.12. Restriction Analyses of DS-2 plasmid
Lane M: 100 bp DNA ladder (Solis BioDyne), Lane1: Undigested DS-2 plasmid, Lane 2: BglII and BstEII digested DS-2 plasmid

4.5 Transformation of Potato Cultivars with DS-1 and DS-2 Constructs

4.5.1 Explant propagation plant material

The shoots culture of both cultivars were established in *in vitro* conditions on basal MS medium containing Murashige and Skoog (1962) mineral salts, sucrose and agar under controlled environmental conditions at 25 ± 2^{o}C with a day length of 16 h light, provided by fluorescent tubes in growth chamber. During transformation, both leaf discs and internode were used.

4.5.2 *Agrobacterium* ınoculum preparation and explants co-cultivation with *Agrobacterium* suspension

Agrobacterium tumefaciens strain (*GV3101*) harboring two different construct combinations (DS-1 and DS-2) were used for genetic transformation of potato cultivars. Prior to co-cultivation with potato tissue, both the *Agrobacterium* strains harboring their respective genes in individual plasmids were cultured overnight in LB medium containing 50mg/mL of kanamycin. Following day, explants (both leaf discs and internodes) were inoculated with the *Agrobacterium* culture suspension grown overnight.

4.5.3 Selection of transformants

The survival percentage of the plantlets of both the cultivars at different growth stages e.g. after cocultivation, shoot and root formation was observed on different MS media.

4.5.3.1 Putative transformed plantlets selection and response on the regeneration selection media

After two days (48 hours) of co-cultivation, explants were transferred to regeneration selection medium i.e. MS medium containing hygromycin (4mg/L) and sulcid (500mg/L) as well as different growth hormones i.e. BAP (2mg/mL), NAA (0.2mg) Kinetin (1mg/mL) and Trans zeatin (2mg/mL). Plants were subculture to fresh selection medium after every 10 days till 6-8 weeks. The transformation efficiency was calculated after six weeks of transformants growing on regeneration selection medium (Figure 4.13). Each transformation experiment was consisting of three replicates, having 20 ex-plants (leaves

and internodes) in each replication. Total 60 explants were used for each experiment. Similar procedure was followed for both cultivars transformed with each construct.

Total 2000 ex-plants of Agria for DS-1 construct, and 1500 ex-plants for DS-2 construct, likewise, 1500 ex-plants of Lady Olympia for each DS-1 and DS-2 constructs were used in transformation experiments. While 1000 ex-plant of each of Agria and Lady Olympia transformed with empty plasmid as positive controls were used.

Callus induction was observed after 3-4 weeks of incubation on regeneration selection media. The mean percent callus induction was calculated as 73.60 % for Agria with DS-1 construct, 69.06 % for Agria with DS-2 construct, 70.46 for Lady Olympia with DS-1 and 69.73% with DS-2 gene construct. Mean percentage number of shoots per callus were also calculated and 2.97% of Agria transformed with DS-1 and 2.57% of Agria transformed with DS-2 gene construct was obtained. Similarly, 2.78 and 2.61 mean percent shoot induction per callus was recorded in Lady Olympia transformed with DS-1 and DS-2 gene constructs respectively (Table 4.1).

The developed shoots obtained from different calli were documented. The ex-plants that were unable to produce callus, changing to dark color was observed after 2 weeks of post inoculation. Those dark colored ex-plants did not develop any calli and were recorded as dead.

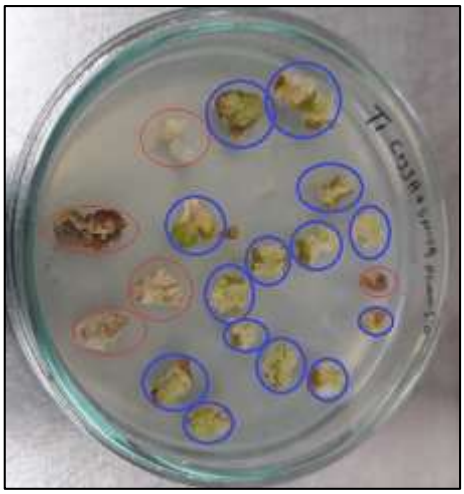


Figure 4.13. Selection of explants on antibiotic medium
Callus formation of explants under *hygromycin* selection (4mg/L). The calli in red circles represent explants that did not survive on the selection medium, the ones labelled in blue circles show successful explants that survived on *hygromycin* selection

4.5.3.2 Transfer and selection on shoot ınduction medium

Six weeks after growing explants on regeneration selection medium, developed calli that were formed, transferred to selective shoot-induction medium. The recipe of the shoot induction medium consisted of MS supplemented with vitamins, sucrose and agarose with 2 mg/L BAP, 0.2 mg/L NAA, 2mg/L of *hygromycin* and 400 mg/L of Sulcid (Figure 4.14, 4.15 and 4.16).

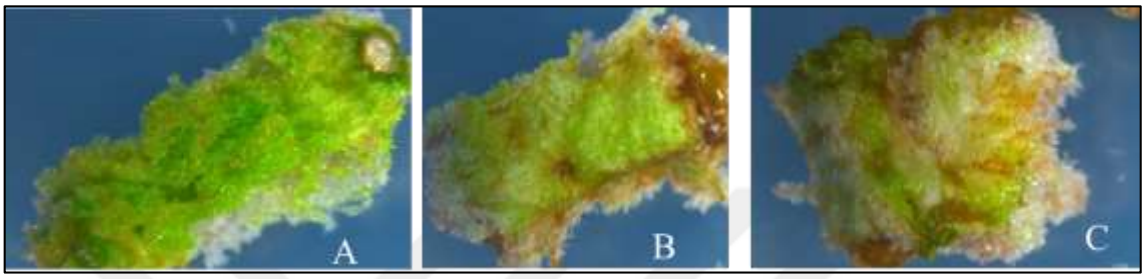


Figure 4.14. Formation of callus on RSM after 3-4 weeks, Agria (a), Lady Olympia (b&c)

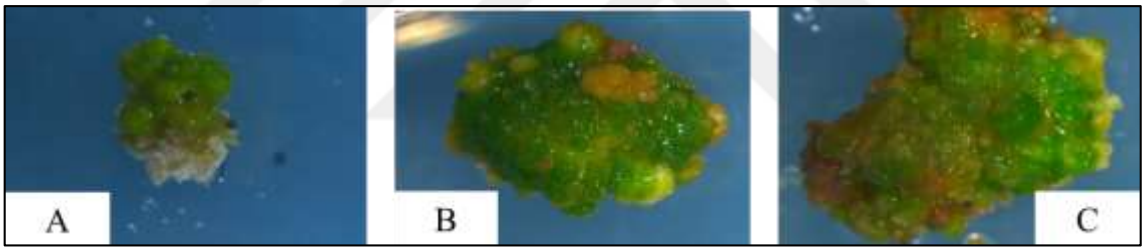


Figure 4.15. Development of Embryoes of Agria (a&b) and Lady Olympia (c)

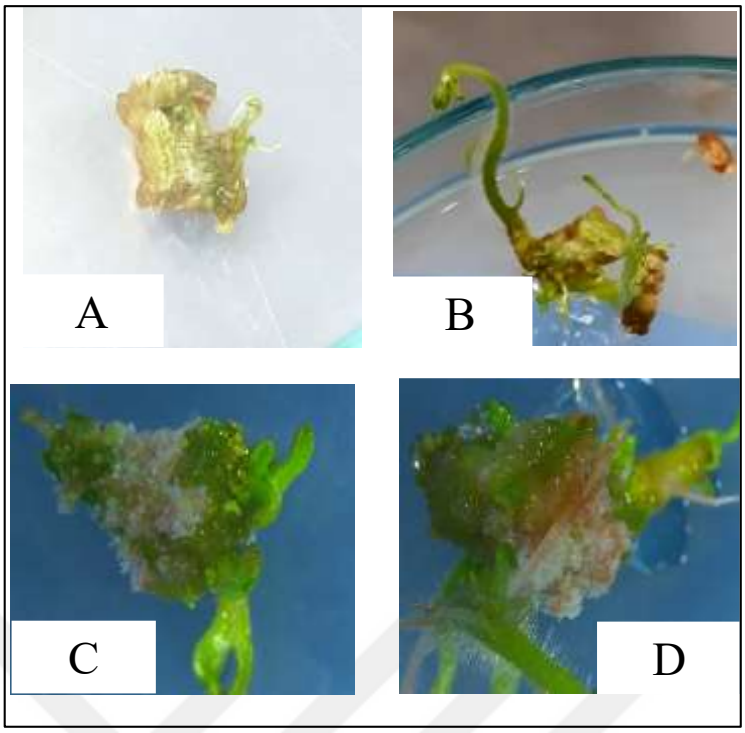


Figure 4.16. Selection of calli and further shoot regeneration on shoot induction medium Agria (a&b) and Lady Olympia (c&d)

4.5.3.3 Transfer and selection on root formation media

After 5-6 weeks, the selected plants with well-developed shoots were subculture to MS normal media i.e. MS supplemented with vitamins, sucrose and agarose, with appropriate antibiotics (Figure 4.17). Plants were continued to subculture on this media for 4-6 weeks.

Figure 4.17. *In vitro* growth of putative transgenic plants of Agria (a) and Lady Olympia (b) on RM (Root Medium)

4.6 Acclimatization

A total of 250 plants were acclimatized in growth chamber. 125 plants of Agria (45, 55 and 25) transformed with DS-1, DS-2 and empty plasmid (control plants) respectively were acclimatized in pots containing soil media consisted of mixture of perlite, peat and vermiculite (1:1:1 v/v/v) in growth chamber at 27 ± 2°C, 16/8 hours light/dark photoperiod. Similarly, 125 plants of Lady Olympia (50, 55 and 20) transformed with of DS-1, DS-2 and empty plasmid (control plants) respectively were acclimatized in the green house in the same way as don with Agria plants (Figure 4.18, 4.19 and 4.20).

Figure 4.18. Acclimatization of putative transgenic plants of Agria and Lady Olympia along with their control in growth chamber

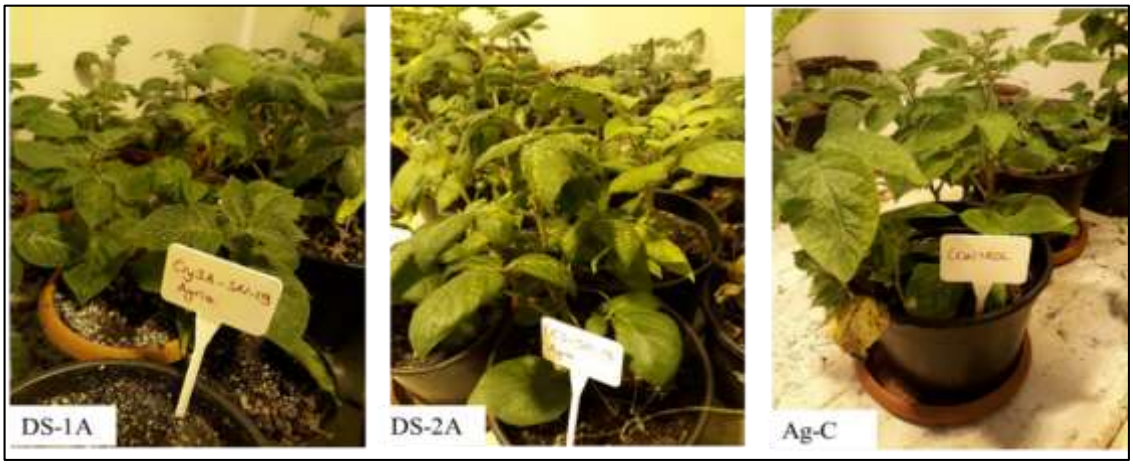


Figure 4.19. A view of the different putative transgenic plants of Agria and their control in growth chamber

Figure 4.20. A view of the different putative transgenic plants of Lady Olympia and their control in growth chamber

4.7 Callus and Shoot Induction Percentage

Callus and shoot induction percentage of potato cultivars Agria and Lady Olympia transformed with DS-1 and DS-2 plasmid constructs are shown in Table 4.1.

Table 4.1. Callus and shoot induction percentage of potato cultivars Agria and Lady Olympia transformed with DS-1 and DS-2 plasmid constructs

Cultivars	Gene Construct					
	DS-1			DS-2		
	No of ex-plants	% Callus formation	% shoot number per callus	No of ex-plants	% Callus formation	% shoot number per callus
Agria	2000	73.6	2.95	1500	69.06	2.57
Lady Olympia	1500	70.46	2.78	1500	69.73	2.61

4.8 Molecular Analysis of Putative Transformants of Both Lady Olympia and Agria

4.8.1 Genomic DNA extraction

The plant genomic DNA were isolated from young leaves of both Lady Olympia and Agria according to method describe by Saha et al. (1997) and Zhang et al. (2000) with some modifications. After DNA extraction, DNA concentrations were checked by running on 1% gel and visualized under UV light (Figure 4.21, 4.22). After measuring the quality of the DNA samples, they were diluted to desirable concentrations. After

making dilutions, plant DNA samples were then used for various molecular analysis in order to confirm proper gene integration and expression in potato genome.

4.8.2 PCR assays

PCR analysis of the primary transformants were carried out with different specific primers to confirm presence of plant selectable marker gene "*hygromycin*" in host genome with hptf-hpt reverse primer (Figure 4.23) gene specific primers {(Partials *cry 3A*, Figure 4.24) *SN19* gene, Figure 4.25) and *OCII* gene, Figure 4.26)}. PCR positive plants samples with gene specific primers were then checked by another PCR using *Chv A* gene specific primers for *Agrobacterium* contamination (Figure 4.27). No plant samples amplified *Chv A* gene (890-bp) fragments in our studies.

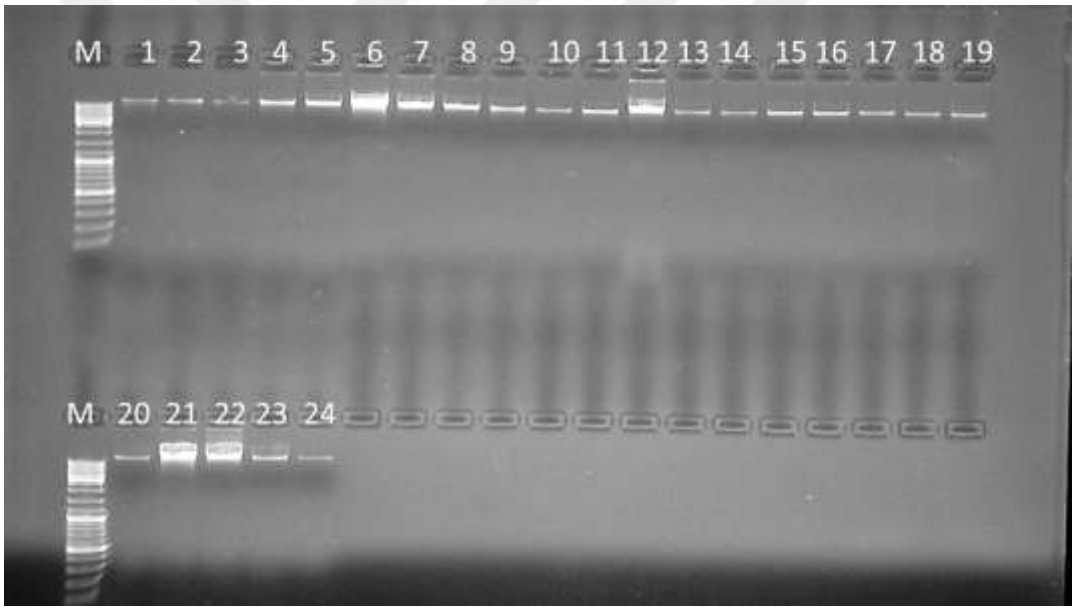


Figure 4.21. Extraction of genomic DNA from primary transformants of Agria with different constructs
Lane1 M: 1 kb plus Gene Ruler DNA Ladder Mix (Thermo Scientific), Lane 1-24: DNA samples of primary transformants of Agria

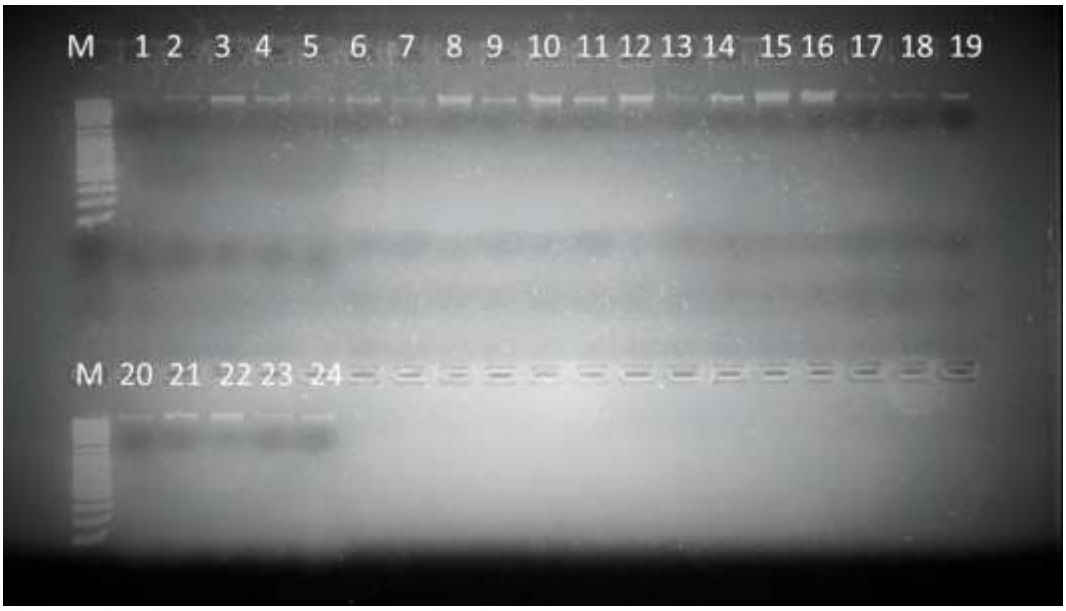


Figure 4.22. Extraction of genomic DNA from primary transformants of Lady Olympia with different constructs
Lane1 M: 1 kb plus Gene Ruler DNA Ladder Mix (Thermo Scientific), Lane 1-24: DNA samples of primary transformants of Lady Olympia

4.8.2.1 Confirmation of *hygromycin* gene in putative transgenic plants of Agria and Lady Olympia

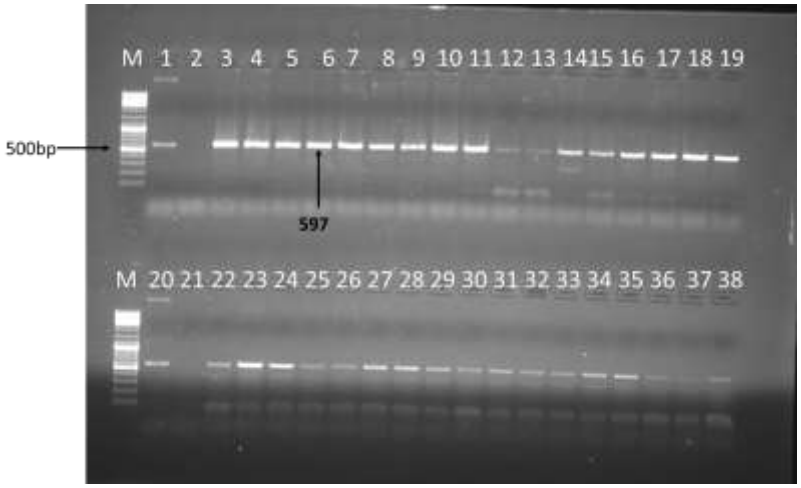


Figure 4.23. PCR assay showed amplification of hptII gene in primary transformants
Lane M: 1 kb plus Gene Ruler DNA Ladder Mix (Thermo Scientific), Lane 1: pCAMBİA 1301 plasmid as positive control, Lane 2: Negative control, Lane 3-19 primary transformants of Agria. Lane 20: pCAMBİA 1301 plasmid as positive control, Lane 21: Negative control, Lane 22-38 primary transformants of Lady Olympia

4.8.2.2 Confirmation of *cry3A* gene in putative transgenic plants of Agria and Lady Olympia

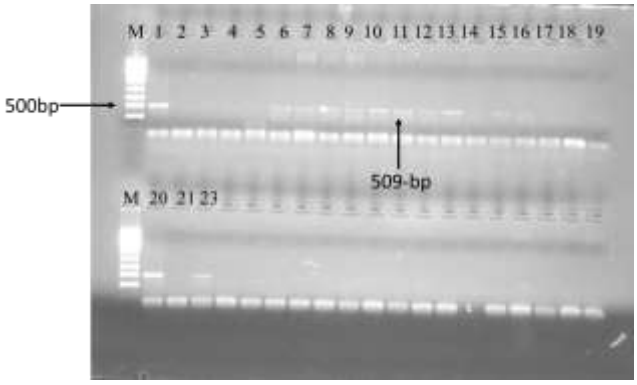


Figure 4.24. Amplification of cry3A gene fragment (509-bp) in putative transgenic plants using gene specific (cry3A partials) primers
Lane M: 1kb plus ladder (Solis BioDyne), Lane 1: DS-1 plasmid as positive control, Lane 2: Negative control, Lane 3-12 primary transformants of Agria with DS-1 construct. Lane 20: DS-1 plasmid as positive. Lane21: Negative control, Lane 13-19 & 21-32 primary transformants of Lady Olympia with DS-1 construct

4.8.2.3 Confirmation of *SN-19* gene fragment in putative transgenic plants of Agria and Lady Olympia

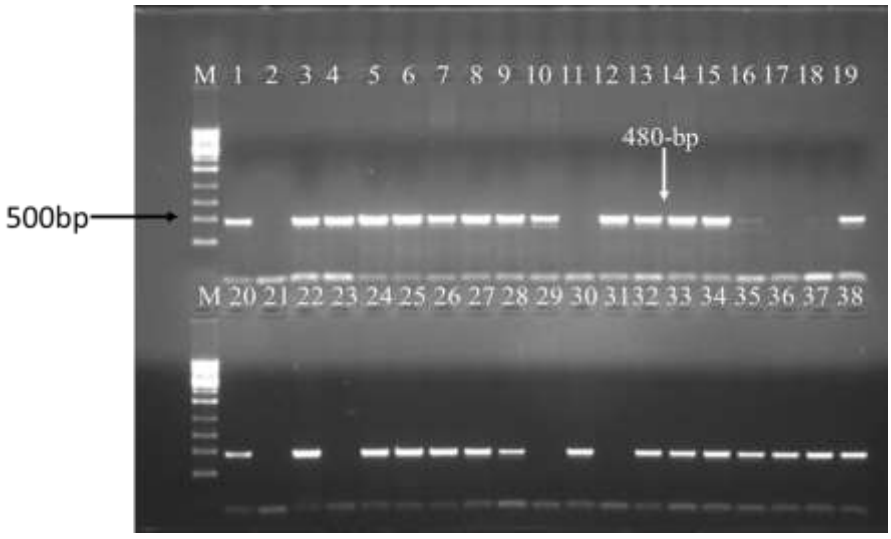


Figure 4.25. Amplification of SN19 gene internal fragment (480-bp) in putative transgenic plants
Lane M: 1kb plus ladder Solis BioDyne, Lane 1: SN19 pCAMBİA 1301 plasmid as positive control, Lane 2: Negative control, Lane 3-10 primary transformants of Agria with DS-1 plasmid construct. Lane 11-19 primary transformants of Agria with DS-2 construct, Lane 20: SN19 pCAMBİA 1301 plasmid as positive control. Lane21: Negative control, Lane 21-30 primary transformants of Lady Olympia with DS-1 construct. Lane 31-38 primary transformants of Lady Olympia with DS-2 construct

4.8.2.4 Confirmation of *OCII* gene in putative transgenic plants of Agria and Lady Olympia

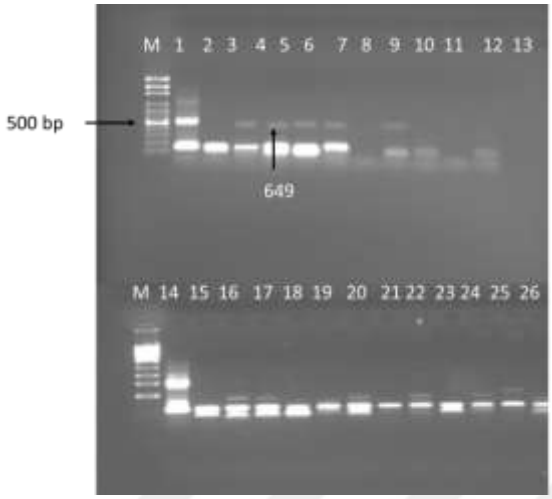


Figure 4.26. Amplification of OCII gene (649-bp) in putative transgenic plants
Lane M: 1kb plus Gene Ruler DNA Ladder Mix (Thermo Scientific), Lane 1: DS-2 plasmid as positive control, Lane 2: Negative control, Lane 3-9 primary transformants of Agria with DS-2 construct. Lane 9-26 primary transformants of Lady Olympia with DS-2 construct, Lane 14: DS-2 construct as positive control. Lane15: Negative control

4.8.2.5 Confirmation of *Chv A* gene in putative transgenic plants of Agria and Lady Olympia

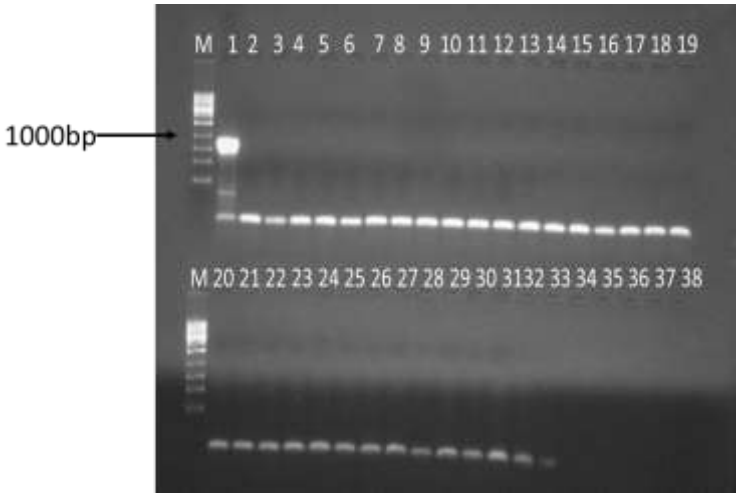


Figure 4.27. Amplification of Chv A gene fragment (890-bp) in putative transgenic plants
Lane M: 1kb plus ladder Solis BioDyne, Lane 1: chromosomal bacterial DNA plasmid as internal positive, Lane 2: Negative control, Lane 3-10 primary transformants of Agria with DS-1 construct. Lane 11-19 primary transformants of Agria with DS-2 construct, Lane 20-30 primary transformants of Lady Olympia with DS-1 construct. Lane 31-38 primary transformants of Lady Olympia with DS-2 construct

4.8.3 Summary of PCR analysis

Detail results of various analyses of different plants transformed with DS-1 and DS-2 constructs in both potato cultivars Agria and Lady Olympia and their control based on PCR analysis with different gene specific primers are listed in Table 4.2.

Table 4.2. Detail results of various analyses of different plants transformed with DS-1 and DS-2 constructs in both potato cultivars Agria and Lady Olympia and their control based on PCR analysis with different gene specific primers

S. No	Plant Name	Constructs	PCR detection of various genes with specific primers				
			hptII	***SN19***	***Cry 3A***	***OCII***	***Chv A***
1	AP1	DS-1	+	+	+	-	-
2	AP2	DS-1	+	+	+	-	-
3	AP3	DS-1	+	+	+	-	-
4	AP4	DS-1	+	+	+	-	-
5	AP5	DS-1	+	+	+	-	-
6	AP6	DS-1	+	+	+	-	-
7	AP7	DS-1	+	+	+	-	-
8	AP8	DS-1	+	+	+	-	-
9	AP1	DS-2	+	+	-	+	-
10	AP2	DS-2	+	+	-	+	-
11	AP3	DS-2	+	+	-	+	-
12	AP4	DS-2	+	+	-	+	-
13	AP5	DS-2	+	+	-	+	-
14	AP6	DS-2	+	+	-	+	-
15	AGC	MA1	+	-	-	-	-
16	AGC	MA2	+	-	-	-	-
17	AGC	MA3	+	-	-	-	-
18	AGC	MA4	+	-	-	-	-
19	AGC	MA5	-	-	-	-	-
20	AGC	MA6	-	-	-	-	-
21	LOP1	DS-1	+	+	+	-	-
22	LOP2	DS-1	+	+	+	-	-
23	LOP3	DS-1	+	+	+	-	-
24	LOP4	DS-1	+	+	+	-	-
25	LOP5	DS-1	+	+	+	-	-
26	LOP6	DS-1	+	+	+	-	-
27	LOP1	DS-2	+	+	-	+	-
28	LOP2	DS-2	+	+	-	+	-
29	LOP3	DS-2	+	+	-	+	-
30	LOP4	DS-2	+	+	-	+	-
31	LOP5	DS-2	+	+	-	+	-
32	LOP6	DS-2	+	+	-	+	-
33	LOP7	DS-2	+	+	-	+	-
34	LOC	ML1	+	-	-	-	-
35	LOC	ML2	+	-	-	-	-
36	LOC	ML3	+	-	-	-	-
37	LOC	ML4	+	-	-	-	-
38	LOC	ML5	-	-	-	-	-
39	LOC	ML6	+				-
40	LOC	ML7	-	-	-	-	-

*S. NO 1-8, AP1-AP8, Agria plants transformed with DS-1 construct, S. NO, 9-14, Agria plants transformed with DS-2 construct, S. NO, 15-20, Mock plants of Agria (Control Plants). S. NO, 21-26, LOP1-LOP6, Lady Olympia plants transformed with DS-1 construct, S. NO, 27-33, Lady Olympia plants transformed with DS-2 construct, S. NO, 34-40, Mock plants of Lady Olympia (Control Plants)

4.8.4 Transformation efficiency

Transformation efficiency calculations of transgenic potato cultivar Agria plants from green house was calculated as 45% and 61.11% for DS-1 and DS-2 gene construct respectively. Similarly, transformation efficiency calculations of transgenic potato cultivar Lady Olympia plants were calculated as 47.61% and 52.38% respectively as mention in (Table 4.3).

Table 4.3. Transformation efficiency calculations of potato cultivars Agria and Lady Olympia transformed with DS-1 and DS-2 constructs based on PCR analysis

Name of cultivar	Total no of transgenic plants	Plasmid constructs			
		DS-1		DS-2	
		T. P	% T. E	T. P	% T. E
Agria	100	45	45 (2.25)	55	55 (3.67)
Lady Olympia	105	50	47.61 (3.17)	55	52.38 (3.49)

4.9 Enzyme-linked Immunosorbent Assay (ELISA) of the Putative Transgenic Plants (Cry3A Protein Expression Analysis)

Different levels of Cry3A protein expression was observed in different plants of Agria and Lady Olympia transformed with DS-1 construct (Figure 4.28). The different levels of Cry3A protein in different plants were divided into three groups (high expression of Cry3A protein, medium expression of Cry3A protein and low expression of Cry3A protein) (Table 4.4).

Table 4.4. Evaluation of different transgenic plants of potato cultivars Agria and Lady Olympia transformed with DS-1 construct for Cry3A protein expression using ELISA reader

Plants No	Quality (color)	Intensity (OD value, wavelength 450)	Remarks[a]
DS-1AP1	Yes	0.934	+++
DS-1AP2	Yes	0.799	++
DS-1AP3	Yes	0.692	++
DS-1AP4	Yes	0.51	+
DS-1AP5	Yes	0.526	+
DS-1AP6	Yes	1.225	+++
DS-1AP7	Yes	1.002	+++
DS-1AP8	Yes	1.29	+++
DS-1LOP1	Yes	1.109	+++
DS-1 LOP2	Yes	0.738	++
DS-1 LOP3	Yes	0.49	+
DS-1 LOP4	Yes	0.493	+
DS-1 LOP5	Yes	0.338	+
DS-1 LOP6	Yes	1.175	+++
Control (+ve, provided in kit)	Yes	0.443	+
Control (-ve)	No	0	-

a* +++ (high expression of cry3A protein), ++ (medium expression of cry3A protein), + (low expression of cry3A protein), - (No cry3A protein)

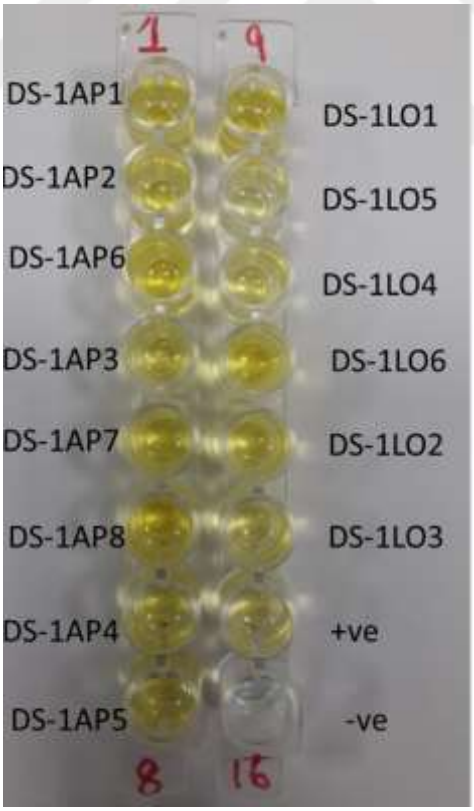


Figure 4.28. Analysis of different transgenic plants of Agria and Lady Olympia transformed with DS-1 construct for Cry3A protein expression
Lane 1-8, different plants of Agria transformed with DS-1 plasmid construct. Lane 9-14, different plants of Lady Olympia transformed with DS-1 construct. Lane 15, positive control provided in the kit. Lane 16, non-transgenic Agria plant

4.10 Southern Blot Analysis

Six transgenic plants of Agria and Lady Olympia transformed with DS-1 gene construct were used for validation of results and to determine gene integration in potato genome using Southern blot analyses. After completing Southern blot, signal detection on membrane confirmed integration of *SN19* gene in potato cultivars Agria and Lady Olympia genomes. Non-transgenic plants of both Agria and Lady Olympia were used as a negative control plants, while DS-1 plasmid was used as positive control (Figure 4.29).

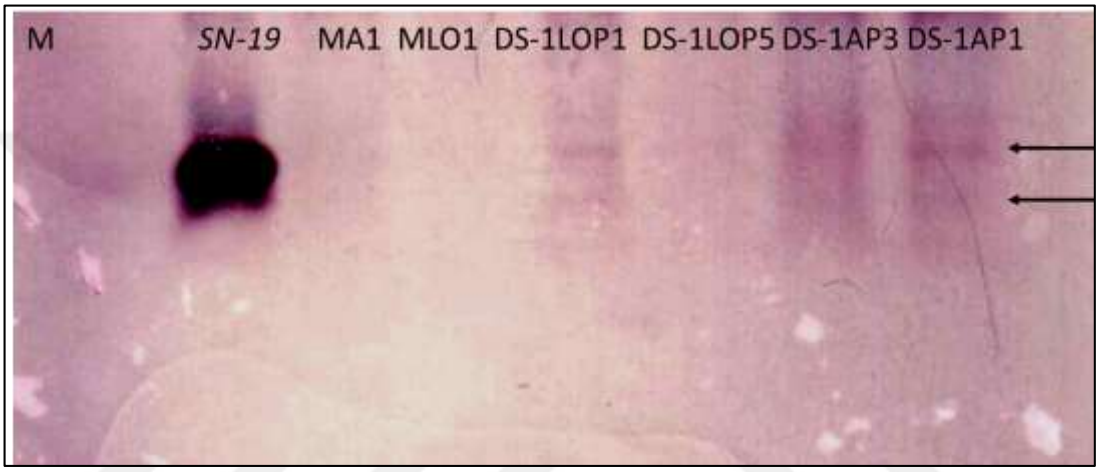


Figure 4.29. Southern blot analyses result of primary transformants of Agria and Lady Olympia
Lane M: Lambda HindIII marker, Lane SN-19: SN19 gene, Lane MA1; Agria control, Lane MLO1: Lady Olympia control, Lane DS-1LOP1 and DS-1LOP5; Lady Olympia transgenic plants and Lane DS-1AP1 and DS-1AP3: Agria putative transgenic plants

4.11 Life Table Studies

4.11.1 Individually reared method

The mean developmental durations of different stages of *L. decemlineata* reared individually on transgenic potato cultivars Agria and Lady Olympia, preadult survival rate, adult longevity, adult preoviposition period (APOP), total preoviposition period (TPOP), female total longevity, male total longevity, oviposition days and fecundity (eggs/female) and their control plants are presented in Table 4.5. The data showed no lifetable data for the transgenic plants as all the insect died before starting any data for different stages, however CPB populations that were reared on control group cultivar Agria had shorter egg developmental duration as compared to CPB populations that were

reared on non-transgenic control group Lady Olympia (*P*= 0.00017), while no significant difference was observed in L1 larval developmental duration between both cultivars (*P*= 0.20667). Further, significantly shorter developmental durations were recorded for L2 (*P*= 0.00) and L3 l stages of CPB that were fed on Lady Olympia as compared to Agria (*P*=0.00). There were no significance differences in the larval developmental duration of L4 stage fed on Lady Olympia and Agria (*P*= 0.1696). A longer mean pupal duration (10.17 days) was recorded in CPB population reared on Agria as compared with Lady Olympia (9.89 days).

CPB population reared on Lady Olympia had significantly higher preadult survival rate (P= 0.00198), higher adult longevity (*P*= 0.00253), higher female total longevity (*P*= 0.00511), oviposition duration (*P*= 0.03914) and higher fecundity (*P*= 0.03404) as compared with CPB reared on Agria. There was no significant difference in the total longevity of male between both CPB populations that were reared on either Lady Olympia or Agria cultivars (*P*= 0.54878).

Table 4.5. Effects of different transgenic potato cultivars and their control plants on different developmental stages of *Leptinotarsa decemlineata* reared individually Values are given as means ± standard error

Stage/parameters	n**	Agria	n	Lady Olympia	*P*-value	Transgenic Agria	Transgenic Lady Olympia
Egg (days)	79	4.07±0.04*	75	4.34±0.06	0.00017	-----	-----
Larval stage 1(days)	79	1.83±0.04	75	1.90±0.04	0.20667	-----	-----
Larval stage 2(days)	77	2.37±0.11*	75	1.48 ±0.06	0.0000	-----	-----
Larval stage 3(days)	71	3 ±0.13*	75	1.68 ±0.06	0.0000	-----	-----
Larval stage 4(days)	69	3.78±0.08	75	3.6 ±0.10	0.1696	-----	-----
Pupal stage (days)	57	10.17±0.11*	68	9.89±0.08	0.04404	-----	-----
Preadult survival rate (%)	79	0.72±0.05*	75	0.90±0.03	0.00198	-----	-----
Adult longevity (days)	57	53.31±2.25*	68	65.91±3.50	0.00253	-----	-----
APOP (days)	29	6.55±0.32*	32	4.78±0.23	0.00004	-----	-----
TPOP (days)	29	31.58±0.39*	32	27.68±0.23	0.0000	-----	-----
Female total longevity (days)	29	75.55±3.09*	32	93.40±5.54	0.00511	-----	-----
Male total longevity (days)	28	81.25 ±3.23	36	84.61±4.53	0.54878	-----	-----
Oviposition days	29	10.72±1.45*	32	15.90±2.04	0.03914	-----	-----
Fecundity (eggs)	29	329.10±63.97*	32	548±80.65	0.03404	-----	-----
Male adult longevity (days)	28	56.21± 3.20	36	61.83±4.45	0.30425	-----	-----
Female adult longevity(days)	29	50.51±3.12*	32	70.5±5.47	0.00159	-----	-----

**n= no of replicates
* indicates significance difference between treatments Agria and Lady Olympia using bootstrap technique The treatments means were compared by using bootstrap technique. Means± standard error in rows followed by different letters are significantly different at $P < 0.05$ by using paired bootstrap test.

The curves for the age-stage specific survival rate of *L. decemlineata* reared individually on potato cultivars Agria and Lady Olympia are shown in Figure 4.30. Curves of s_{xj} showed significant overlapping due to the different developmental rates that occurred among individuals. The total developmental time was longer and survival rate was higher in Lady Olympia than they were in Agria. The first male and female emerged on the 22nd day on Lady Olympia while on the 23rd day on Agria.

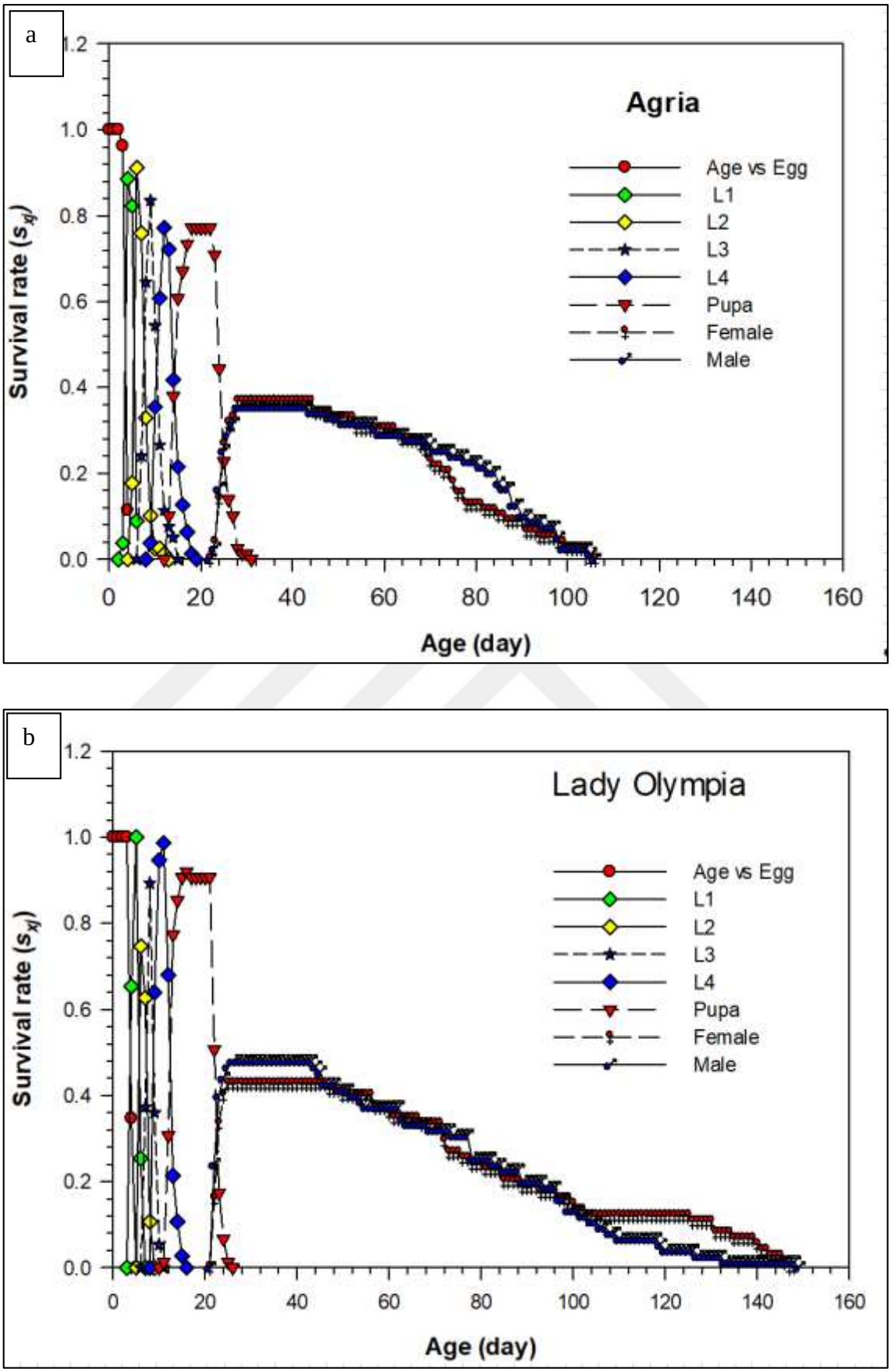


Figure 4.30. Survival rate of different development stages of *Leptinotarsa decemlineata* reared individually on potato cultivars Agria (a) and Lady Olympia (b) respectively

Figure 4.31 curves show the age-specific survival rate (l_x), the female age-stage specific fecundity (f_x), the age-specific fecundity (m_x), and the age-specific maternity ($l_x m_x$) versus age of *Leptinotarsa decemlineata* reared individually on potato cultivars Agria and Lady Olympia respectively. The survival rate (l_x) curve for CPB population reared individually on potato cultivar Agria dropped more rapidly at the early stages of development than it did on potato cultivar Lady Olympia indicating that the mortality rate at this stage was high in CPB populations that were reared on potato cultivar Agria (Figure 4.31). The curve of the age-stage specific fecundity (f_x) showed peak value of 38.96 offspring on 29th day on potato cultivar Lady Olympia (B) which was higher than the peak value of the age-stage specific fecundity recorded for Agria (28 offspring) on 95th day (A). The curve also showed early start of egg laying by female reared on Lady Olympia (25th day) as compared to Agria where the female started egg laying on 28th day.

The curve of age specific fecundity (m_x) showed egg laying of CPB adults that were fed on Lady Olympia started at 25 days of age while on that of Agria began at 28 days of age. The fecundity of CPB females reared on Agria was significantly less than females that were reared on Lady Olympia cultivar (P= 0.03404; Table 4.5). The m_x curve further showed peak value of 15 offspring on day 99 on Agria cultivar (A) and peak curve value on day 29 with 18.33 offspring on Lady Olympia (B). The figure also showed a higher peak value of 16.62 offspring of the age-specific maternity ($l_x m_x$) for Lady Olympia (B) than Agria on which the peak value of curve for the age-specific maternity was 7.78 offspring (A).

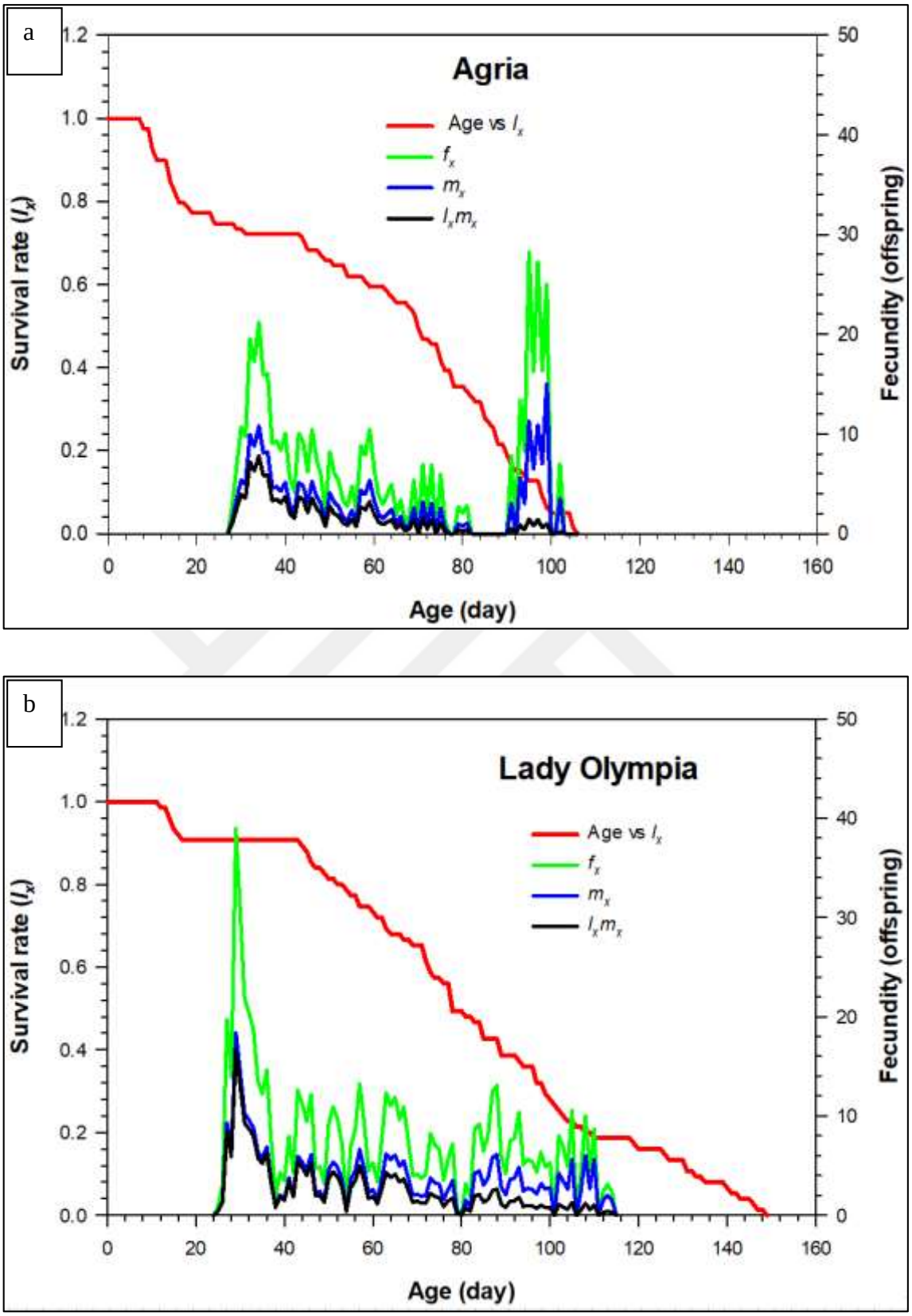


Figure 4.31. The age-specific survival rate (l_x), female age-specific fecundity (f_x), age-specific fecundity (m_x), and the net maternity (l_xm_x) versus age of *Leptinotarsa decemlineata* reared individually on potato cultivars Agria (a) and Lady Olympia (b) respectively

Effects of different potato cultivars on life table parameters viz the net reproductive rate (R_0), the intrinsic rate of increase (r), the finite rate of increase (λ), gross reproductive rate (GRR) and the mean generation time (T) of *L. decemlineata* reared individually on potato cultivars Agria and Lady Olympia is shown in Table 4.6. The data showed no life table data for CPB reared on different transgenic plants of both Agria and Lay Olympia, however CPB that were reared on control plants Agria had significantly lower values of r, λ, and R_0 (0.12, 1.13 and 120.81) respectively as compared with CPB population that was reared on control plants of potato cultivar Lady Olympia. The value of mean generation time (T) 39.75 for CPB population fed on potato cultivar Agria was not significantly different from that of CPB population that were reared on potato cultivar Lady Olympia (37.43) i.e. P= 0.17064. The difference between values for gross reproductive rate (GRR) in both treatments was also found non-significant (P= 0.29438).

Table 4.6. Effect of different potato cultivars on life table parameters of *Leptinotarsa decemlineata* reared individually on potato cultivars Agria and Lady Olympia
Values are given as mean± standard error

Parameters	n**	Agria	n	Lady Olympia	*P*- value
The intrinsic rate of increase (*r)* per day	79	0.12 ±0.01*	75	0.15 ±0.01	0.00396
The finite rate of increase (λ) per day	79	1.13±0.01*	75	1.16±0.01	0.00382
The net reproductive rate (R_0)	79	120.81±29.29*	75	233.81±46.20	0.03921
The mean generation time (T) in days	79	39.75±1.29	75	37.43±1.03	0.17064
Gross reproductive rate (GRR)	79	237.15±73.93	75	349.12±77.77	0.29438

n**= no of replicates
* indicates significance difference between treatments Agria and Lady Olympia using bootstrap technique. The treatments means were compared by using bootstrap technique. Standard error of the means was calculated with 100000 resampling using bootstrap technique. Means+ standard error in rows followed by different letters are significantly different at P <0.05 by using paired bootstrap test.

The age-stage life expectancy (e_{xj}) of *L. decemlineata* reared individually on potato cultivars Agria and Lady Olympia respectively is shown in Figure 4.32 A and B. The longevity of *L. decemlineata* population from age zero (e_{01}) was 60.84 days on Agria which was shorter than the longevity of *L. decemlineata* reared individually on Lady Olympia (81.84 days) (Figure 4.32 a and b).

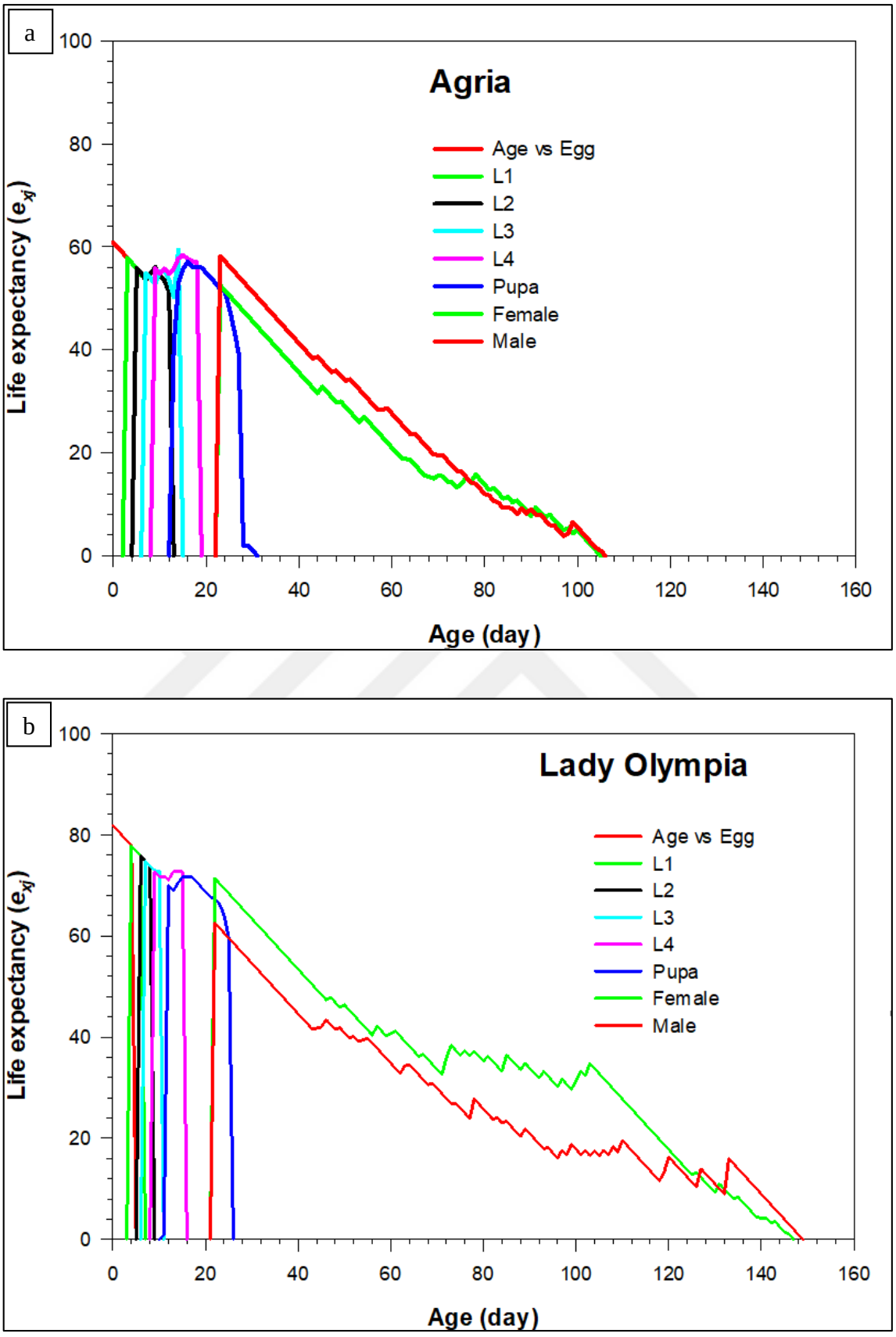
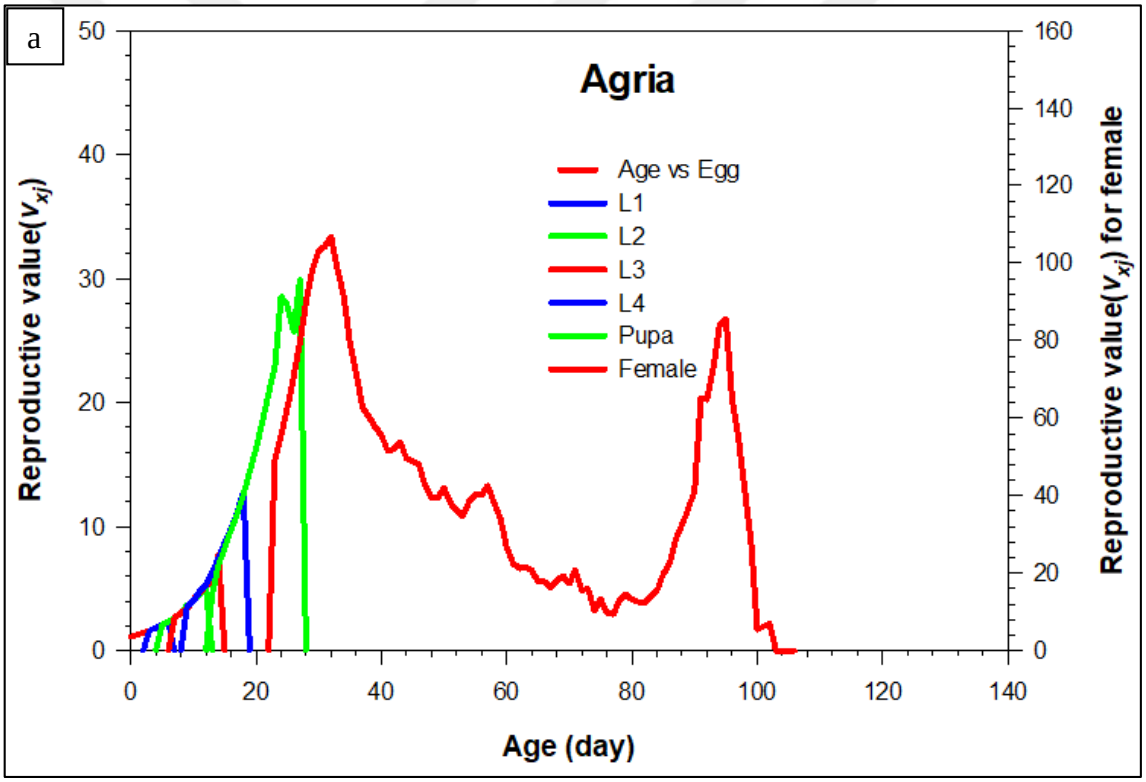


Figure 4.32. Age-stage life expectancy (e_{xj}) of *Leptinotarsa decemlineata* reared individually on potato cultivars Agria (a) and Lady Olympia (b) respectively

The age-stage reproductive value (v_{xj}) of *L. decemlineata* reared individually on potato cultivars Agria and Lady Olympia respectively is shown in Figure 4.33 A and B. The reproductive values (v_{01}) is exactly the same as the finite rate of increase (λ). Figure 4.34 A and B showed that the curves for reproductive value significantly increased when reproduction started. The reproductive value on potato cultivar Agria at age zero was 1.13 offspring per day, which was significantly smaller than the reproductive value of CPB population reared on potato cultivar Lady Olympia on which the reproductive value at age zero was 1.16 offspring per day. The reproductive value (v_{xj}) on potato cultivar Agria reached the peak value of 106.75 offspring per day on day 32 when female emerged and when reared on potato cultivar Lady Olympia reached the peak value of 136.57 offspring per day on day 29 when female emerged (Figure 4.33 a and b).



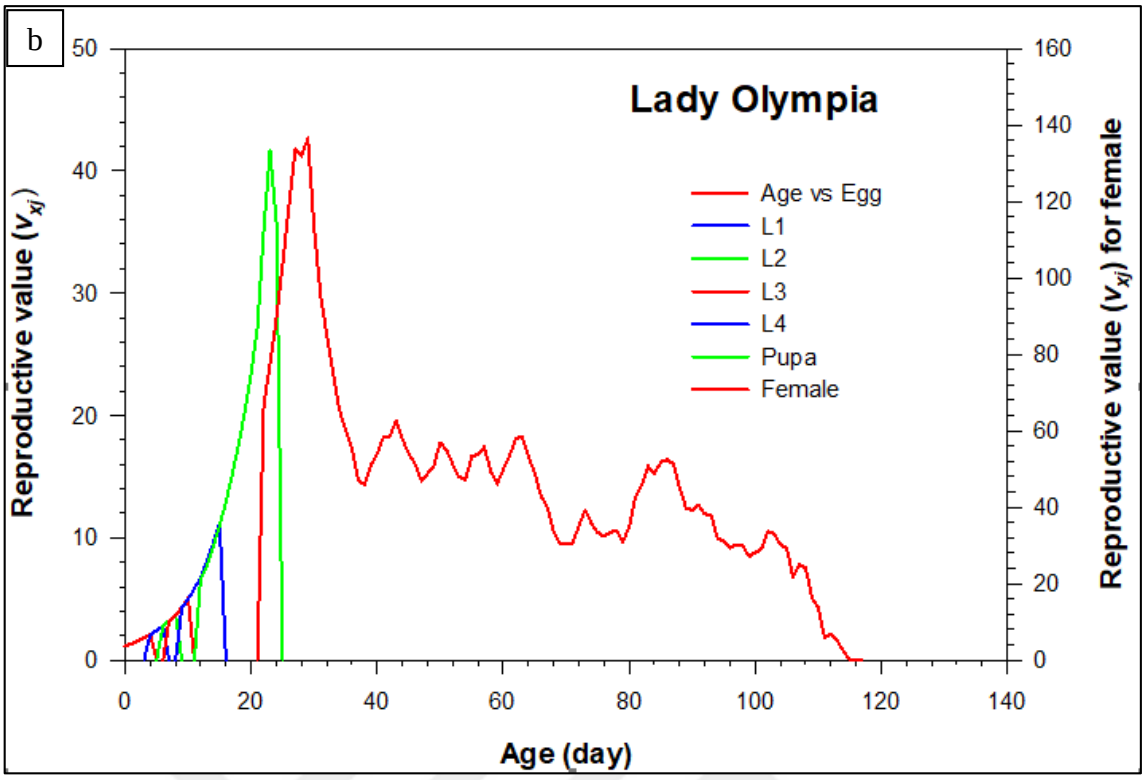


Figure 4.33. Age-stage reproductive value (v_{xj}) of *Leptinotarsa decemlineata* reared individually on potato cultivars Agria (a) and Lady Olympia (b) respectively

The population projections of CPB on Agria and Lady Olympia with an initial population of 10 eggs based on the calculated parameters e.g. mean generation time, fecundity etc. are shown in Figure 4.34 A and B. According to the simulation result, CPB populations on potato cultivar Agria reached to 1014 adults after 60 days. Similarly, the population size on potato cultivar Lady Olympia reached to approximately 18552 adults i.e. approximately 17538 more CPB adults as compared to Agria.

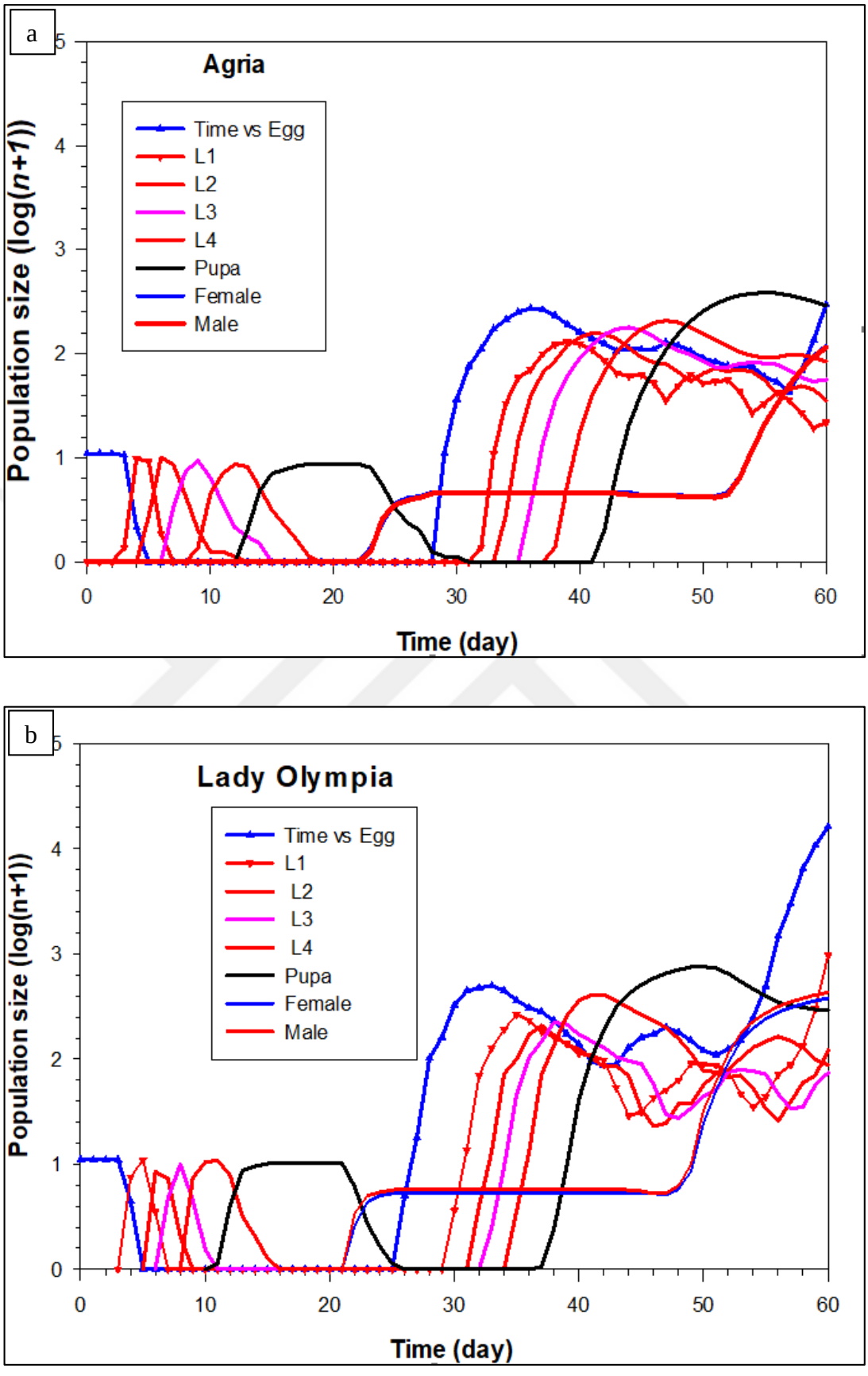


Figure 4.34. The population growth projection of *Leptinotarsa decemlineata* based on parameters calculated from individually reared on potato cultivars Agria (a) and Lady Olympia (b) respectively

4.11.2 Group reared method

Effects of different transgenic potato cultivars and their respective control plants on life table parameters of CPB e.g. the intrinsic rate of increase (*r*), the finite rate of increase (λ), the net reproductive rate (R_0), the mean generation time (*T*) and gross reproductive rate (*GRR*) of *Leptinotarsa decemlineata* reared in groups are shown in Table 4.7. The data showed no significance differences in the intrinsic rate of increase (*r*) and the finite rate of increase (λ) values for CPB population that were fed on potato cultivar Agria and Lady Olympia (*P*-value (*r*)= 0.27385; *P*-value (λ) = 0.27385). The CPB population that was fed on Agria had significantly lower values of R_0, *T* and *GRR* (84.20, 39.15 and 212.08) respectively as compared to CPB population that was fed on potato cultivar Lady Olympia ($P \leq 0.05$).

Table 4.7. Effects of different transgenic potato cultivars Agria and Lady Olympia and their respective control plants on life table parameters of *Leptinotarsa decemlineata* reared in groups
Values are given as mean± standard error

Parameters	n**	Agria	Lady Olympia	*P*- value	Transgenic Agria	Transgenic Lady Olympia
The intrinsic rate of increase (*r)* per day	70	0.11±0.01	0.12±0.00	0.27385	----	----
The finite rate of increase (λ) per day	70	1.12±0.01	1.13±0.01	0.27385	----	----
The net reproductive rate (R_0)	70	84.20±18.48*	204.31±34.05	0.00205	----	----
The mean generation time (*T*) in day	70	39.15±0.55*	43.86±0.55	0.000	----	----
Gross reproductive rate (*GRR*)	70	212.08±39.90*	373.62±57.76	0.02027	----	----

n** = no of replicates
* indicates significance between treatments Agria and Lady Olympia using bootstrap technique.
Means+ standard error in rows followed by different letters are significantly different at $P < 0.05$ by using paired bootstrap test.

The curves for the age-stage specific survival rate (s_{xj}) of *L. decemlineata* reared in groups on potato cultivars Agria and Lady Olympia are shown in Figure 4.35 A and B. Curves of s_{xj} showed significant overlapping due to the different developmental rate that occurred

among individuals (Figure 4.35 A and B). The total developmental time was longer and survival rate was higher on Lady Olympia than on Agria. The first males and females emerged on 22nd day on Agria while on 23rd day on Lady Olympia.

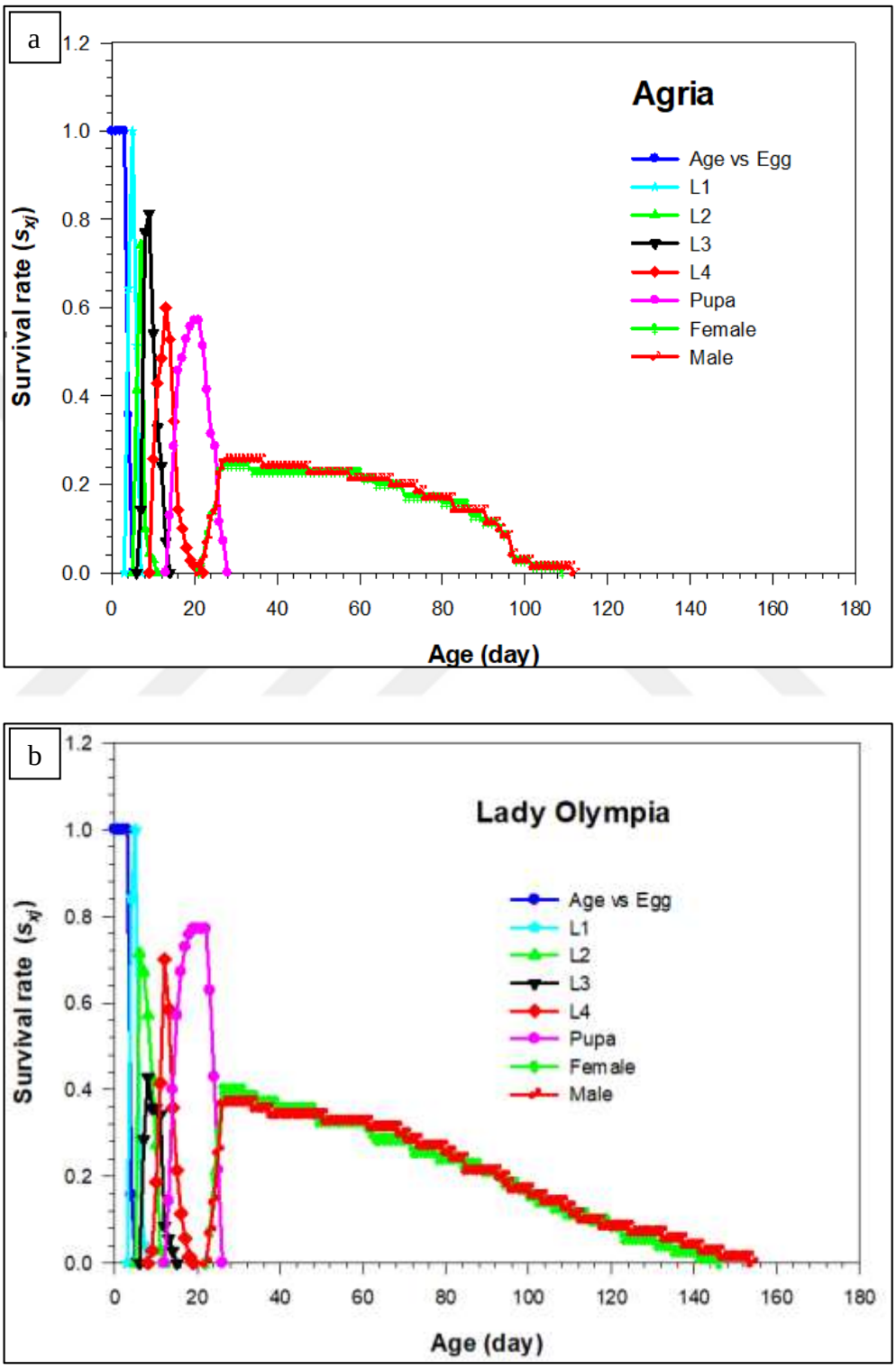


Figure 4.35. Survival rate of different development stages of *Leptinotarsa decemlineata* reared in group on potato cultivars Agria (a) and Lady Olympia (b) respectively

Figure 4.36 A and B curves showed the age-specific survival rate (l_x), the female age-stage specific fecundity (f_x), the age-specific fecundity (m_x), and the age-specific maternity ($l_x m_x$) versus age of *L. decemlineata* reared in groups on potato cultivars Agria and Lady Olympia respectively. Almost a similar trend was noticed as in the case of individual reared method. The survival rate (l_x) curve for CPB population reared in groups on potato cultivar Agria dropped more rapidly at the early stages of development than it did on potato cultivar Lady Olympia indicating that the mortality rate at this stage was high in CPB populations that were fed on potato cultivar Agria. The curve line of the age-stage specific fecundity (f_x) showed peak value of 28.65 offspring on day 29 on potato cultivar Lady Olympia (B) which was higher than the peak value of the age-stage specific fecundity recorded for Agria (27.5 offspring) on day 100 (A). The curve also showed early start of egg laying by female adult reared on Agria (day 26) as compared to Lady Olympia where the female started egg laying on day 28. The curve line of age specific fecundity (m_x) showed that reproduction of CPB populations that were fed on Lady Olympia began at 28 days of age while on that of Agria began at 27 days of age. The m_x curve further showed peak value of 13.75 offspring on day 100 on Agria (A) and peak curve value on day 38 with 14.90 offspring on Lady Olympia (B). The figure further showed higher peak value of 10.64 offspring of the age-specific maternity ($l_x m_x$) for Lady Olympia (B) than Agria where peak value of curve for the age-specific maternity was 6.28 offspring (A).

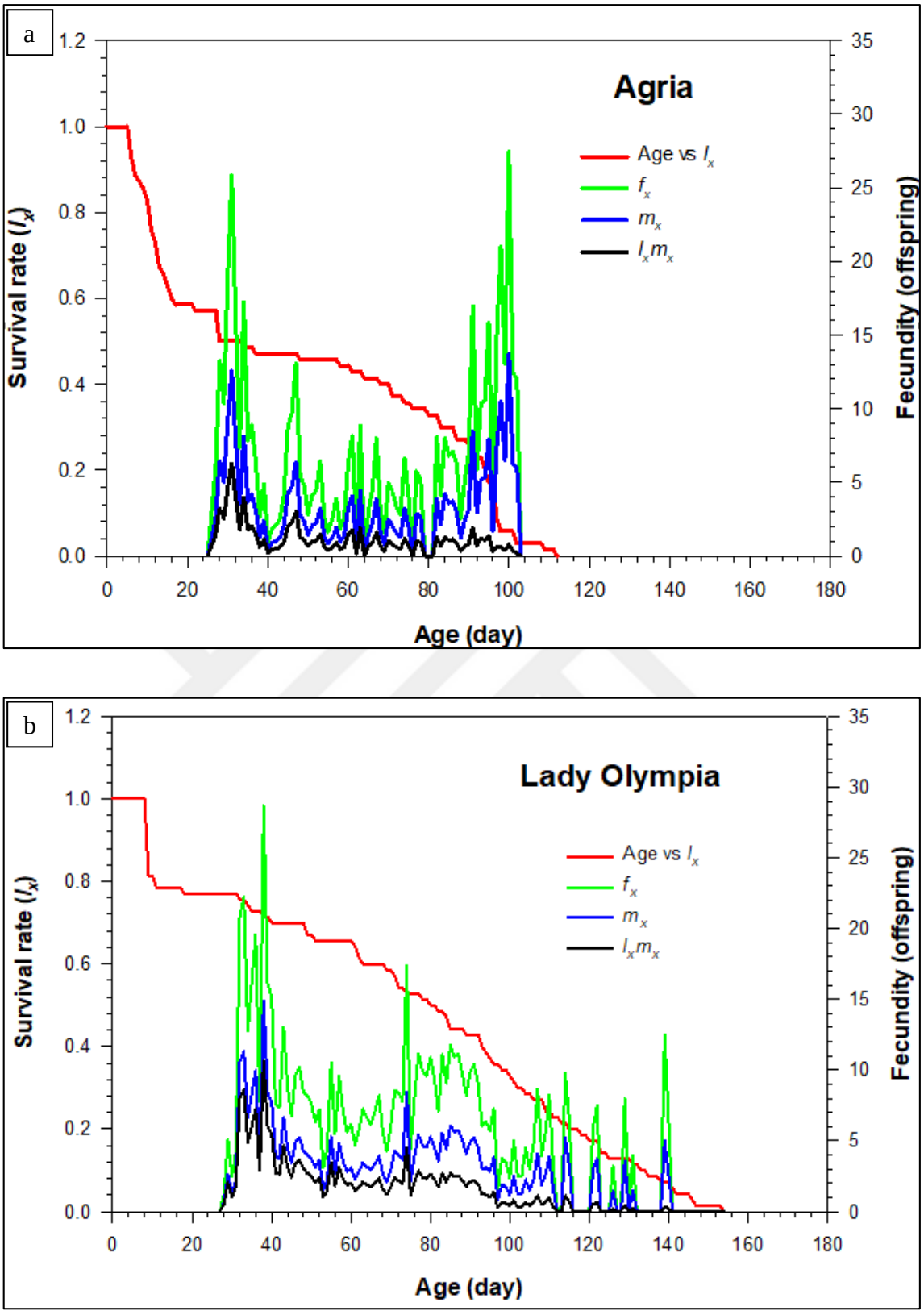


Figure 4.36. The age-specific survival rate (l_x), female age-specific fecundity (f_x), age-specific fecundity (m_x), and age-specific maternity ($l_x m_x$) versus age of *Leptinotarsa decemlineata* reared in groups on potato cultivars Agria (A) and Lady Olympia (B) respectively

The age-stage life expectancy (e_x) of *L. decemlineata* reared in groups on potato cultivars Agria and Lady Olympia respectively is shown in Figure 4.37. The longevity of *L. decemlineata* population from age zero (e_0) was 48.70 days on Agria, which was shorter than the longevity of *L. decemlineata* reared on Lady Olympia (74.13 days) (Figure 4.37).

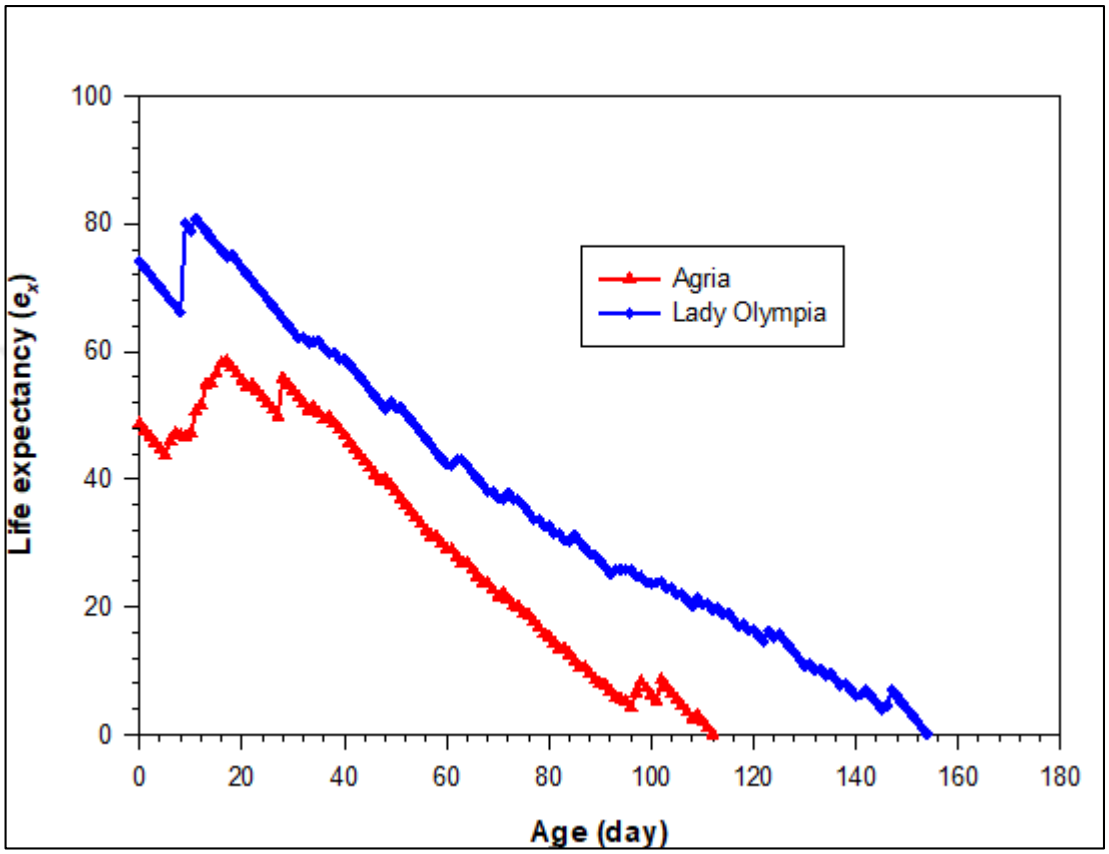


Figure 4.37. Age specific life expectancy (e_x) of *Leptinotarsa decemlineata* reared in groups on potato cultivars Agria and Lady Olympia

The age specific reproductive value (v_x) of *L. decemlineata* reared in groups on potato cultivars Agria and Lady Olympia is shown in Figure 4.38. The reproductive values (v_0) is exactly the same as the finite rate of increase (λ). The curves for reproductive value significantly increased when reproduction started (Figure 4.38). The reproductive value on potato cultivar Agria at age zero was 1.12 offspring per day, which was non-significant than the reproductive value of CPB population reared on potato cultivar Lady Olympia on which the reproductive value at age zero was 1.13 offspring per day. However, the reproductive value (v_x) on potato cultivar Agria reached the peak value of 48.30 offspring per day on day 28 as it reached the peak value of 63.00 offspring per day on day 32 on potato cultivar Lady Olympia when female emerged (Figure 4.38).

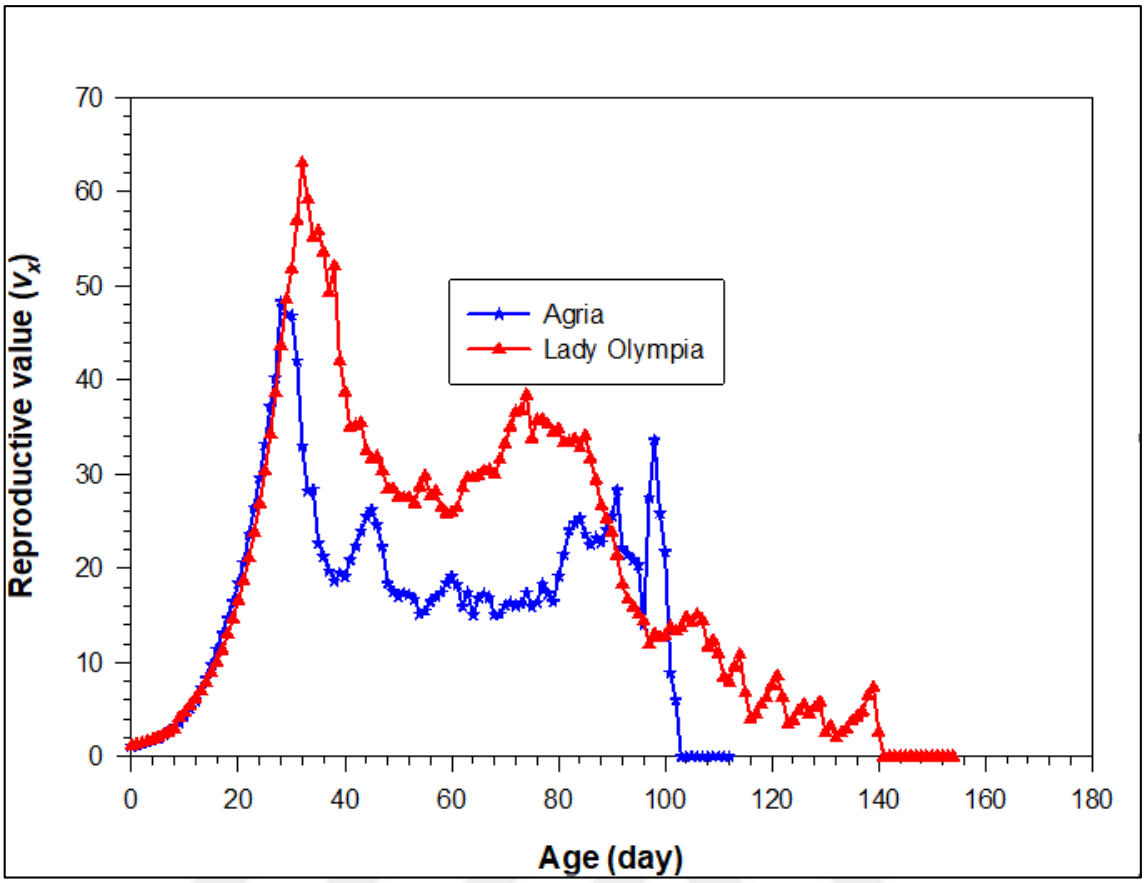


Figure 4.38. Age-stage reproductive value (v_x) of *Leptinotarsa decemlineata* reared in groups on potato cultivars Agria and Lady Olympia

4.11.3 Life table study of CPB with transgenic potato plants

Life table study of CPB with transgenic potato plants of both Agria and Lady Olympia from low to high expression levels were tried first with freshly emerged adults and then directly with egg clusters collected from their respective non-transgenic plants. In both cases, the life table studies were not initiated due to the fact that CPB adult beetles were found reluctant to feed on transgenic plants as compared with the larval stages. But when they started feeding on transgenic plants at different expressions levels, they all died before egg laying. Life table studies from egg clusters were also not recorded because after hatching of eggs, 100 percent mortality of 1st instar larvae were recorded when they started to feed on transgenic plants of both Lady Olympia and Agria cultivars at different expressions levels. Therefore, no valid data was obtained in the study.

4.12 Leaf Bioassay with Putative Transgenic Plants

The toxicity potential of introduced insecticidal genes in T_0 progeny transgenic potato plants were evaluated against Colorado potato beetle by laboratory bioassays.

4.12.1.1 Leaf bioassay of CPB 1st ınstar larvae fed on Agria plants transformed with DS-1 construct

The percent mortality of CPB 1st instar larvae fed on different transgenic potato plants of Agria transformed with DS-1 construct is shown in Table 4.8. Leaf bioassay results with CBP 1st instar larvae showed that there was significant difference in larval mortality among different transgenic plants ($P \leq 0.05$) in the first 72 hours of incubation. Larval mortality was recorded in different transgenic plants within 12 hours (Table 4.8). Significantly higher mortality was recorded in all Agria plants transformed with DS-1 gene construct as compared to non-transgenic control plants. The mortality rate of CPB 1st instar larvae fed on different transgenic plants progressively increased with increasing time of feeding. After 48 hours of feeding, 100 percent mortality was recorded AP1 plants, this was followed by AP2, AP3, AP4 and AP5 transgenic plants by recording mean percent mortality of 76.82, 70.33, 35.35 and 53.35 % respectively, while only 1.14 percent mean mortality was recorded in the control plant which was significantly lower than all the transgenic plants of Agria transformed with DS-1 construct. High insect larval mortality rate was observed after 120 hours of feeding, where all tested larvae were dead in all transgenic plants. Contrarily, only 1.14 percent mean larval mortality was observed in larvae fed on control plant leaves.

Table 4.8. Mortality rate (%Mean ± SEM)* of **1st** larval stage CPB fed on different transgenic potato plants of Agria transformed with DS-1 construct

Construct	Plants	Incubation time (hour)					
		12	24	48	72	96	120
DS-1	AP1	16.36±0.22a	56.84±0.81a	100±0.00a	100±0.00a	100±0.00a	100±0.00a
	AP2	13.02±0.22a	43.31±0.11ab	76.82±0.15b	100±0.00a	100±0.00a	100±0.00a
	AP3	13.02±0.22a	36.59±0.13ab	70.33±0.41b	90.74±8.85ab	100±0.00a	100±0.00a
	AP4	4.53±1.14ab	26.52±0.15b	53.35± 1.92b	63.40± 2.00bc	93.30± 0.11ab	100±0.00a
	AP5	4.53±1.14ab	23.18± 0.15b	53.35± 0.11b	60.13± 3.40c	80.68±0.55b	100±0.00a
Control	AgC	0.00±0.00b	0.00±0.00c	4.53±1.14c	4.53±0.14d	4.53±1.14c	4.53±1.14b

*Means within column followed by different letters are significantly different at α=0.05, determined using the Tukey test. Mortality data (% Mean± SEM) are means of three replicates.

4.12.1.2 Leaf bioassay of CPB 1st ınstar larvae fed on Agria plants transformed with DS-2 construct

The percent mortality of CPB 1st instar larvae fed on different transgenic potato plants of Agria transformed with DS-2 construct is shown in Table 4.9. Leaf bioassays results with CBP 1st instar larvae showed that there was significance difference in larval mortality among different transgenic plants except after 12 and 120 hours of incubation ($P \leq 0.05$). The mortality rate of CPB 1st instar larvae fed on different transgenic plants progressively increased with increasing time of feeding. After 72 hours of feeding, mean percent mortality was much more variable ranging from 70.34 to 100 % among the positive transgenic plants of Agria transformed with DS-2 construct. While only 1.14 % mean mortality data was observed in the control plants.

Table 4.9. Mortality rate (%Mean ± SEM)* of **1st** larval stage CPB fed on different transgenic potato plants of Agria transformed with DS-2 construct

Construct	Plants	Incubation time (hour)					
		12	24	48	72	96	120
DS-2	AP1	13.02±2.71a	43.31±1.92a	70.33±3.65a	100±0.00a	100±0.00a	100±0.00a
	AP2	10.01±0.00a	39.84±3.40ab	66.73±2.00a	98.85±6.14a	100±0.00a	100±0.00a
	AP3	4.53±1.14a	25.44±6.17ab	53.35±3.84a	76.82±2.21b	100±0.00a	100±0.00a
	AP4	4.53±1.14a	16.36±2.71b	43.30±1.92a	66.74±2.00b	98.85±6.14ab	100±0.00a
	AP5	4.53±1.14a	16.36±2.71b	43.16±3.93a	70.34±3.65b	86.99±2.71b	100±0.00a
Control	AgC	0.00±0.00b	0.00±0.00c	4.53±1.14b	4.53±1.14c	4.53±1.14c	4.53±1.14b

*Means within column followed by different letters are significantly different at α=0.05, determined using the Tukey test. Mortality data (% Mean± SEM) are means of three replicates.

4.12.1.3 Leaf bioassay of CPB 1st ınstar larvae fed on Lady Olympia plants transformed with DS-1 construct

The percent mortality of CPB 1st instar larvae fed on different transgenic potato plants of Lady Olympia transformed with DS-1 construct is shown in Table 4.10. Same pattern of mean percent mortality data was observed as in case of Agria plants transformed with DS-1 gene construct. Leaf bioassays results with CBP 1st instar larvae showed that there was significance difference in larval mortality among different transgenic plants at 24, 48 and 72 hours of incubation ($P \leq 0.05$). Significantly higher mortality of 95.47% was recorded in LOP1 plant after 48 hours incubation period, this was followed by LOP2 and LOP3 plants where 84.28 and 70.33 % mean mortality was recorded. While LOP4 and LOP5 showed 67.08 and 50 % mortality data of CPB 1st instar larvae after 48 hours of feeding. No mortality data was recorded in the control plants. This mortality data of CPB 1st instar larvae fed on different transgenic plants progressively increased with increasing time of feeding and after 120 hours incubation, 100 percent mortality was recorded in all Lady Olympia plants transformed with DS-1 construct. The control plants of Lady Olympia showed no mortality even after 120 hours of feeding.

Table 4.10. Mortality rate (%Mean ± SEM)* of **1st** larval stage CPB fed on different transgenic potato plants of Lady Olympia transformed with DS-1 construct

Construct	Plants	Incubation time (hour)					
		12	24	48	72	96	120
DS-1	LOP1	13.02±2.71a	50.00±3.33a	95.47±6.14a	100±0.00a	100±0.00a	100±0.00a
	LOP2	13.02±2.71a	39.84±3.40a	84.28±4.92ab	100±0.00a	100±0.00a	100±0.00a
	LOP3	16.36 ±2.71a	36.59±2.006a	70.33±3.65ab	93.30±7.85ab	97.63± 8.85a	100±0.00a
	LOP4	10.01± 0.00a	33.24±2.00ab	67.08± 4.22b	80.69±4.27b	98.85 ±6.14a	100±0.00a
	LOP5	4.53±1.14ab	16.36±2.71b	50.00±3.33b	77.54± 4.92b	97.63± 8.85a	100±0.00a
Control	LOC	0.00±0.00b	0.00±0.00c	0.00±0.00c	0.00±0.00c	0.00±0.00b	0.00±0.00b

*Means within column followed by different letters are significantly different at α=0.05, determined using the Tukey test. Mortality data (% Mean± SEM) are means of three replicates.

Leaf Bioassay of CPB 1st ınstar larvae fed on Lady Olympia plants transformed with DS-2 construct

4.12.1.4 Leaf bioassay of CPB 1st ınstar larvae fed on Lady Olympia plants transformed with DS-2 construct

The percent mortality of CPB 1st instar larvae fed on different transgenic potato plants of Lady Olympia transformed with DS-2 construct is shown in Table 4.11. Results with CBP 1st instar larvae showed that there was significance difference in larval mortality among different transgenic plants after 24 and 48 hours of incubation ($P \leq 0.05$). The data showed initially a little slower toxic action of DS-2 construct against CPB 1st instar larvae as compared to DS-1 gene construct. However, with the passage of time, this mortality rate of CPB 1st instar larvae progressively increased, and 100 percent mortality of CPB larvae were recorded in all Lady Olympia plants transformed with DS-2 construct after 120 hours of incubation period. There was no mortality in the control plants of Lady Olympia even after 120 hours of incubation period.

Table 4.11. Mortality rate (%Mean ± SEM)* of **1st** larval stage CPB fed on different transgenic potato plants of Lady Olympia with DS-2 construct

Construct	Plants	Incubation time (hour)					
		12hr	24hr	48hr	72hr	96hr	120hr
DS-2	LOP1	16.36 ±2.71a	36.59±2.006a	70.33±3.65a	95.47± 6.14a	98.85 ±6.14a	100±0.00a
	LOP2	13.02±2.71a	43.31±1.92ab	83.65 ± 2.71ab	98.85± 6.14a	100±0.00a	100±0.00a
	LOP3	10.01± 0.00a	40.00 ±0.00abc	66.74±2.00b	93.30± 7.85a	97.63±8.85a	100±0.00a
	LOP4	10.01 ±0.00a	30.18 ±0.00bc	63.40 ±2.00b	80.69±4.27a	84.28± 4.92a	100±0.00a
	LOP5	4.53±1.14ab	26.51 ± 2.21c	56.69 ±1.92b	80.69±4.27a	90.75± 8.85a	100±0.00a
Control	LOC	0.00±0.00b	0.00±0.00d	0.00±0.00c	0.00±0.00b	0.00±0.00b	0.00±0.00b

*Means within column followed by different letters are significantly different at α=0.05, determined using the Tukey test. Mortality data (% Mean± SEM) are means of three replicates

4.12.2 Leaf bioassay analysis with CPB 2nd ınstar larvae

4.12.2.1 Leaf bioassay of CPB 2nd ınstar larvae fed on Agria plants transformed with DS-1 construct

The percent mortality of CPB 2nd instar larvae fed on different transgenic potato plants of Agria transformed with DS-1 construct is shown in Table 4.12. Results with CBP 2nd instar larvae showed that there was significance difference in larval mortality among different transgenic plants only after 24 hours of incubation ($P \leq 0.05$). Larval mortality was recorded in different transgenic plants within 12 hours. The mortality rate of CPB 2nd instar larvae fed on different transgenic plants also progressively increased with increasing time of feeding. After 72 hours of feeding, mean percent mortality was much more variable ranging from 83.64 to 100 % among the positive transgenic plants of Agria transformed with DS-1 construct.

Table 4.12. Mortality rate (%Mean ± SEM* of 2nd larval stage CPB fed on different transgenic potato plants of Agria transformed with DS-1 construct

Construct	Plants	Incubation time (hour)					
		12	24	48	72	96	120
DS-1	AP1	26.51±2.21a	53.35±1.92a	83.64±2.71a	100±0.00a	100±0.00a	100±0.00a
	AP2	23.18±2.21a	46.63±1.92a	76.82±2.21a	100±0.00a	100±0.00a	100±0.00a
	AP3	19.99±0.00a	39.84±3.40ab	60.63±6.98a	93.30±7.85a	100±0.00a	100±0.00a
	AP4	16.35±2.71a	40.00±0.00ab	70.33±3.65a	93.30±7.85a	100±0.00a	100±0.00a
	AP5	16.35±2.71a	30.00±0.00b	60.14±3.40a	83.64±2.71a	100±0.00a	100±0.00a
Control	AgC	0.00±0.00b	0.00±0.00c	0.00±0.00b	0.00±0.00b	0.00±0.00b	0.00±0.00b

*Means within column followed by different letters are significantly different at α=0.05, determined using the Tukey test. Mortality data (% Mean± SEM) are means three replicates.

4.12.2.2 Leaf bioassay of CPB 2nd ınstar larvae fed on Agria plants transformed with DS-2 construct

The percent mortality of CPB 2nd instar larvae fed on different transgenic plants of Agria transformed with DS-2 construct is shown in Table 4.13. The data showed increased in mean percent mortality of CPB 2nd instar larvae with increasing time of feeding. After 72 hours of feeding, mean percent mortality was much more variable ranging from 80.69 to 93.30 % among the positive transgenic plants of Agria transformed with DS-2 construct. High insect larval mortality rate was recorded after 120 hours of feeding, where 100 percent mortality was recorded in all transgenic plants. No larval mortality was observed in larvae fed on control plants leaves even after 120 hours of incubation period.

Table 4.13. Mortality rate (%Mean ± SEM)* of 2nd larval stage CPB fed on different transgenic potato plants of Agria transformed with DS-2 construct

Construct	Plants	Incubation time (hour)					
		12	24	48	72	96	120
DS-2	AP1	19.99±0.00a	46.63±1.92a	73.47±2.21a	93.30±7.85a	98.85±6.14a	100±0.00a
	AP2	16.35±2.71a	33.24±2.00a	63.40±2.00a	90.75±8.85a	98.85±6.14a	100±0.00a
	AP3	16.35±2.71a	39.84±3.40a	63.92±5.44a	90.75±8.85a	97.63±8.85a	100±0.00a
	AP4	16.35±2.71a	36.59±2.00a	59.99±0.00a	80.69±4.27a	95.47±6.14a	100±0.00a
	AP5	13.37±2.71a	36.59±2.00a	59.99±0.00a	83.64±2.71a	97.63±8.85a	100±0.00a
Control	AgC	0.00±0.00b	0.00±0.00b	0.00±0.00b	0.00±0.00b	0.00±0.00b	0.00±0.00b

*Means within column followed by different letters are significantly different at α=0.05, determined using the Tukey test. Mortality data (% Mean± SEM) are means of three replicates.

4.12.2.3 Leaf bioassay of CPB 2nd ınstar larvae fed on Lady Olympia plants transformed with DS-1 construct

The percent mortality of CPB 2nd instar larvae fed on different transgenic potato plants of Lady Olympia transformed with DS-1 construct is shown in Table 4.14. The mortality data showed significance difference among different transgenic plants of Lady Olympia transformed with DS-1 construct after 12 hours of feeding ($P \leq 0.05$). Significantly higher larval mortality of CPB 2nd instar larvae was recorded in LOP1 transgenic plants within 12 hours. The data regarding mortality rate of CPB 2nd instar larvae fed on different transgenic plants showed increasing pattern with increasing time of incubation. High insect larval mortality rate was recorded after 120 hours of feeding, where 100 percent mortality was recorded in all transgenic plants. No larval mortality was observed in larvae fed on control plants leaves.

Table 4.14. Mortality rate (%Mean ± SEM)* of 2nd larval stage CPB fed on different transgenic potato plants of Lady Olympia transformed with DS-1 construct

Construct	Plants	Incubation time (hour)					
		12	24	48	72	96	120
DS-1	LOP1	33.24±2.00a	53.35±1.92a	80.69±4.27a	100±0.00a	100±0.00a	100±0.00a
	LOP2	26.51±2.21ab	43.16±3.92a	70.80±6.14a	97.63±8.85a	100±0.00a	100±0.00a
	LOP3	29.66±3.65abc	43.16±5.15a	71.57±7.68a	93.30±7.85a	98.85±6.14a	100±0.00a
	LOP4	16.34±2.71bc	36.45±3.92a	63.88±5.44a	93.30±7.85a	98.85±6.14a	100±0.00a
	LOP5	13.00±2.71c	36.45±3.92a	63.88±5.44a	93.30±7.85a	98.85±6.14a	100±0.00a
Control	LOC	0.00±0.00d	0.00±0.00b	0.00±0.00b	0.00±0.00b	0.00±0.00b	0.00±0.00b

*Means within column followed by different letters are significantly different at α=0.05, determined using the Tukey test. Mortality data (% Mean± SEM) are means of three replicates.

4.12.2.4 Leaf bioassay of CPB 2nd ınstar larvae fed on Lady Olympia plants transformed with DS-2 construct

The percent mortality of CPB 2nd instar larvae fed on different transgenic potato plants of Lady Olympia transformed with DS-2 construct is shown in Table 4.15. The mortality data showed significance difference among different transgenic plants of Lady Olympia transformed with DS-2 construct only after 48 and 72-hours incubation ($P \leq 0.05$). The mortality rate of CPB 2nd instar larvae fed on different transgenic plants also progressively increased with increasing time of feeding. After 72 hours of feeding, mean percent mortality was much more variable ranging from 77.84 to 100 % among the positive transgenic plants of Lady Olympia transformed with DS-2 construct. High larval mortality rate was recorded after 120 hours of feeding, where 100 percent mortality was recorded in all transgenic plants.

Table 4.15. Mortality rate (%Mean ± SEM)* of 2nd larval stage CPB fed on different transgenic potato plants of Lady Olympia transformed with DS-2 construct

Construct	Plants	Incubation time (hour)					
		12	24	48	72	96	120
DS-2	LOP1	26.51±2.21a	43.16±3.92a	70.32±3.65ab	98.85±6.14ab	100±0.00a	100±0.00a
	LOP2	23.17±2.21a	46.63±3.84a	73.47±2.21a	100±0.00a	100±0.00a	100±0.00a
	LOP3	23.17±2.21a	46.63±3.84a	73.47±2.21a	86.98±2.71bc	93.30±7.85a	100±0.00a
	LOP4	16.34±2.71a	36.58±2.00a	56.68±1.92b	77.84±6.05c	83.63±2.71a	100±0.00a
	LOP5	16.34±2.71a	29.99±0.00a	56.68±1.92b	79.99±0.00c	90.74±8.85a	100±0.00a
Control	LOC	0.00±0.00b	0.00±0.00b	0.00±0.00c	0.00±0.00d	0.00±0.00b	0.00±0.00b

*Means within column followed by different letters are significantly different at α=0.05, determined using the Tukey test. Mortality data (% Mean± SEM) are means of three replicates.

4.12.3 Leaf bioassay analysis with CPB 3rd ınstar larvae

4.12.3.1 Leaf bioassay of CPB 3rd ınstar larvae fed on Agria plants transformed with DS-1 construct

The percent mortality of CPB 3rd instar larvae fed on different transgenic potato plants of Agria transformed with DS-1 construct is shown in Table 4.16. The mortality data showed significance difference among different transgenic plants of Agria transformed with DS-1 gene construct except after 96 to 144 hours incubation ($P \leq 0.05$). The data revealed higher larval mortality of CPB 3rd instar larvae in AP1 plants transformed with DS-1 gene construct (33.24%) recorded within 12 hours of incubation, followed by AP2, AP3 and AP4 plants by recording 26.51, 26.51 and 19.99 % mortality respectively, while lower mean percent mortality of 16.35% was recorded within 12 hours of feeding in AP5 plants transformed with DS-1 construct. At this stage, no mortality was observed in the control plants. The mortality data further showed mortality rate of CPB 3rd instar larvae increased with increasing time of incubation. After 72 hours of feeding, mean percent mortality was much more variable ranging from 73.47 to 100 % among the positive transgenic plants of Agria transformed with DS-1 construct. In case of CPB 3rd instar larvae, it took one day more to reach 100 percent mortality as compared to the early CPB larval instars i.e. high insect larval mortality rate was recorded after 144 hours of feeding. No larval mortality was observed in larvae fed on control plants leaves.

Table 4.16. Mortality rate (%Mean ± SEM) * of 3rd larval stage CPB fed on different transgenic potato plants of Agria transformed with DS-1 construct

Construct	Plants	Incubation time (hour)						
		12	24	48	72	96	120	144
DS-1	AP1	33.24±2.00a	66.74±2.00a	100±0.00a	100±0.00a	100±0.00a	100±0.00a	100±0.00a
	AP2	26.51±2.21ab	56.68±1.92ab	97.63±8.85ab	100±0.00a	100±0.00a	100±0.00a	100±0.00a
	AP3	26.51±2.21ab	53.35±3.84ab	80.69±4.27bc	98.85±6.14ab	100±0.00a	100±0.00a	100±0.00a
	AP4	19.99±0.00ab	53.35±1.92ab	76.82±2.21bc	90.75±8.85ab	95.47±6.14ab	100±0.00a	100±0.00a
	AP5	16.35±2.71b	43.16±3.92b	66.74±2.00c	73.47±2.21b	86.98±2.71b	98.85±6.14a	100±0.00a
Control	AgC	0.00±0.00c	0.00±0.00c	0.00±0.00d	0.00±0.00c	0.00±0.00c	0.00±0.00b	0.00±0.00b

*Means within column followed by different letters are significantly different at α=0.05, determined using the Tukey test. Mortality data (% Mean± SEM) are means of three replicates.

4.12.3.2 Leaf bioassay of CPB 3rd ınstar larvae fed on Agria plants transformed with DS-2 construct

The percent mortality of CPB 3rd instar larvae fed on different transgenic potato plants of Agria transformed with DS-2 construct is shown in Table 4.17. The data revealed no significant differences in the larval mortality rate of CPB 3rd instar larvae that were fed on different plants of Agria transformed with DS-2 construct within 12 hours of incubation, however this mortality data was significantly different from that of the control plants ($P \leq 0.05$). The mortality data further revealed increasing trends in larval mortality rate of CPB 3rd instar larvae with increasing time of incubation. After 72 hours of feeding, mean percent mortality was much more variable ranging from 73.47 to 85.80 % among the positive transgenic plants of Agria transformed with DS-2 construct. High insect larval mortality rate (100%) was recorded in all the positive transgenic plants of Agria transformed with DS-2 construct after 144 hours of feeding. There was no larval mortality in larvae fed on control plants leaves.

Table 4.17. Mortality rate (%Mean ± SEM)* of 3rd larval stage CPB fed on different transgenic potato plants of Agria transformed with DS-2 construct

Construct	Plants	Incubation time (hour)						
		12	24	48	72	96	120	144
DS-2	AP1	26.51±2.21a	56.68±1.92a	73.47±2.21a	83.64±2.71a	86.98±2.71a	100±0.00a	100±0.00a
	AP2	23.18±2.21a	53.35±1.92ab	77.54±4.92a	84.28±4.92a	86.98±2.71a	98.85±6.14ab	100±0.00a
	AP3	19.99±0.00a	50.00±0.00ab	73.47±2.21a	85.80±11.07a	95.46±6.14a	98.85±6.14ab	100±0.00a
	AP4	16.35±2.71a	36.59±2.00ab	59.99±0.00a	73.47±2.21a	86.98±2.71a	89.99±0.00b	100±0.00a
	AP5	16.35±2.71a	39.84±3.40b	60.14±3.40a	73.47±2.21a	86.98±2.71a	89.99±0.00b	100±0.00a
Control	AgC	0.00±0.00b	0.00±0.00c	0.00±0.00b	0.00±0.00b	0.00±0.00b	0.00±0.00c	0.00±0.00b

*Means within column followed by different letters are significantly different at α=0.05, determined using Tukey test. Mortality data (% Mean± SEM) are means of three replicates.

4.12.3.3 Leaf bioassay of CPB 3rd ınstar larvae fed on Lady Olympia plants transformed with DS-1 construct

The percent mortality of CPB 3rd instar larvae fed on different transgenic potato plants of Lady Olympia transformed with DS-1 construct is shown in Table 4.18. The data revealed significantly higher larval mortality of CPB 3rd instar larvae in LOP plants transformed with DS-1 construct (36.59%) recorded within 12 hours of incubation, while significantly lower mean percent mortality of 19.99 and 16.35% was recorded within 12 hours of feeding in LOP4 and LOP5 plants transformed with DS-1 construct. At this stage, no mortality was observed in the control plants. The mortality data further showed mortality rate of CPB 3rd instar larvae increased with increasing time of incubation. After 72 hours of feeding, mean percent mortality was much more variable ranging from 73.47 to 100 % among the positive transgenic plants of Lady Olympia transformed with DS-1 construct. CPB 3rd instar larvae took longer time to reach 100 % mortality in all the positive transformed plants as compared with the 1st and 2nd larval instars i.e. the greatest larval mortality rate (100%) was recorded in all positive transgenic plants of Lady Olympia after 144 hours of feeding. No larval mortality was observed in larvae fed on control plants leaves.

Table 4.18. Mortality rate (%Mean ± SEM)* of 3rd larval stage CPB fed on different transgenic potato plants of Lady Olympia transformed with DS-1 construct

Construct	Plants	Incubation time (hour)						
		12	24	48	72	96	120	144
DS-1	LOP1	36.59±2.00a	66.74±2.00a	98.85±6.14a	100±0.00a	100±0.00a	100±0.00a	100±0.00a
	LOP2	30.00±0.00ab	59.99±0.00ab	80.69±4.27ab	90.75±8.85ab	98.85±6.14a	100±0.00a	100±0.00a
	LOP3	29.66±3.65ab	50.00±3.33bc	74.54±6.17b	88.38±10.14ab	98.85±6.14a	98.85±6.14a	100±0.00a
	LOP4	19.99±0.00b	40.00±0.00c	63.40±2.00b	76.82±2.21ab	93.30±7.85a	95.47±6.14a	100±0.00a
	LOP5	16.35±2.71b	36.59±2.00c	56.68±1.92b	73.47±2.21b	86.98±2.71a	95.47±6.14a	100±0.00a
Control	LOC	0.00±0.00c	0.00±0.00d	0.00±0.00c	0.00±0.00c	0.00±0.00b	0.00±0.00b	0.00±0.00b

*Means within column followed by different letters are significantly different at α=0.05, determined using Tukey test. Mortality data (% Mean± SEM) are means of three replicates.

4.12.3.4 Leaf bioassay of CPB 3rd ınstar larvae fed on Lady Olympia plants transformed with DS-2 construct

The percent mortality of CPB 3rd instar larvae fed on different transgenic potato plants of Lady Olympia transformed with DS-2 construct is shown in Table 4.19. The mortality data revealed increasing trends in larval mortality rate of CPB 3rd instar larvae with increasing time of incubation. After 72 hours of feeding, mean percent mortality was much more variable ranging from 70.32 to 97.63 % among the positive transgenic plants of Lady Olympia transformed with DS-2 construct. One hundred percent larval mortality rate was recorded in all the positive transgenic plants of Lady Olympia transformed with DS-2 construct after 144 hours of feeding. There was no mortality in the control.

Table 4.19. Mortality rate (%Mean ± SEM)* of 3rd larval stage CPB fed on different transgenic potato plants of Lady Olympia transformed with DS-2 construct

Construct	Plants	Incubation time (hour)						
		12	24	48	72	96	120	144
DS-2	LOP1	30.00±0.00a	59.99±0.00a	86.98±2.71a	97.63±8.85a	100±0.00a	100±0.00a	100±0.00a
	LOP2	26.51±2.21ab	50.00±3.33ab	73.80±4.22ab	77.84±6.05a	97.63±8.85ab	98.85±6.14a	100±0.00a
	LOP3	26.51±2.21ab	50.00±3.33ab	69.99±0.00bc	80.69±4.27a	89.99±0.00ab	95.46±6.14a	100±0.00a
	LOP4	19.99±0.00ab	43.30±1.92ab	59.99±0.00bc	83.64±2.71a	95.46±6.14ab	98.85±6.14a	100±0.00a
	LOP5	16.35±2.71b	33.24±2.00b	53.35±1.92c	70.32±3.65a	86.98±2.71b	89.99±0.00a	100±0.00a
Co	LOC	0.00±0.00c	0.00±0.00c	0.00±0.00d	0.00±0.00b	0.00±0.00c	0.00±0.00b	0.00±0.00b

*Means within column followed by different letters are significantly different at α=0.05, determined using the Tukey test. Mortality data (% Mean± SEM) are means of three replicates.

4.12.4 Leaf bioassay analysis with CPB 4th ınstar larvae

4.12.4.1 Leaf bioassay of CPB 4th ınstar larvae fed on Agria plants transformed with DS-1 construct

The percent mortality of CPB 4th instar larvae fed on different transgenic plants of Agria transformed with DS-1 construct is shown in Table 4.20. Mean percent mortality of the CPB 4th larval instars were found with gradual increase with increased in incubation time. After 72 hours of feeding, mean percent mortality was much more significantly variable ranging from 43.30 to 76.82% among the positive transgenic plants of Agria transformed with DS-1 construct. Significantly higher (100%) mean percent mortality was recorded after 96 hours of feeding in AP1 and AP2 plants of Agria transformed with DS-1 gene construct, followed by significantly lower mean percent mortality of 73.79, 59.99 and 56.68 mean percent mortality in AP3, AP4 and AP5 plants respectively. While no mortality data was recorded in the control plants even after 96 hours of feeding. Remaining alive CPB 4th instar larvae feeding after 96 hours incubation went to pupation in the soil. They were regularly checked and kept moisture until adult emergence. After 15 days, all the larvae, that were fed on control plants were emerged as adults, while none of a single adult were emerged from the larvae that were fed on transgenic plants of Agria transformed with DS-1 gene construct.

Table 4.20. Mortality rate (%Mean ± SEM)* of 4th larval stage CPB fed on different transgenic potato plants of Agria transformed with DS-1 construct

Construct	Plants	Incubation time (hour)					
		12	24	48	72	96	456
DS-1	AP1	26.51±2.21a	36.58±2.00a	53.34±1.92a	76.82±2.21a	100±0.00a	100±0.00a
	AP2	23.17±2.21a	30.00±0.00ab	46.63±1.92ab	73.43±2.21a	100±0.00a	100±0.00a
	AP3	23.17±2.21a	30.00±0.00ab	43.16±3.92ab	63.54±3.92ab	73.79±4.22b	100±0.00a
	AP4	16.35±2.71a	23.18±2.21bc	33.24±2.00b	43.30±1.92b	59.99±0.00bc	100±0.00a
	AP5	13.00±2.71a	19.99±0.00c	33.24±2.00b	49.99±3.33b	56.68±1.92c	100±0.00a
Control	AgC	0.00±0.00b	0.00±0.00d	0.00±0.00c	0.00±0.00c	0.00±0.00d	4.53±1.14b

*Means within column followed by different letters are significantly different at α=0.05, determined using the Tukey test. Mortality data (% Mean± SEM) are means of three replicates.

4.12.4.2 Leaf bioassay of CPB 4th ınstar larvae fed on Agria plants transformed with DS-2 construct

The percent mortality of CPB 4th instar larvae fed on different transgenic plants of Agria transformed with DS-2 construct is shown in Table 4.21. The data revealed gradual increase in CPB 4th instar larvae with increased in incubation time. After 72 hours of feeding, mean percent mortality was much more significantly variable ranging from 50 to 70.32% among the positive transgenic plants of Agria transformed with DS-2 construct as compared to the control plants ($P \leq 0.05$). Significantly higher (98.85%) mean percent mortality was recorded after 96 hours of feeding in AP2 plant of Agria transformed with DS-2 gene construct, followed by significantly lower variable mean percent mortality of 76.82, 67.08 and 66.74 mean percent mortality in AP3, AP4 and AP5 plants respectively. Remaining alive CPB 4th instar larvae feeding after 96 hours incubation went to pupation in the soil. They were regularly checked and kept moisture until adult emergence. After 15 days, all the larvae, that were fed on control plants were emerged as adults, while none of a single adult were emerged from the larvae that were fed on transgenic plants of Agria transformed with DS-2 construct.

Table 4.21. Mortality rate (%Mean ± SEM)* of 4th larval stage CPB fed on different transgenic potato plants of Agria transformed with DS-2 construct

Construct	Plants	Incubation time (hour)					
		12hr	24hr	48hr	72hr	96hr	456hr
DS-2	AP1	23.18±2.21a	33.24±2.00a	50.00±3.33a	70.32±3.65ab	93.30±7.85ab	100±0.00a
	AP2	19.99±0.00a	30.00±0.00a	50.00±0.00a	73.47±2.21a	98.85±6.14a	100±0.00a
	AP3	16.35±2.71a	26.51±2.21a	39.84±3.40a	56.68±1.92abc	76.82±2.21bc	100±0.00a
	AP4	13.01±2.71a	23.18±2.21a	39.84±3.40a	53.35±1.92bc	67.08±4.22c	100±0.00a
	AP5	13.01±2.71a	23.18±2.21a	40.00±0.00a	50.00±0.00c	66.74±2.00c	100±0.00a
Control	AgC	0.00±0.00b	0.00±0.00b	0.00±0.00b	0.00±0.00d	0.00±0.00d	4.53±1.14 b

*Means within column followed by different letters are significantly different at α=0.05, determined using the Tukey test. Mortality data (% Mean± SEM) are means of three replicates.

4.12.4.3 Leaf bioassay of CPB 4th ınstar larvae fed on Lady Olympia plants transformed with DS-1 construct

The percent mortality of CPB 4th instar larvae fed on different transgenic plants of Lady Olympia transformed with DS-1 construct is shown in Table 4.22. The data revealed gradual increase in CPB 4th instar larval mortality with increased in incubation time. After 72 hours of feeding, mean percent mortality was much more significantly variable ranging from 53.35 to 77.54% among the positive transgenic plants of Lady Olympia transformed with DS-1 construct, while no mortality was recorded in the control plants. Significantly higher (100%) mean percent mortality was recorded after 96 hours of feeding in LOP1 plant of Lady Olympia transformed with DS-1 construct, followed by significantly lower variable mean percent mortality of 76.82, 70.32 and 66.74 mean percent mortality in LOP3, LOP4 and LOP5 plants respectively. While no mortality data was recorded in the control plants even after 96 hours of feeding. Remaining alive CPB 4th instar larvae feeding after 96 hours went to pupation in the soil. After 15 days, all the larvae, that were fed on control plants were emerged as adults, while none of a single adult were emerged from the larvae that were fed on transgenic plants of Lady Olympia transformed with DS-1 construct.

Table 4.22. Mortality rate (%Mean ± SEM)* of 4th larval stage CPB fed on different transgenic potato plants of Lady Olympia transformed with DS-1 construct

Construct	Plants	Incubation time (hour)					
		12	24	48	72	96	456
DS-1	LOP1	30.00±0.00a	43.30±1.92a	60.14±3.40a	77.54±4.92a	100±0.00a	100±0.00a
	LOP2	23.18±2.21ab	33.24±2.00b	46.63±1.92ab	66.74±2.00a	93.30±7.85ab	100±0.00a
	LOP3	23.18±2.21ab	30.00±0.00b	43.30±1.92ab	56.68±1.92a	76.82±2.21bc	100±0.00a
	LOP4	19.99±0.00b	30.00±0.00b	39.84±3.40b	56.84±3.92a	70.32±3.65bc	100±0.00a
	LOP5	19.99±0.00b	30.00±0.00b	36.59±2.00b	53.35±3.84a	66.74±2.00c	100±0.00a
Control	LOC	0.00±0.00c	0.00±0.00c	0.00±0.00c	0.00±0.00b	0.00±0.00d	4.53±1.14 b

Means within column followed by different letters are significantly different at α=0.05, determined using the Tukey test. Mortality data (% Mean± SEM) are means of three replicates.

4.12.4.4 Leaf bioassay of CPB 4th ınstar larvae fed on Lady Olympia plants transformed with DS-2 construct

The percent mortality of CPB 4th instar larvae fed on different transgenic plants of Lady Olympia transformed with DS-2 construct is shown in Table 4.23. Larval mortality of CPB 4th instars larvae ranging from 13.01 to 26.51 % was recorded in different plants of Lady Olympia transformed with DS-2 construct within 12 hours of feeding, while no mortality was recorded in the control plants. Gradual increase was observed in CPB 4th instar larval mortality with increased in incubation time. Significantly higher (98.85%) mean percent mortality was recorded after 96 hours of incubation in LOP1 plant of Lady Olympia transformed with DS-2 construct, followed by significantly lower variable mean percent mortality of 77.54, 70.32 and 66.74 mean percent mortality in LOP3, LOP4 and LOP5 plants respectively. Remaining alive CPB 4th instar larvae feeding after 96 hours incubation went to pupation in the soil. After 15 days, all the larvae, that were fed on control plants were emerged as adults, while none of a single adult were emerged from the larvae that were fed on transgenic plants of Lady Olympia transformed with DS-2 construct.

Table 4.23. Mortality rate (%Mean ± SEM)* of 4th larval stage CPB fed on different transgenic potato plants of Lady Olympia transformed with DS-2 construct

Construct	Plants	Incubation time (hour)					
		12	24	48	72	96	456
DS-2	LOP1	26.51±2.21a	36.59±2.00a	53.35±1.92a	73.43±2.21a	98.85±6.14a	100±0.00a
	LOP2	23.18±2.21a	33.24±2.00a	43.30±1.92ab	60.14±3.40a	83.64±2.71ab	100±0.00a
	LOP3	23.18±2.21a	26.51±2.21a	43.30±1.92ab	56.84±3.92a	77.54±4.92b	100±0.00a
	LOP4	19.99±0.00a	26.51±2.21a	39.84±3.40ab	56.84±3.92a	70.32±3.65b	100±0.00a
	LOP5	13.01±2.71a	23.18±2.21a	33.24±2.00b	53.35±1.92a	66.74±2.00b	100±0.00a
Control	LOC	0.00±0.00b	0.00±0.00b	0.00±0.00c	0.00±0.00b	0.00±0.00c	4.53±1.14b

*Means within column followed by different letters are significantly different at α=0.05, determined using the Tukey test. Mortality data (% Mean± SEM) are means of three replicates.

4.12.5 Leaf bioassay analysis with CPB adults

4.12.5.1 Leaf bioassay of CPB adult fed on Agria plants transformed with DS-1 construct

The percent mortality of CPB adults fed on different transgenic plants of Agria transformed with DS-1 construct and their control plants is shown in Table 4.24. CPB adults analysis showed no mortality after 24 hours of feeding showing slower mortality rate as compared to the larval stages. It may due to the fact that CPB adults were reluctant to fed on the leaves of transgenic plants and were resistant to hunger than larval stages. Hence the adult stage was found to resist eating leaves of transgenic plants. When the incubation time was extended, they started feeding on the transgenic plants, but no egg laying was recorded in CPB reared on transgenic plants as compared to control plants. As feeding continued, the mortality was observed. The data revealed significantly variable data for CPB adult mortality after 72 hours of feeding means ranging from 0 to 13.01 percent among the positive transgenic plants of Agria ($P \leq 0.05$). High frequency of CPB adult mortality rate was observed after 336 hours of feeding, where 100 percent mortality was recorded in all transgenic plants of Agria transformed with DS-1 construct. Contrarily, significantly very low mortality (4.53%) was recorded in the control plants after 336 hours of incubation.

Table 4.24. Mortality rate (%Mean ± SEM)* of Adult stage CPB fed on different transgenic potato plants of Agria transformed with DS-1 construct

Construct		DS-1					Control
Plant		AP1	AP2	AP3	AP4	AP5	AgC
Incubation time (hours)	72	13.01±2.71a	4.53±1.14ab	6.70±1.87ab	0.00±0.00b	0.00±0.00b	0.00±0.00b
	96	23.18±2.21a	13.01±2.71a	16.35±2.71a	4.53±1.14ab	4.53±1.14ab	0.00±0.00b
	120	39.84±3.40a	30.00±0.00ab	23.18±2.21bc	16.35±2.71bc	19.99±0.00c	0.00±0.00d
	144	63.55±3.92a	53.35±1.92ab	39.84±3.40bc	30.00±0.00bc	36.59±2.00c	0.00±0.00d
	168	79.99±0.00a	73.47±2.21a	53.35±1.92b	50.00±0.00b	50.00±0.00b	0.00±0.00c
	192	93.30±7.85a	86.98±2.71a	60.14±3.40b	56.68±1.92b	59.99±0.00b	0.00±0.00c
	216	98.85±6.14a	95.47±6.14ab	73.47±2.21bc	67.08±4.22c	66.74±2.00c	0.00±0.00d
	240	100.00±0.00a	98.85±6.14a	83.64±2.71ab	79.99±0.00ab	77.60±4.92b	0.00±0.00c
	264	100.00±0.00a	100.00±0.00a	97.63±8.85a	93.30±7.85a	90.75±8.85a	0.00±0.00b
	288	100.00±0.00a	100.00±0.00a	100.00±0.00a	98.85±6.14a	98.85±6.14a	4.53±1.14b
	312	100.00±0.00a	100.00±0.00a	100.00±0.00a	100.00±0.00a	100.00±0.00a	4.53±1.14b
	336	100.00±0.00a	100.00±0.00a	100.00±0.00a	100.00±0.00a	100.00±0.00a	4.53±1.14b

*Means within column followed by different letters are significantly different at α=0.05, determined using the Tukey test. Mortality data (% Mean± SEM) are means of three replicates.

4.12.5.2 Leaf bioassay of CPB adult fed on Agria plants transformed with DS-2 construct

The percent mortality of CPB adults fed on different transgenic plants of Agria transformed with DS-2 construct and their control plants is shown in Table 4.25. CPB adults analysis showed no mortality after 24 hours of feeding showing slower mortality rate as compared to the larval stages. The adult stage was found to resist eating leaves of transgenic plants. When the incubation time was extended, they started feeding on the transgenic plants, but no egg laying was recorded in CPB reared on transgenic plants as compared to control plants. As feeding continued, the mortality was observed. The data further showed gradual increase in CPB adult mortality with increased in incubation time. A hundred percent adult mortality was recorded on AP1 and AP2 plants of Agria transformed with DS-2 construct followed by AP3, AP4 and AP5 plants where 97.63, 86.98 and 80.69% mortality respectively were recorded after 264 hours of incubation. High rate of CPB adult mortality was observed after 336 hours of feeding, where 100% mortality was recorded in all transgenic plants of Agria transformed with DS-2 construct. Significantly very low mortality (4.53%) was recorded in the control plants after 336 hours feeding.

Table 4.25. Mortality rate (%Mean ± SEM)* of Adult stage CPB fed on different transgenic potato plants of Agria transformed with DS-2 construct

Construct		DS-2					Control
Plant		AP1	AP2	AP3	AP4	AP5	AgC
Incubation time (hours)	72	4.53±1.14a	4.53±1.14a	4.53±1.14a	0.00±0.00a	0.00±0.00a	0.00±0.00a
	96	4.53±	13.01±2.71a	13.01±2.71a	6.70±1.87ab	4.53±1.14ab	0.00±0.00b
	120	16.35±2.71a	26.19±4.22ab	23.18±2.21ab	19.99±0.00b	19.99±0.00b	0.00±0.00c
	144	36.59±2.00a	39.84±3.40ab	43.30±1.92ab	43.30±1.92ab	36.59±2.00b	0.00±0.00c
	168	60.14±3.40a	63.55±3.92ab	66.74±2.00ab	63.40±2.00ab	56.68±1.92b	0.00±0.00c
	192	80.69±4.27a	73.47±2.21ab	73.47±2.21ab	73.47±2.21ab	60.14±3.40b	0.00±0.00c
	216	90.75±8.85a	86.98±2.71a	88.36±10.14a	79.99±0.00a	70.32±3.65a	0.00±0.00b
	240	97.63±8.85a	93.30±7.85a	90.75±8.85a	79.99±0.00a	73.47±2.21a	0.00±0.00b
	264	100.00±0.00a	100.00±0.00a	97.63±8.85ab	86.98±2.71ab	80.69±4.27b	0.00±0.00c
	288	100.00±0.00a	100.00±0.00a	98.85±6.14a	98.85±6.14a	83.64±2.71	4.53±1.14b
	312	100.00±0.00a	100.00±0.00a	98.85±6.14a	100.00±0.00a	93.30±7.85a	4.53±1.14b
	336	100.00±0.00a	100.00±0.00a	100.00±0.00a	100.00±0.00a	100.00±0.00a	4.53±1.14b

*Means within column followed by different letters are significantly different at α=0.05, determined using the Tukey test. Mortality data (% Mean± SEM) are means of three replicates.

4.12.5.3 Leaf bioassay of CPB adult fed on Lady Olympia plants transformed with DS-1 construct

The percent mortality of CPB adults fed on different transgenic plants of Lady Olympia transformed with DS-1 construct and their control plants is shown in Table 4.26. CPB adults analysis showed no mortality after 24 hours of feeding showing slower mortality rate as compared to the larval stages. The adult stage was found to resist eating leaves of transgenic plants. When the incubation time was extended, they started feeding on the transgenic plants, but no egg laying was recorded in CPB reared on transgenic plants as compared to control plants. As feeding continued, the mortality was observed. The data revealed a gradual increase in CPB adult mortality with increased in incubation time. A hundred percent mortality was recorded in all transgenic plants of Lady Olympia transformed with DS-1 construct after 36 hours of feeding. Significantly very low mortality rate (4.53%) was recorded in the control plants after 336 hours feeding.

Table 4.26. Mortality rate (%Mean ± SEM)* of Adult stage CPB fed on different transgenic potato plants of Lady Olympia transformed with DS-1 construct

Construct		DS-1					Control
Plant		LOP1	LOP2	LOP3	LOP4	LOP5	LOC
Incubation time (hours)	72hr	9.99±0.00a	4.53±1.14ab	4.53±1.14ab	0.00±0.00b	0.00±0.00b	0.00±0.00b
	96hr	16.35±2.71a	13.01±2.71a	9.99±0.00a	4.53±1.14a	9.99±0.00ab	0.00±0.00b
	120hr	36.45±3.92a	26.51±2.21ab	19.99±0.00ab	23.18±2.21ab	23.18±2.21b	0.00±0.00c
	144hr	63.40±2.00a	46.63±1.92b	43.30±1.92b	43.16±3.92b	46.63±1.92b	0.00±0.00c
	168hr	79.99±0.00a	66.74±2.00ab	60.14±3.40b	60.14±3.40b	63.40±2.00b	0.00±0.00c
	192hr	86.98±2.71a	77.54±4.92a	69.99±0.00a	70.32±3.65a	69.99±0.00a	0.00±0.00b
	216hr	93.30±7.85a	80.69±4.27a	76.82±2.21a	80.69±4.27a	76.82±2.21a	0.00±0.00b
	240hr	97.63±8.85a	86.98±2.71a	88.38±10.14a	86.98±2.71a	83.64±2.71a	0.00±0.00b
	264hr	100.00±0.00a	97.63±8.85a	93.30±7.85a	97.63±8.85a	89.99±0.00a	0.00±0.00b
	288hr	100.00±0.00a	97.63±8.85a	97.63±8.85a	98.85±6.14a	95.47±6.14a	4.53±1.14b
	312hr	100.00±0.00a	100.00±0.00a	98.85±6.14a	98.85±6.14a	98.85±6.14a	4.53±1.14b
	336hr	100.00±0.00a	100.00±0.00a	100.00±0.00a	100.00±0.00a	100.00±0.00a	4.53±1.14b

*Means within column followed by different letters are significantly different at α=0.05, determined using the Tukey test. Mortality data (% Mean± SEM) are means of three replicates.

4.12.5.4 Leaf bioassay of CPB adult fed on Lady Olympia plants transformed with DS-2 construct

The percent mortality of CPB adults fed on different transgenic plants of Lady Olympia transformed with DS-2 construct and their control plants is shown in Table 4.27. The data revealed significantly variable number of CPB adult mortality data after 72 hours of feeding means ranging from 0 to nearly 10 percent among the positive transgenic plants of Lady Olympia ($P \leq 0.05$). Gradual increase in CPB adult mortality was observed with increased in incubation time. A hundred percent mortality was recorded in all transgenic plants of Lady Olympia transformed with DS-2 construct after 336 hours of incubation. No egg laying was recorded in CPB reared on transgenic plants as compared to control plants. Significantly very low mortality rate (4.53%) was recorded in the control plants after 336 hours feeding.

Table 4.27. Mortality rate (%Mean ± SEM)* of Adult stage CPB fed on different transgenic potato plants of Lady Olympia transformed with DS-2 construct

Construct		DS-2					Control
Plant		LOP1	LOP2	LOP3	LOP4	LOP5	LOC
Incubation time (hours)	72	9.99±0.00a	4.53±1.14ab	4.53±1.14ab	0.00±0.00b	0.00±0.00b	0.00±0.00b
	96	16.35±2.71a	13.01±2.71a	9.99±0.00a	4.53±1.14ab	4.53±1.14ab	0.00±0.00b
	120	36.10±5.44a	26.51±2.21a	23.18±2.21a	23.18±2.21a	23.18±2.21a	0.00±0.00b
	144	58.80±5.39a	46.63±1.92ab	39.84±3.40b	36.59±2.00b	40.00±0.00b	0.00±0.00c
	168	79.99±0.00a	63.55±3.92ab	63.55±3.84ab	63.55±3.84b	59.99±0.00b	0.00±0.00c
	192	90.758.85a	70.32±3.65a	69.99±0.00a	60.14±3.40a	63.40±2.00a	0.00±0.00b
	216	97.63±8.85a	73.80±4.22b	76.82±2.21b	67.08±4.22b	66.74±2.00b	0.00±0.00c
	240	98.85±6.14a	86.04±11.56ab	80.69±4.27ab	73.80±4.22ab	70.32±3.65b	0.00±0.00c
	264	100.00±0.00a	97.63±8.85ab	95.47±6.14ab	79.99±0.00b	76.82±2.21b	0.00±0.00c
	288	100.00±0.00a	100.00±0.00a	98.85±6.14ab	83.64±2.71b	83.64±2.71b	4.53±1.14c
	312	100.00±0.00a	100.00±0.00a	100.00±0.00a	86.98±2.71b	86.98±2.71b	4.53±1.14c
	336	100.00±0.00a	100.00±0.00a	100.00±0.00a	100.00±0.00a	100.00±0.00a	4.53±1.14b

*Means within column followed by different letters are significantly different at α=0.05, determined using the Tukey test. Mortality data (% Mean± SEM) are means of three replicates.

4.13 Foliage Consumption of CPB on Transgenic Plants

4.13.1 Foliage consumption by CPB 1st ınstar larva

4.13.1.1 Foliage consumption by CPB 1st ınstar larva fed per day on potato cultivar Agria transformed with DS-1 construct

Foliage consumption by individual CPB 1st instar larva fed per day on different transgenic plants of Agria transformed with DS-1 construct and their control plants are shown in Figure 4.39. The data showed significant differences in the feeding behavior of 1st instars larvae that were fed on different transgenic plants as compared to non-transgenic plants ($P \leq 0.05$). Significantly higher leaf area consumption was recorded in Agria control plants where 0.88 cm^2 mean leaf area consumption per day was recorded. The transgenic plants DS-1AP1 and DS-1AP2 of Agria caused significance reduction in foliage consumption of CPB 1st instar larvae with consumption of only 0.73 and 0.75 cm^2 which was significantly lower than the control plants. The data showed no significant difference amongst the transgenic plants in terms of leaf area consumption.

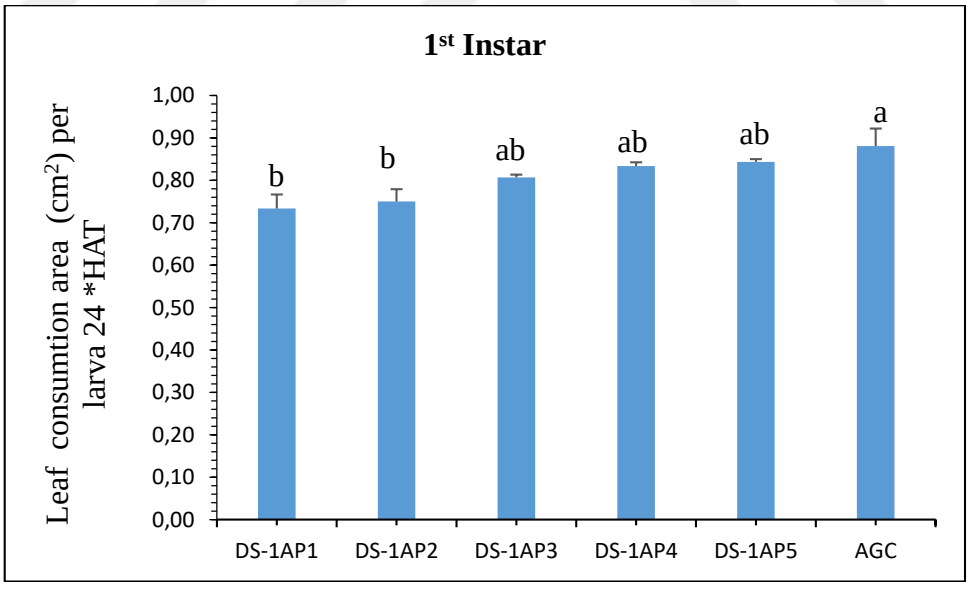


Figure 4.39. Foliage consumption by individual CPB 1st instar larva fed per day on different transgenic potato plants of Agria transformed with DS-1 construct
Each data point denotes the means of three replicates
*HAT, Hours after treatment

4.13.1.2 Foliage consumption by CPB 1st ınstar larva fed per day on potato cultivar Agria transformed with DS-2 construct

Foliage consumption by individual CPB 1st instar larva fed per day on different transgenic plants of Agria transformed with DS-2 construct and their control plants are shown in Figure 4.40. The data showed significant differences in the feeding behavior of 1st instars larvae that were fed on different transgenic plants as compared to non-transgenic plants ($P \leq 0.05$). High foliage consumption area was recorded in the Lady Olympia control plant (0.88 cm^2), while low mean of leaf consumed area (0.75 cm^2) was observed in DS-2AP1 plant of Agria. This was followed by other transgenic plants where mean leaf consumed area per larva after 24 hours were in the range from 0.78 to 0.86 cm^2.

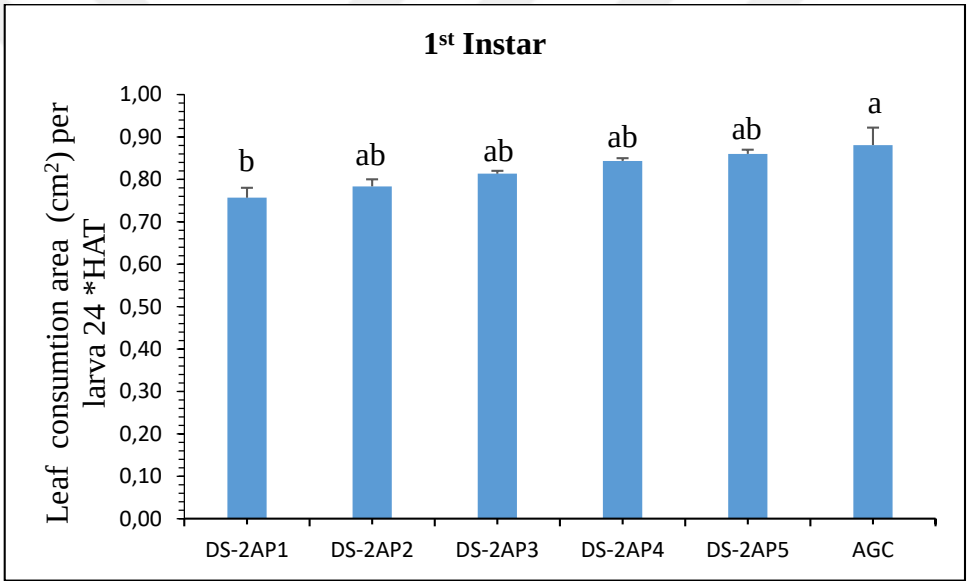


Figure 4.40. Foliage consumption by individual CPB 1st instar larva fed per day on different transgenic potato plants of Agria transformed with DS-2 construct
Each data point denotes the means of three replicates
*HAT, Hours after treatment

4.13.1.3 Foliage consumption by CPB 1st ınstar larva fed per day on potato cultivar Lady Olympia transformed with DS-1 construct

Foliage consumption by individual CPB 1st instar larva fed per day on different transgenic plants of Lady Olympia transformed with DS-1 construct and their control plants are shown in Figure 4.41. The data showed significant differences in the feeding behavior of 1st instars larvae that were fed on DS-1LOP1 plant as compared to non-transgenic plants

($P \leq 0.05$). Higher leaf area consumption in Lady Olympia control plants was 1.08 cm^2 while significantly lower 0.88 $cm^2$was recorded on DS-1LOP1 after 24 hours. The data further showed no significant differences amongst the transgenic plants in terms of leaf area consumption.

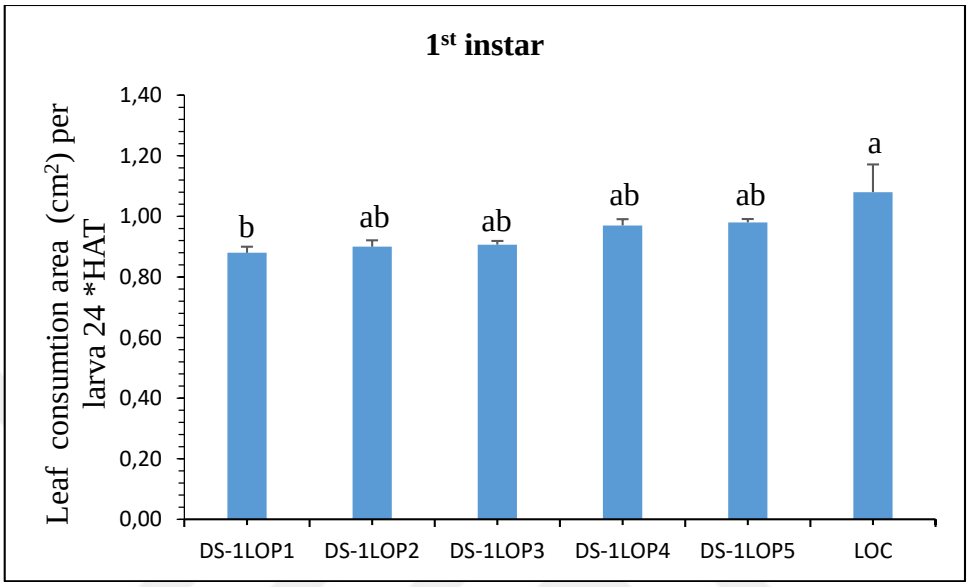


Figure 4.41. Foliage consumption by individual CPB 1st instar larva fed per day on different transgenic potato plants of Lady Olympia transformed with DS-1 construct Each data point denotes the means of three replicates

*HAT, Hours after treatment

4.13.1.4 Foliage consumption by CPB 1st ınstar larva fed per day on potato cultivar Lady Olympia transformed with DS-2 construct

Foliage consumption by individual CPB 1st instar larva fed per day on different transgenic plants of Lady Olympia transformed with DS-2 construct and their control plants are shown in Figure 4.42. Higher leaf area consumption of 1.08 cm^2 was recorded in Lady Olympia control plants. The transgenic plants DS-2LOP1 of Lady Olympia caused significance reduction to the CPB 1st instar larva with consumption area of only 0.90 cm^2 which was significantly lower than the control plants. The data also showed no significant difference amongst the transgenic plants in terms of leaf area consumption.

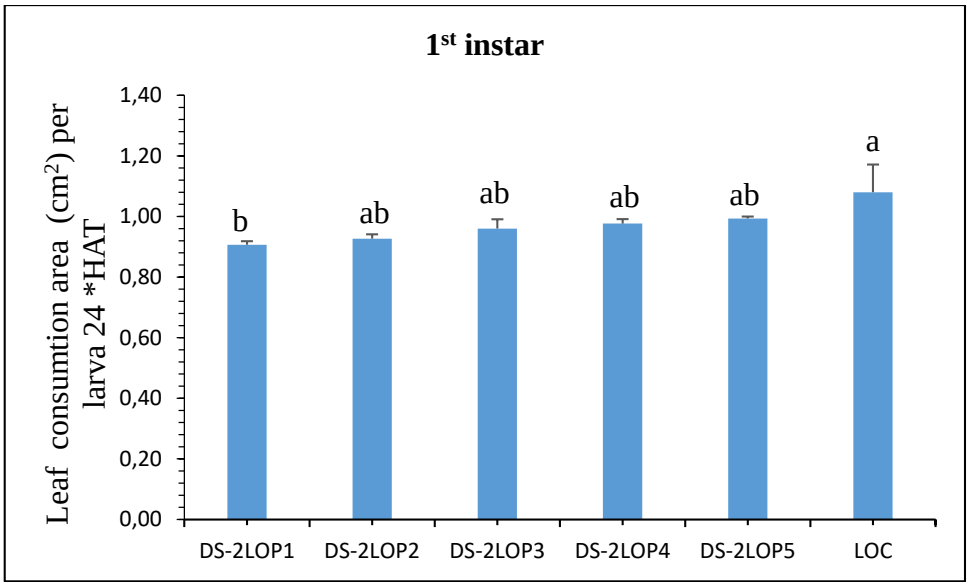


Figure 4.42. Foliage consumption by individual CPB 1st instar larva fed per day on different transgenic potato plants of Lady Olympia transformed with DS-2 construct Each data point denotes the means of three replicates

*HAT, Hours after treatment

4.13.2 Foliage consumption by CPB 2nd ınstar larva

4.13.2.1 Foliage consumption by CPB 2nd ınstar larva fed per day on potato cultivar Agria transformed with DS-1 construct

Foliage consumption by individual CPB 2nd instar larva fed per day on different transgenic plants of Agria transformed with DS-1 construct and their control plants are shown in Figure 4.43. The data showed significant differences in the feeding behavior of 2nd instars larvae that were fed on different transgenic plants as compared to non-transgenic plants ($P \leq 0.05$). Significantly higher leaf area consumption of 1.96 cm^2 was recorded in Agria control plants after 24 hours of feeding. The data revealed no significant difference in terms of leaf area consumption by CPB 2nd instar larva amongst the transgenic plants of Agria transformed with DS-1 construct after 24 hours of feeding.

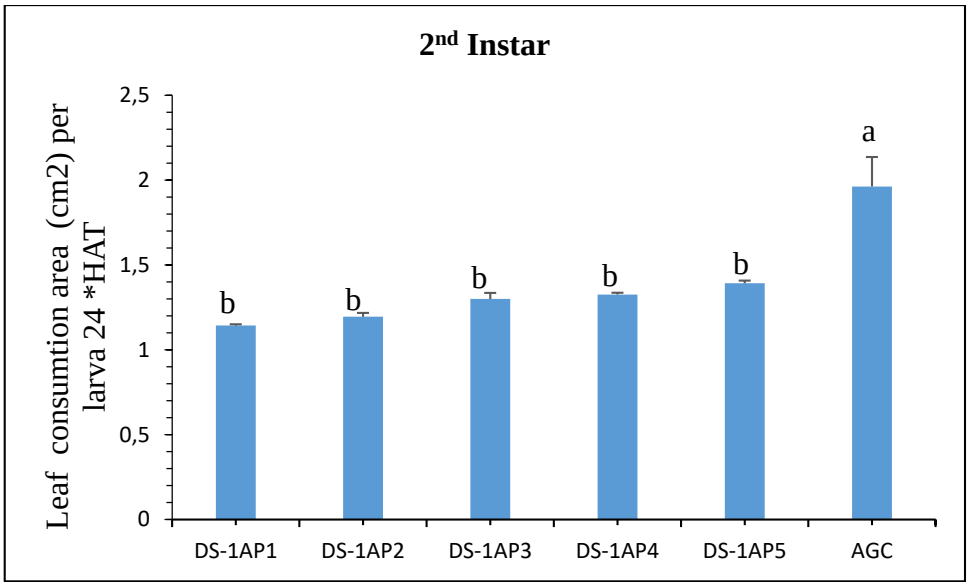


Figure 4.43. Foliage consumption by individual CPB 2nd instar larva fed per day on different transgenic potato plants of Agria transformed with DS-1 construct
Each data point denotes the means of three replicates
*HAT, Hours after treatment

4.13.2.2 Foliage consumption by CPB 2nd ınstar larva fed per day on potato cultivar Agria transformed with DS-2 construct

Foliage consumption by individual CPB 2nd instar larva fed per day on different transgenic plants of Agria transformed with DS-2 construct and their control plants are shown in Figure 4.44. The data showed significant differences in the feeding behavior of 2nd instars larvae that were fed on different transgenic plants of Agria as compared to non-transgenic plants ($P \leq 0.05$). Significantly higher leaf area consumption of 1.96 cm^2 was recorded in Agria control plants after 24 hours of feeding. Leaf consumption area by CPB 2nd instar larva was in the range from 1.23 to 1.44 cm^2 and there was no significant difference in terms of leaf area consumption amongst the transgenic plants of Agria transformed with DS-2 construct.

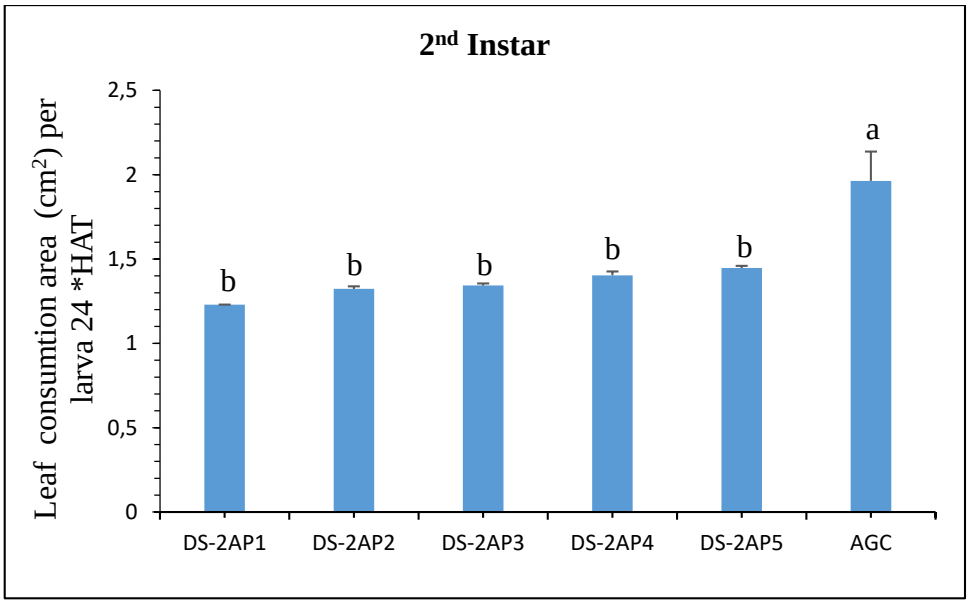


Figure 4.44. Foliage consumption by individual CPB 2nd instar larva fed per day on different transgenic potato plants of Agria transformed with DS-2 construct
Each data point denotes the means of three replicates
*HAT, Hours after treatment

4.13.2.3 Foliage consumption by CPB 2nd ınstar larva fed per day on potato cultivar Lady Olympia transformed with DS-1 construct

Foliage consumption by individual CPB 2nd instar larva fed per day on different transgenic plants of Lady Olympia transformed with DS-1 construct and their control plants are shown in Figure 4.45. Significant differences were observed in the feeding behavior of 2nd instars larvae that were fed on different transgenic plants as compared to control plants ($P \leq 0.05$). Significantly higher leaf consumption of 2.39 $cm^2$was recorded in the Lady Olympia control plants after 24 hours of feeding. The leaf consumption area by CPB 2nd instar larva in different transgenic plants of Lady Olympia were in the range from 1.25 to 1.41 cm^2 and there were no significant differences in terms of leaf area consumption amongst the transgenic plants of Lady Olympia transformed with DS-1 construct.

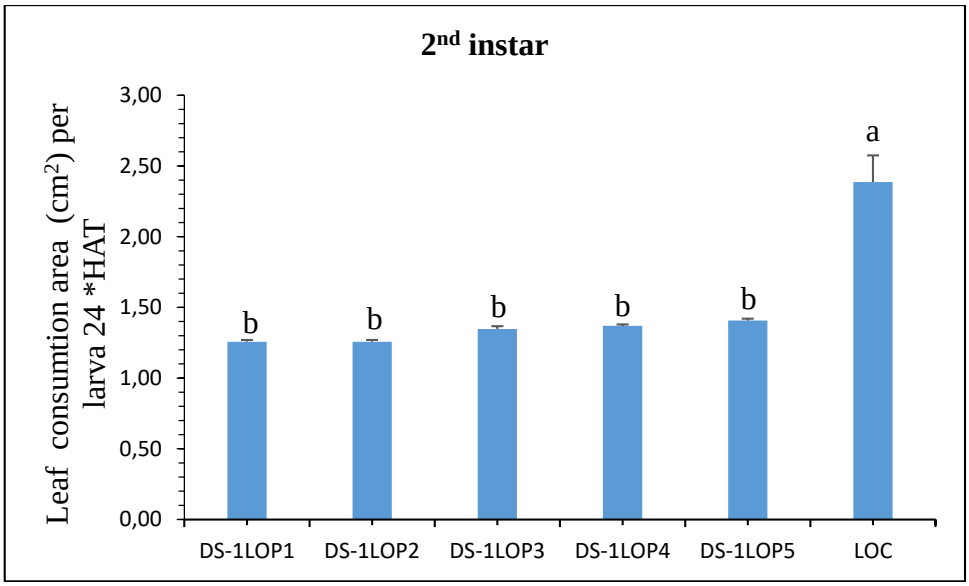


Figure 4.45. Foliage consumption by individual CPB 2nd instar larva fed per day on different transgenic potato plants of Lady Olympia transformed with DS-1 construct
Each data point denotes the means of three replicates
*HAT, Hours after treatment

4.13.2.4 Foliage consumption by CPB 2nd ınstar larva fed per day on potato cultivar Lady Olympia transformed with DS-2 construct

Foliage consumption by individual CPB 2nd instar larva fed per day on different transgenic plants of Lady Olympia transformed with DS-2 construct and their control plants are shown in Figure 4.46. The data showed significant differences in the feeding behavior of 2nd instars larvae that were fed on different transgenic plants of Lady Olympia as compared to non-transgenic plants ($P \leq 0.05$). Significantly higher leaf area consumption area of 2.38 $cm^2$was recorded in control plants after 24 hours of feeding. The leaf consumption area of transgenic plants of Lady Olympia transformed with DS-2 construct by CPB 2nd instar larva per day were in the range from 1.27 to 1.46 cm^2 after 24 hours of feeding and there was no significant difference in terms of leaf area consumption amongst the transgenic plants of Lady Olympia transformed with DS-2 construct.

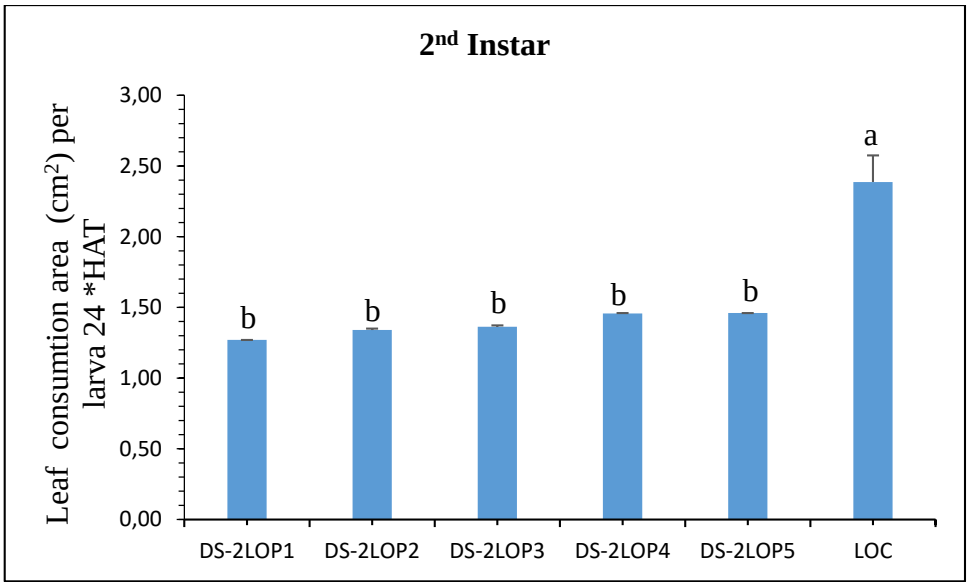


Figure 4.46. Foliage consumption by individual CPB 2nd instar larva fed per day on different transgenic potato plants of Lady Olympia transformed with DS-2 construct Each data point denotes the means of three replicates

*HAT, Hours after treatment

4.13.3 Foliage consumption by CPB 3rd ınstar larva

4.13.3.1 Foliage consumption by CPB 3rd ınstar larva fed per day on potato cultivar Agria transformed with DS-1 construct

Foliage consumption by individual CPB 3rd instar larva fed per day on different transgenic plants of Agria transformed with DS-1 construct and their control plants are shown in Figure 4.47. The data showed variable significant difference in the feeding behavior of 3rd instars larvae that were fed on different transgenic and non-transgenic plants of Agria ($P \leq 0.05$). Due to its bigger size, CPB 3rd instar consume more foliage as compared to the early 1st and 2nd larval instars. Significantly higher leaf consumption area of 4.29 cm^2 was recorded in control by CPB 3rd instar larva after 24 hours of feeding. Significantly lower leaf consumption area of 2.22 cm^2 was recorded in DS-1AP1 plants of Agria, this was followed by DS-1AP2 where 2.56 cm^2 leaf consumption area by CPB 3rd instar larva per day was recorded. The leaf consumption area per day per 3rd instar larva in plants DS-1AP3, DS-1AP4 and DS-1AP5 of Agria were 2.67, 2.78 and 3 cm^2 respectively (Figure 4.47).

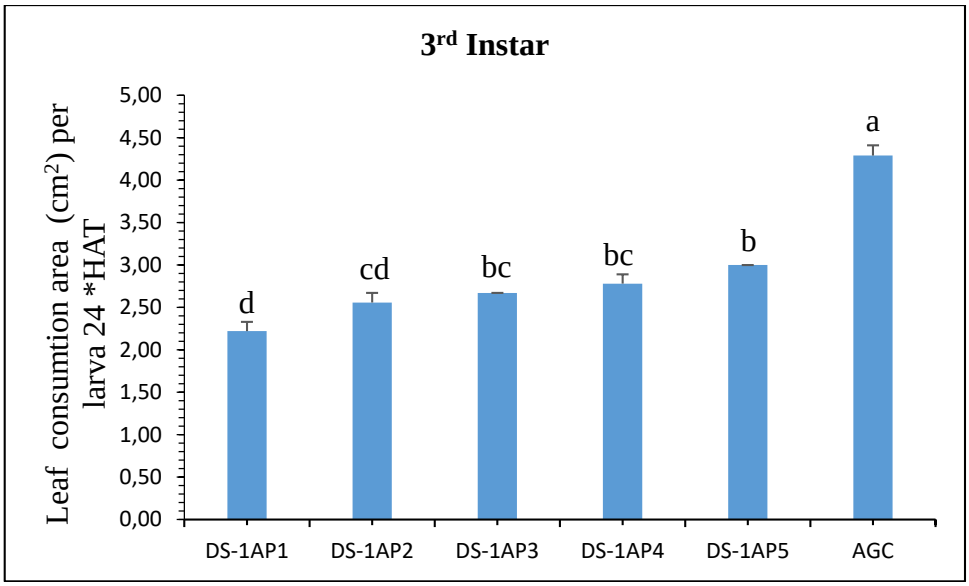


Figure 4.47. Foliage consumption by individual CPB 3rd instar larva fed per day on different transgenic potato plants of Agria transformed with DS-1 construct
Each data point denotes the means of three replicates
*HAT, Hours after treatment

4.13.3.2 Foliage consumption by CPB 3rd ınstar larva fed per day on potato cultivar Agria transformed with DS-2 construct

Foliage consumption by individual CPB 3rd instar larva fed per day on different transgenic plants of Agria transformed with DS-2 construct and their control plants are shown in Figure 4.48. Significant difference was observed in the feeding behavior of 3rd instars larvae that were fed on different transgenic and non-transgenic plants of Agria ($P \leq 0.05$). Significantly higher leaf consumption area of 4.29 cm^2 was recorded in control by CPB 3rd instar larva after 24 hours of feeding. Significantly lower leaf consumption area of 2.33, 2.33 and 2.36 cm^2 was recorded in DS-2AP1, DS-2AP2 and DS-2AP3 plants of Agria which were significantly lower than the leaf consumption area of DS-2AP4 and DS-2AP5, where significantly higher leaf consumption area of 2.78 and 3.13 cm^2 respectively were recorded after 24 hours of feeding (Figure 4.48).

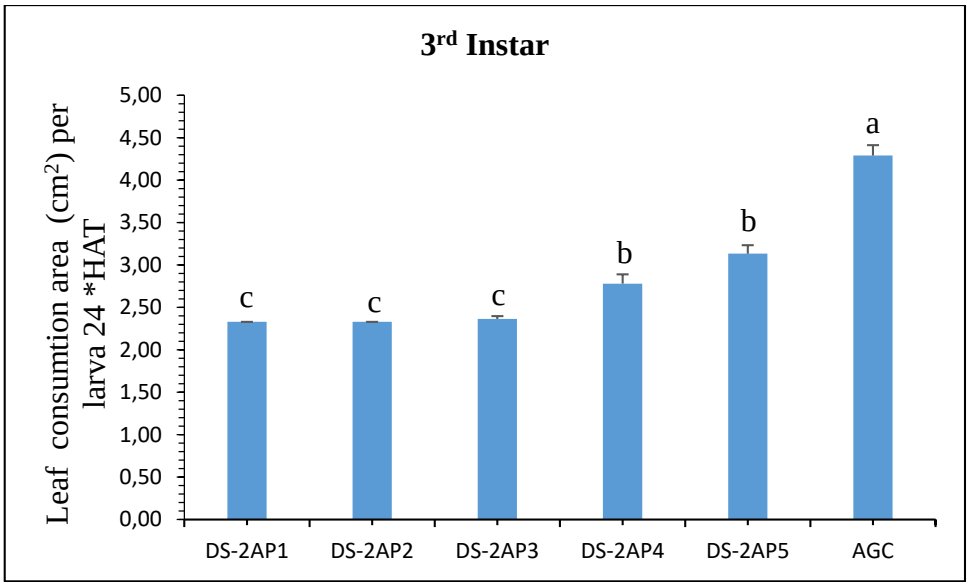


Figure 4.48. Foliage consumption by individual CPB 3rd instar larva fed per day on different transgenic potato plants of Agria transformed with DS-2 construct
Each data point denotes the means of three replicates
*HAT, Hours after treatment

4.13.3.3 Foliage consumption by CPB 3rd ınstar larva fed per day on potato cultivar Lady Olympia transformed with DS-1 construct

Foliage consumption by individual CPB 3rd instar larva fed per day on different transgenic plants of Lady Olympia transformed with DS-1 construct and their control plants are shown in Figure 4.49. The data revealed variable significant differences in the feeding behavior of 3rd instars larvae that were fed on different transgenic and non-transgenic plants of Lady Olympia ($P \leq 0.05$). Significantly higher leaf consumption area of 5.21 cm^2 was recorded in control by CPB 3rd instar larva after 24 hours of feeding. Lower leaf consumption area of 2.22 cm^2 was recorded in DS-1LOP1 plants of Lady Olympia, this was followed by DS-1LOP2, DS-1LOP3 and DS-1LOP4 where 2.67, 2.67 and 2.89 cm^2 leaf consumption area by CPB 3rd instar larva was recorded after 24 hours of feeding. No significant difference was observed among the transgenic plants in terms of leaf area consumption after 24 hours of feeding (Figure 4.49).

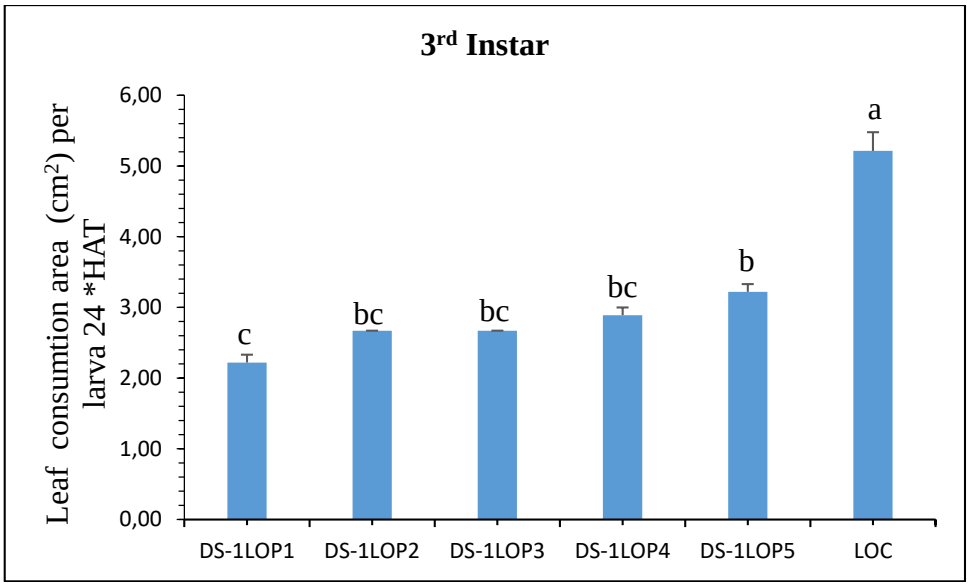


Figure 4.49. Foliage consumption by individual CPB 3rd instar larva fed per day on different transgenic potato plants of Lady Olympia transformed with DS-1 construct Each data point denotes the means of three replicates

*HAT, Hours after treatment

4.13.3.4 Foliage consumption by CPB 3rd ınstar larva fed per day on potato cultivar Lady Olympia transformed with DS-2 construct

Foliage consumption by individual CPB 3rd instar larva fed per day on different transgenic plants of Lady Olympia transformed with DS-2 construct and their control plants are shown in Figure 4.50. The data showed variable significant differences in the feeding behavior of 3rd instars larvae that were fed on different transgenic and non-transgenic plants of Lady Olympia ($P \leq 0.05$). Significantly higher leaf consumption area of 5.21 cm^2 by CPB 3rd instar larva was recorded in control plant after 24 hours of feeding. Lower leaf area consumption area of 2.44 cm^2 was recorded in DS-2LOP1 plant after 24 hours of feeding. The leaf consumption area in rest of the transgenic plants were in the range of 2.89 cm^2 in DS-2LOP2 plant to 3.78 cm^2 in DS-2LOP5 plant (Figure 4.50).

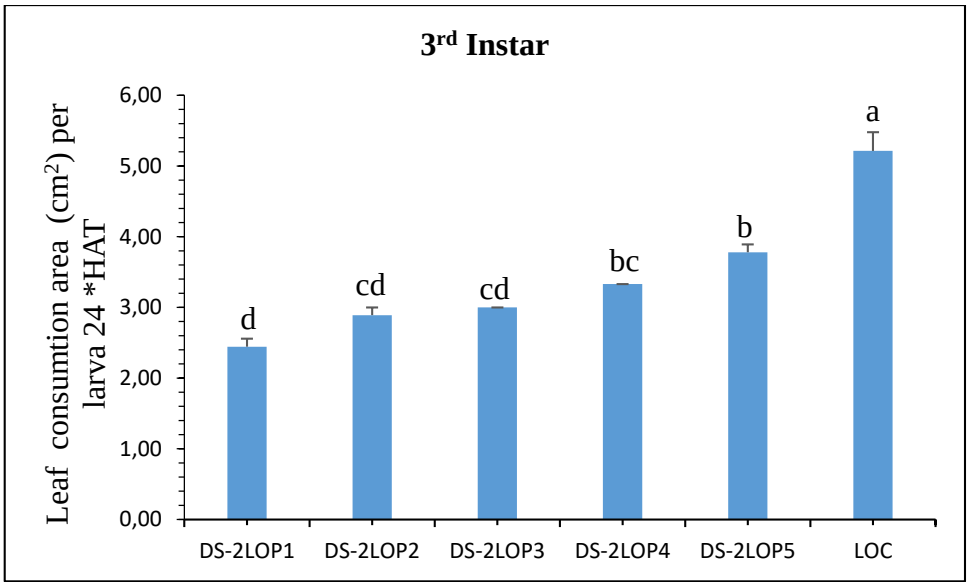


Figure 4.50. Foliage consumption by individual CPB 3rd instar larva fed per day on different transgenic potato plants of Lady Olympia transformed with DS-2 construct Each data point denotes the means of three replicates

*HAT, Hours after treatment

4.13.4 Foliage consumption by CPB 4th ınstar larva

4.13.4.1 Foliage consumption by CPB 4th ınstar larva fed per day on potato cultivar Agria transformed with DS-1 construct

Foliage consumption by individual CPB 4th instar larva fed per day on different transgenic plants of Agria transformed with DS-1 construct and their control plants are shown in Figure 4.51. The data revealed variable significant difference in the feeding behavior of 4th instars larvae that were fed on different transgenic and non-transgenic plants of Agria ($P \leq 0.05$). Significantly higher leaf consumption area of 8.55 cm^2 by CPB 4th instar larva was recorded in control plant after 24 hours of feeding. This was followed by significantly lower leaf consumption area of 5.08 and 5 cm^2 in DS-1AP5 and DS-1AP4 plants of Agria respectively, while significantly lower leaf consumption area by CPB 4th instar larva was recorded in DS-1AP1 and DS-1AP2 plants where only 4.03 and 4.25 cm^2 leaf consumption area was recorded (Figure 4.51).

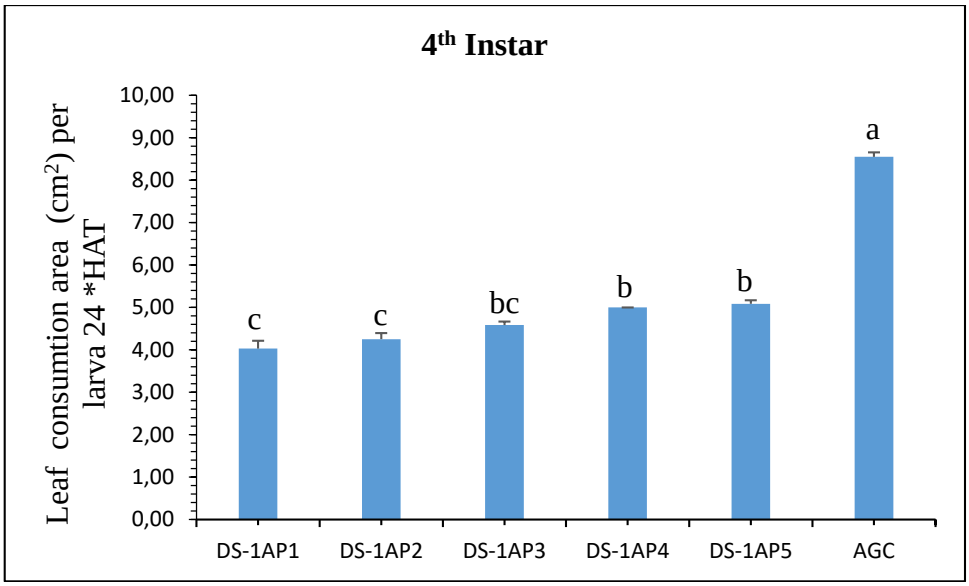


Figure 4.51. Foliage consumption by individual CPB 4th instar larva fed per day on different transgenic potato plants of Agria transformed with DS-1 construct
Each data point denotes the means of three replicates
*HAT, Hours after treatment

4.13.4.2 Foliage consumption by CPB 4th ınstar larva fed per day on potato cultivar Agria transformed with DS-2 construct

Foliage consumption by individual CPB 4th instar larva fed per day on different transgenic plants of Agria transformed with DS-2 construct and their control plants are shown in Figure 4.52. The data showed variable significant difference in the feeding behavior of 4th instars larvae that were fed on different transgenic and non-transgenic plants of Agria ($P \leq 0.05$). Significantly higher leaf consumption area of 8.55 cm^2 by CPB 4th instar larva was recorded in control plant after 24 hours of feeding. This was followed by significantly lower leaf consumption area of 5.67 and 5.08 cm^2 in DS-2AP5 and DS-2AP4 plants of Agria respectively, while significantly lower leaf consumption area by CPB 4th instar larva was recorded in DS-2AP1 and DS-2AP2 plants where only 4.33 and 4.58 cm^2 leaf consumption area were recorded after 24 hours of feeding (Figure 4.52).

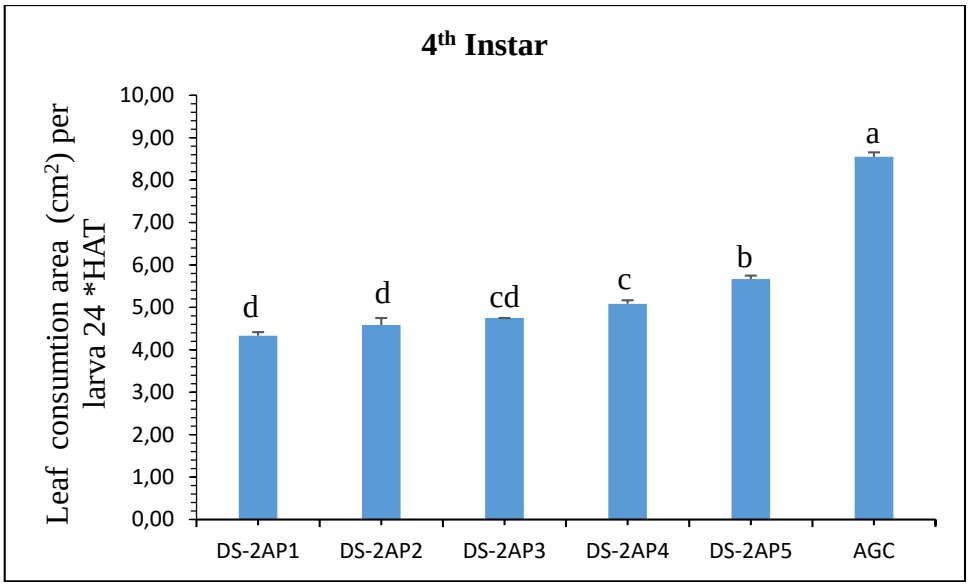


Figure 4.52. Foliage consumption by individual CPB 4th instar larva fed per day on different transgenic potato plants of Agria transformed with DS-2 construct
Each data point denotes the means of three replicates
*HAT, Hours after treatment

4.13.4.3 Foliage consumption by CPB 4th ınstar larva fed per day on potato cultivar Lady Olympia transformed with DS-1 construct

Foliage consumption by individual CPB 4th instar larva fed per day on different transgenic plants of Lady Olympia transformed with DS-1 construct and their control plants are shown in Figure 4.53. The data displayed variable significant difference in the feeding behavior of 4th instars larvae that were fed on different transgenic and non-transgenic plants of Lady Olympia ($P \leq 0.05$). Significantly higher leaf consumption area of 9.64 cm^2 by CPB 4th instar larva was recorded in control plant after 24 hours of feeding. This was followed by significantly lower leaf consumption area of 5.32 and 5 cm^2 in DS-1LOP5 and DS-1LOP4 plants of Lady Olympia respectively, while significantly lower leaf consumption area by CPB 4th instar larva was recorded in DS-1LOP1 and DS-1LOP2 plants where only 4.25 cm^2 leaf consumption area were recorded in both the plants which were significantly lower than the rest of all transgenic and non-transgenic control plants (Figure 4.53).

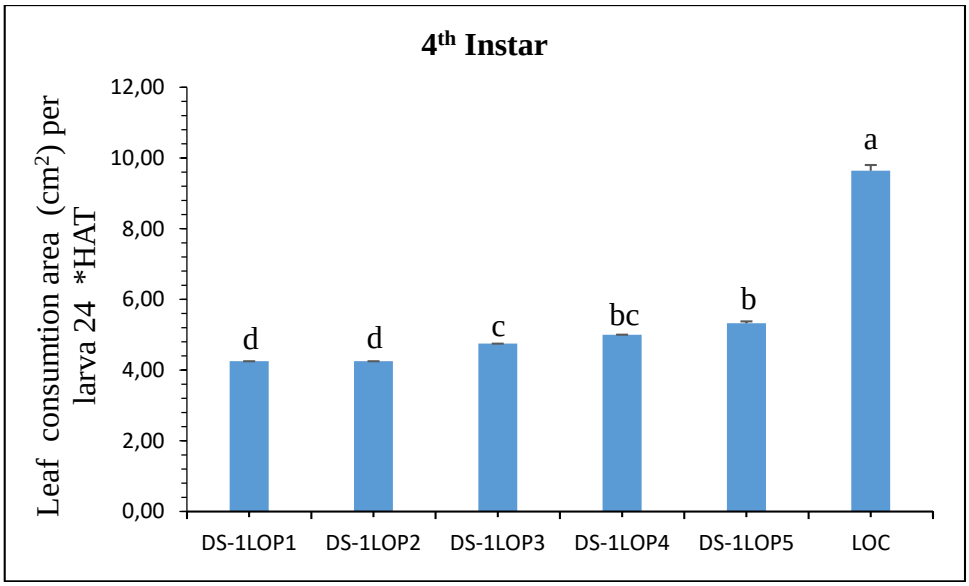


Figure 4.53. Foliage consumption by individual CPB 4th instar larva fed per day on different transgenic potato plants of Lady Olympia transformed with DS-1 construct Each data point denotes the means of three replicates

*HAT, Hours after treatment

4.13.4.4 Foliage consumption by CPB 4th ınstar larva fed per day on potato cultivar Lady Olympia transformed with DS-2 construct

Foliage consumption by individual CPB 4th instar larva fed per day on different transgenic plants of Lady Olympia transformed with DS-2 construct and their control plants are shown in Figure 4.54. The data revealed variable significant difference in the feeding behavior of 4th instars larvae that were fed on different transgenic and non-transgenic plants of Lady Olympia ($P \leq 0.05$). Significantly higher leaf consumption area of 9.64 cm^2 by CPB 4th instar larva per day was recorded in control plant. This was followed by significantly lower leaf consumption area of 5.39 and 5.92 cm^2 in DS-2LOP5 and DS-2LOP4 plants of Lady Olympia respectively, while significantly lower leaf consumption area by CPB 4th instar larva was recorded in DS-2LOP1 and DS-2LOP2 plants where only 4.31 and 4.36 cm^2 leaf consumption area were recorded respectively (Figure 4.54).

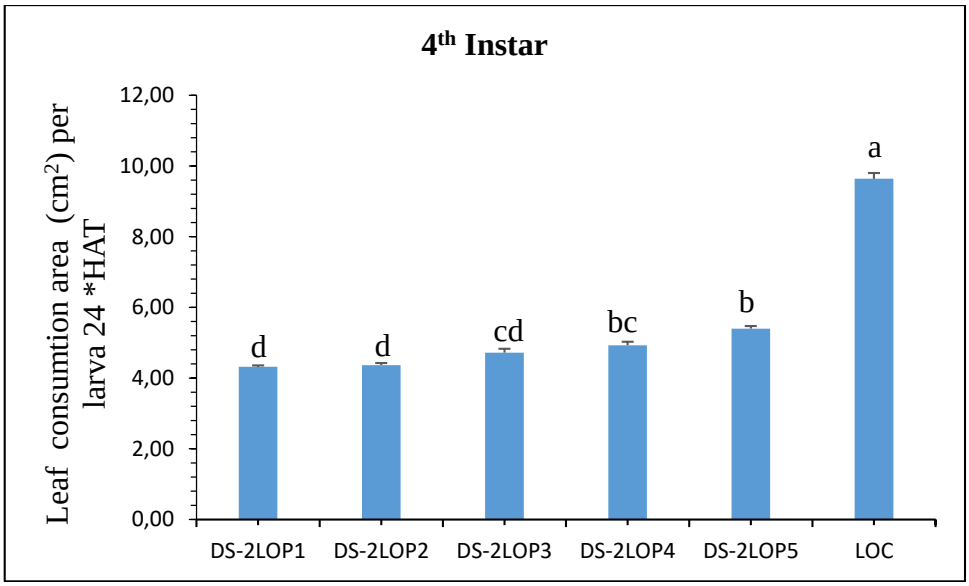


Figure 4.54. Foliage consumption by individual CPB 4th instar larva fed per day on different transgenic potato plants of Lady Olympia transformed with DS-2 construct Each data point denotes the means of three replicates

*HAT, Hours after treatment

4.13.5 Foliage consumption by CPB adults

4.13.5.1 Foliage consumption by CPB adult beetle fed for 3 days on potato cultivar Agria transformed with DS-1 construct

Foliage consumption by individual CPB adult beetle fed per on different transgenic plants of Agria transformed with DS-1 construct and their control plants are shown in Figure 4.55. The data showed variable significant difference in the feeding behavior of CPB adult beetles that were fed on different transgenic and non-transgenic plants of Agria ($P \leq 0.05$). The adult beetles were seen reluctant to feed on the foliage of all the transgenic plants of Agria as compared to their respective control plants. Significantly higher leaf consumption area of 15.97 cm^2 was recorded in Agria control plants, this was followed by DS-1A5 where 8.46 cm^2 leaf consumption area was recorded. Among the transgenic plants, DS-1A1caused significance reduction to foliage consumption by CPB adult beetle and resulted in significantly lower leaf area consumption of about 4.45 cm^2. This was followed by DS-1A2 and DS-1A3 where leaf consumption area of about 6.63 and 6.88 cm^2 was recorded (Figure 4.55).

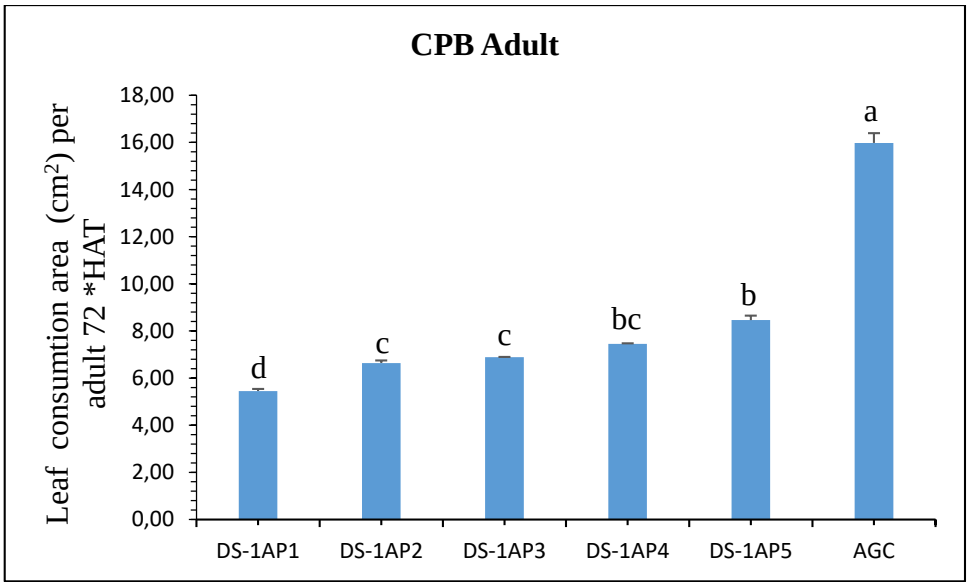


Figure 4.55. Foliage consumption by individual CPB adult fed per 3 days on different transgenic potato plants of Agria transformed with DS-1 construct
Each data point denotes the means of three replicates of 3 days
*HAT, Hours after treatment

4.13.5.2 Foliage consumption by CPB adult beetle fed for 3 days on potato cultivar Agria transformed with DS-2 construct

Foliage consumption by individual CPB adult beetle fed per day on different transgenic plants of Agria transformed with DS-2 construct and their control plants are shown in Figure 4.56. The data showed variable significant differences in the feeding behavior of CPB adult beetles that were fed on different transgenic and non-transgenic plants of Agria ($P \leq 0.05$). Significantly higher leaf consumption area of 15.97 cm^2 was recorded in Agria control plants. Among the transgenic plants, mean leaf consumption area per CPB adult were in the range from 6.06 cm^2 in DS-1A1 to 8.72 cm^2 in DS-2A5 after 48 hours of feeding (Figure 4.56).

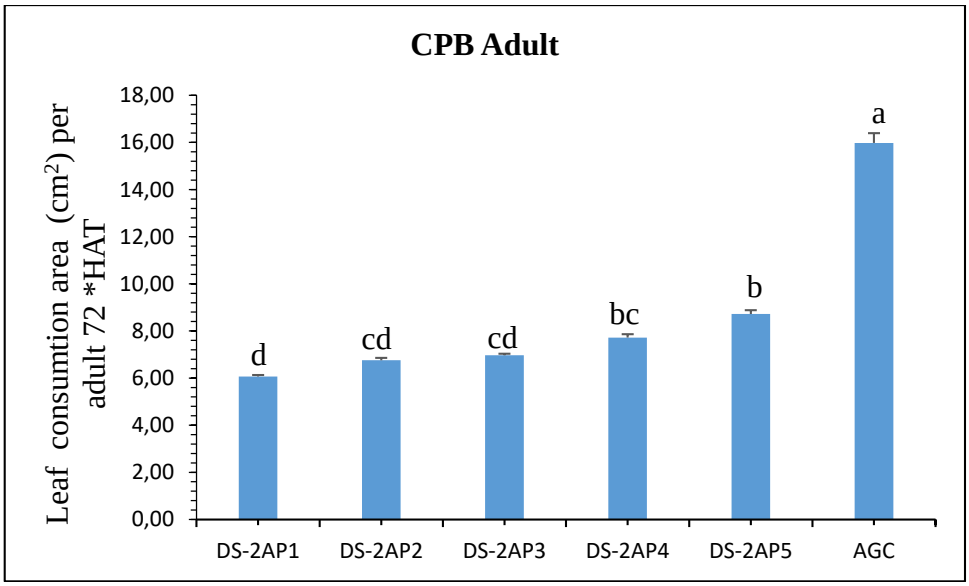


Figure 4.56. Foliage consumption by individual CPB adult fed per 3 days on different transgenic potato plants of Agria transformed with DS-2 construct
Each data point denotes the means of three replicates of 3 days
*HAT, Hours after treatment

4.13.5.3 Foliage consumption by CPB adult beetle fed for 3 days on potato cultivar Lady Olympia transformed with DS-1 construct

Foliage consumption by individual CPB adult beetle fed on different transgenic plants of Lady Olympia transformed with DS-1 construct and their control plants are shown in Figure 4.57. The data showed variable significant differences in the feeding behavior of CPB adult beetles that were fed on different transgenic and non-transgenic plants of Lady Olympia ($P \leq 0.05$). Significantly higher leaf consumption area of 18.45 cm^2 was recorded in Lady Olympia control plants, this was followed by significantly lower leaf consumption area in DS-1A5 where 8.58 cm^2 leaf consumption area was recorded. Among the transgenic plants, DS-1LO1 caused significance reduction in foliage consumption by CPB adult beetle and resulted in significantly lower leaf area consumption of about 6.76 cm^2 after 48 hours of feeding. This was followed by DS-1LO2 and DS-1LO3 where mean leaf consumption area of about 7.39 and 7.90 cm^2 was recorded (Figure 4.57).

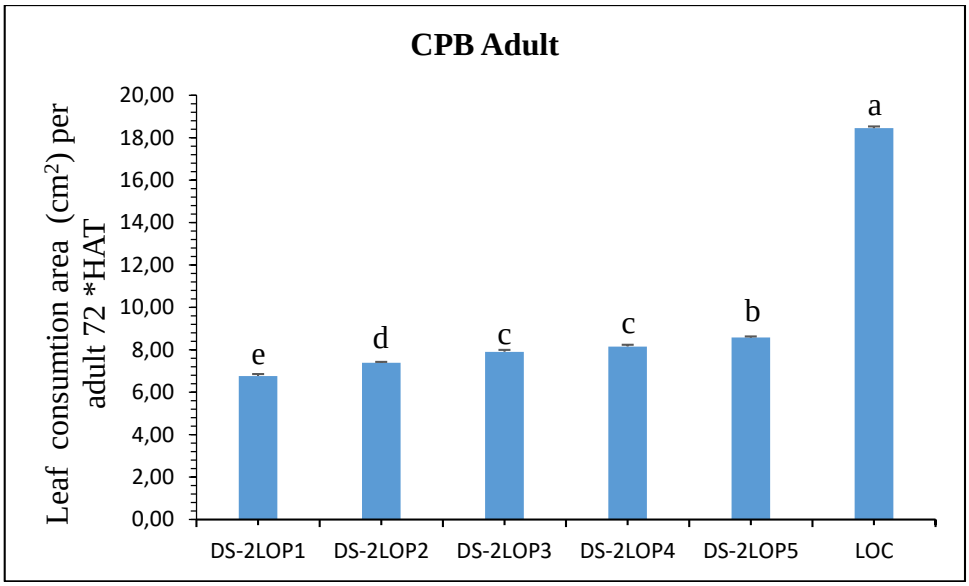


Figure 4.57. Foliage consumption by individual CPB adult fed per 3 days on different transgenic potato plants of Lady Olympia transformed with DS-1 construct
Each data point denotes the means of three replicates of 3 days
*HAT, Hours after treatment

4.13.5.4 Foliage consumption by CPB adult beetle fed for 3 days on potato cultivar Lady Olympia transformed with DS-2 construct

Foliage consumption by individual CPB adult beetle fed per day on different transgenic plants of Lady Olympia transformed with DS-2 construct and their control plants are shown in Figure 4.58. The data showed variable significant differences in the feeding behavior of CPB adult beetles that were fed on different transgenic and non-transgenic plants of Lady Olympia ($P \leq 0.05$). Significantly higher leaf consumption area of 18.45 cm^2 was recorded in Lady Olympia control plants. Among the transgenic plants, mean leaf consumption area per CPB adult were in the range from 6.06 cm^2 in DS-1LO1 to 8.59 cm^2 in DS-2LO5 which was significantly higher than the rest of the transgenic plants except DS-1LO2 (Figure 4.58).

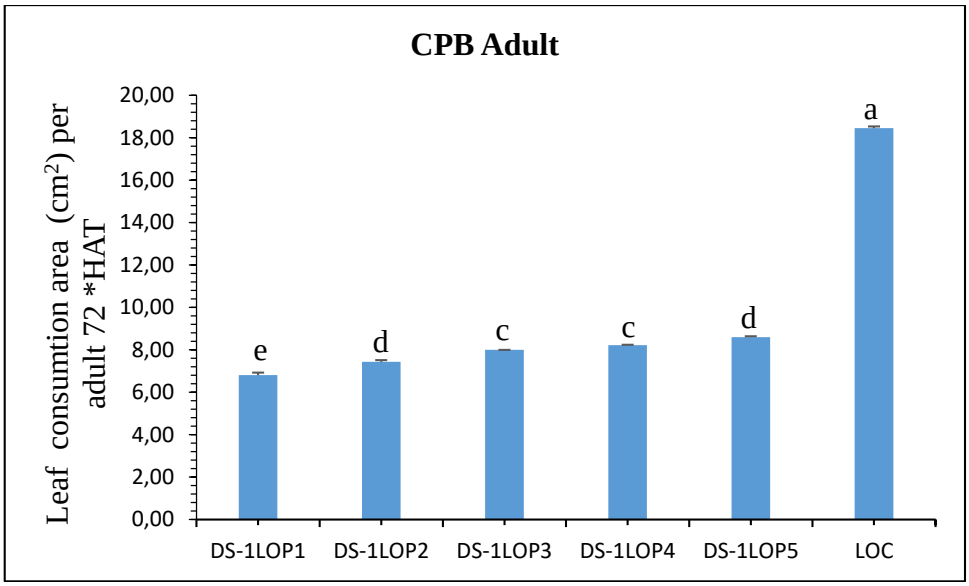


Figure 4.58. Foliage consumption by individual CPB adult fed per 3 days on different transgenic potato plants of Lady Olympia transformed with DS-2 construct
Each data point denotes the means of three replicates of 3 days
*HAT, Hours after treatment

CHAPTER V

DISCUSSION

Currently plant transformation with genes for resistance against insect pest species depends on various genes including those from *Bt* and PIs. In the present study, potato cultivars Agria and Lady Olympia were co-transformed with one *SN19* and *cry3A* genes (DS-1 plasmid) and *SN19* and *OCII* genes (DS-2 plasmid), each cultivar was co-transformed with two constructs i.e. DS-1 and DS-2 constructs using *Agrobacterium*-mediated transformation technique as suggested by De Block et al. (1988) and Beaujean et al. (1998) with some modifications. *Agrobacterium tumefaciens* strain *GV3101* was used for transformation of DS-1 and DS-2 constructs for genetic transformation of potato cultivars. After co-transformation, the platelets were shifted to MS medium supplemented with different growth hormones. This media was also optimized for *hygromycin* concentrations. Overall transformation efficiency in our experiments was calculated as 3.15 %. Many scientists have reported different data regarding potato transformation efficiency in their studies while using different explants (Veale et al., 2012; Soto et al., 2007; Hameed et al. 2017). Halpin (2005) documented different methods of gene pyramiding strategies and found that co-transformation method is one of the best methodologies employed for pyramiding of multiple foreign genes. There are many studies showing the effects of the transformation conditions on the co-transformation and/or co-integration. McCormac et al. (2001) reported 100 % co-transformation frequencies in tobacco using two T-DNA. Successful pyramiding of genes through co-transformation methods have been reported in many GM maize, rice varieties as well as some cruciferous crops (Ye et al., 2000; Maqbool et al., 2001; Halpin, 2005).

The primary transformants developed out of both cultivars (Agria and Lady Olympia) were analyzed for integrative (stable) transformation of the insecticidal genes within T-DNA region through PCR, ELISA, Southern blotting and leaf bioassays with CPB larval and adult stages. PCR analysis of the primary transformants with different specific primers confirmed the presence of plant selectable marker gene "*npt* II" in host genome with specific primers, gene specific primers (Partials *cry3A, SN19* gene and *OCII* gene). These results are in line with the finding of several other researchers including Cousins et al. (1991), Dhaliwal et al. (2004) and Bakhsh (2011), who used PCR analyses to

confirm the integration of foreign DNA in plant genome. PCR positive plants samples with gene specific primers were then checked by another PCR using *ChvA* gene specific primers for *Agrobacterium* contamination. Our finding reports no amplification with *ChvA* gene confirming that there was no *Agrobacterium* contamination because if there is any contamination, PCR should amplify *Chv A* gene of about 890 bp using specific primers (Nain et al., 2005).

Putative transgenic plants produced in both Lady Olympia and Agria cultivars were further tested for validation and confirmation of results with Southern blot analysis. The Nylon membrane showed a recognition of about 480-bp fragment of *SN19* probe. The presence of this fragments on membrane confirmed that our plants are successfully transplants with both gene constructs. Similar results with expected hybridization signal were also reported by Zha et al. (2011). The expression of *cry3A* gene was also confirmed through ELISA which is in line with the findings of Bashir et al. (2004) who studied the expression of Cry proteins in transgenic plants of indica Basmati rice through ELISA. Several factors may alter expression of *cry* gene in plant genome. These includes some internal and external environmental factors as well as the nucleotide sequences of gene and point of insertion in DNA of transgenic variety, copy numbers of transgenes and the kind of promotor use in the study. Therefore, in many studies alterations in the level of gene expression have been reported by other scientists (Agaisse et al., 1995; Sunilkumar et al., 2002; Rao, 2005; Guo et al., 2015).

The main finding of this study is that both potato cultivars i.e. Agria and Lady Olympia were successfully transformed with double gene constructs and resulted in greater resistance to all stages of CPB than the non-transgenic control plants. After confirming gene integration and expression in various potato plants, leaf bioassays with both transgenic and non- transgenic control plants of both Agria and Lady Olympia plants confirmed the successful transformation of targeted genes.

The effect of pyramiding of insecticidal genes in potato to encode resistance against imidacloprid CPB colony was studies. Before starting the bioassays with transgenic plants, complete life history parameters of CPB were studied on both commercial verities of Agria and Lady Olympia (non-transgenic) as well as the transgenic counterparts of these cultivars. Both individually and group reared methods were used to study various

life history parameters including survival, development and reproduction in detail. All tested insects reared on transgenic plants died before obtaining any life table parameter so that the parameters were calculated the insects reared on non-transgenic plants. For comparing life history parameters of CPB fed on non-transgenic cultivars, the appropriate and most useful parameter is the intrinsic rate of increase. The intrinsic rate of increase is helpful for comparing the fitness of various CPB populations feed on different potato cultivars or under different climatic conditions (Southwood, 1966; Smith, 1991). During this study, the potato cultivar "Agria" was found less suitable host than Lady Olympia to CPB population parameters with lower values of r, λ, and R_0 respectively as compared to CPB population that were reared on potato cultivar Lady Olympia. CPB populations that were reared on potato cultivar Agria had shorter egg developmental duration as compared to CPB populations that were reared on potato cultivar Lady Olympia, while no significant difference was observed in the first stage larval developmental duration in both cultivars. Further, significantly shorter developmental durations were recorded for the second and third larval stages of CPB that were reared on Lady Olympia as compared to Agria. There were no significance differences in the larval developmental duration of the fourth stage fed on Lady Olympia and Agria. Longer mean pupal duration was recorded in CPB population reared on Agria as compared to Lady Olympia. These results confirm the finding of Asghar Fathi et al. (2013), who studied life history parameters of *L. decemlineata* on seven commercial potato varieties including Agria and reported significantly lower developmental time of immature stages on Agria as compared to other potato varieties. They further reported lower survival rate of different stages of CPB that were fed on Agria as compared to other varieties.

Feeding CPB on Lady Olympia had significantly increased preadult survival rate, adult longevity, female total longevity and fecundity as compared to CPB reared on Agria. However, there was no significant difference in the total longevity of male in both CPB populations that were reared on Lady Olympia and Agria cultivars. These results are in line with Yaş and Güngör (2005) findings, who studied life history parameters of *L. decemlineata* on five different potato varieties including Agria and reported adult longevity ranged from 79.06 to 87.48 days. They further reported total fecundity of 303.75 eggs on Agria, which was close to the results observed in the current study i.e. 329.10 eggs. These results further confirm the findings of other researchers, who reported that different host plants can significantly affect the growth, development, survival, and

reproduction of insects and these can be due to differences in plant secondary metabolite, difference in plant morphological characters such as trichomes and nutrient content of host plants (Hantas, 1995; Lyytinen et al., 2007).

Besides individually reared method, the demographic analysis of CPB population reared in groups on potato cultivars Agria and Lady Olympia using age-stage two-sex life table was also studied and compared. The result showed lower survival rate on both the cultivars as compared to the individual reared method. This may be due to the cannibalism behaviors of the CPB because cannibalistic behavior observed more often in parallel to increasing population densities both in larva and adult stages. These results confirmed the findings of Goodbrod and Goff, (1990) and Takashi et al. (2009), who reported in their studies that insect activities such as larval growth and development, adult longevity, copulation, fecundity etc. are significantly affected by population level. Our results further showed higher mortality on different growth stages when reared in groups on both cultivars. These results contrast with the finding of Li et al. (2015) and Wharton et al. (1968), who reported in their studies that if larvae are reared in groups, they grow more quickly and have lower mortality than when reared alone. However, they used different insect species (Subterranean insect, *Bradysia odoriphaga* and American cockroach, *Periplaneta americana*) and there is no cannibalism amongst the tested species in those studies.

After studying the complete life history parameters on non-transgenic potato cultivars, leaf bioassays with their transgenic plants were also performed on larval and adult stages of CPB. First, second, third and fourth instar larvae of CPB as well as their adults were allowed to feed on the fresh detaches leaves of different transgenic plants of Agria and Lady Olympia cultivars. The mortality and leaf consumption pattern were almost similar in the transgenic plants transformed with two different constructs (DS-1 and DS-2). The data from ELISA, mean percent mortality and leaf consumption area showed that higher expression level of *cry3A* gene in DS-1 construct in both cultivars in various plants may directly be linked to CPB toxicity. The plants with higher gene expression level could produce less foliage consumption and early total mortality in all the stages of CPB. Early 100% mortality in DS-1AP1 in a short period of time could be the result of high expression of *cry3A* gene because in this plant, it has high expression of gene (1.29 OD). The results further showed that in all the larval stages, mortalities started within 12 hours

of feeding. Among different larval instars, CPB 1st and 2nd instar larvae resulted in early death upon feeding as compared to 3rd and 4th instar larvae. The adult stage took even longer time as compared to the larval stages. This may be due to the fact that the adults can tolerate hunger more than the larval stages because stored extra energy as fat help them to survive when food is not available, while the larval stages need to continue feeding to support their energy requirements for molting and preparation for pupal stage. Similar mortality results were reported in potato transformed with only *SN19* gene construct, where 100 % mortality of CPB larval instars was reported in primary transformants of potato (Ahmed et al., 2017). Guo et al. (2016), developed a selectable marker-free transgenic *cry3A* potato for controlling damages by CPB to protect potato produce under biosafety assessment of the environment and toxicology. They used selectable marker-free transgenic potato (CT) lines in a resistance bioassay against CPB in the laboratory and reported that CT lines exhibited high toxicity to CPB, and 100% mortality of first-instar larvae was observed within 6 days after incubation. Similarly, in another study conducted by Naimov et al. (2006) it was reported that the construction of this hybrid gene (*SN19* gene) had broaden the spectrum of pest control. In that study, they transferred the Hybrid delta endotoxin gene into Desiree potato plants via *Agrobacterium* transformation and the resultant transgenic plants resisted attack by *P. opercullela, O. nubilalus* as well as *L. decemlineata* larvae and adults. These were the first transgenic potato plants that exhibited resistance to variety of different insects. In the current study both *Bt* genes and Proteinase inhibitor *Oryza cystatins II* genes in staked form were used. The plants that were co-transformed with these genes constructs also showed similar mortality and foliage consumption as in the case of potato plants transformed with only *Bt* genes. These results are in accord with the finding of Cingel et al. (2014) who reported that expression of proteinase inhibitors *Oryza cystatins I* and *II* (*OCI* and *OCII*) in potato genome resulted in increasing resistance against CPB with causing significant reduction in adult body weight and foliage consumption. Foliage consumption area by CPB 1st, 2nd, 3rd, 4th instar larvae and adults on different transgenic plants and their control plants showed significant differences as the feeding activity of larval instars and adults fed on different transgenic plants reduced when compared with non-transgenic plants. Significantly high consumed foliage area was recorded in both control plants, Lady Olympia and Agria in all growth stages of CPB while significantly lower leaf area consumptions were recorded in all the transgenic plants of both Agria and Lady Olympia. These results are in similarity with the finding of Zhou et al. (2012), who studied CPB

resistance against high, medium and low doses of transgenic potato plants expressing Cry3A toxin. Toxicity of transgenic plants to CPB raised with increasing gene expression levels of the *cry3A* in potato plants. They reported that the transgenic potato plants with high and medium dose of cry3A toxin caused early 100 % mortality as compared to the low dose transgenic plants on which only 50 % mortality was recorded. They reported low leaf consumption area and higher yield in high and medium dose cry3A transgenic plants as compared to the low dose cry3A transgenic plants. Similarly, in another study, Me et al. (2015) reported that expressing *cry3A* gene in transgenic potato plants signify toxic effect against Colorado potato beetle. Inoculation with the first instar larvae on the transgenic plants produced higher mortality in the larvae, lower insect biomass accumulation and foliage consumption as compared to non-transgenic control plants.

Potato crop is attacked by many insect pests belonging to different insect orders. The present study results demonstrate that the combination of these new double gene construct can be used against CPB and other insect pests in coleopteran as well as lepidopteran insect pests attacking potato. Furthermore, this toxin combination along with other tactics such as biocontrol agents, biopesticides including natural insecticides and chemical insecticides will enhance pest management programmes against insect pests in the diversified agricultural cropping system.

CHAPTER VI

CONCLUSION

The main aim of this PhD dissertation work was to study the lethal and sub lethal effects of transgenic potato plants produced by gene pyramiding technique on various life stages of Colorado potato beetle. Plant expression vector (*pCAMBIA 1301*) was used to develop two different double stacked constructs (DS-1 & DS-2). The *cry3A* gene in *pGT-22-b* vector was obtained from Czech Republic and *OCII* gene was amplified from rice cultivar namely Edirne. The Synthetic hybrid *SN19* gene was obtained from the Plant Transformation Laboratory, department Agriculture and Genetic engineering, Nigde University. Potato cultivars Agria and Lady Olympia cultivars were co-transformed with both *Bt* and PIs (*OCII* gene), each cultivar was co-transformed with two different constructs i.e. DS-1 and DS-2 constructs using *Agrobacterium*-mediated transformation technique.

This is the very first study of using both *Bt* genes and proteinase inhibitors together against CPB which is one of the important pests of potato in Turkey and the rest of the world. The primary transformants developed out of both cultivars (Agria and Lady Olympia) were analyzed for integrative (stable) transformation of the insecticidal genes within T-DNA region through PCR, Southern blotting and leaf bioassays with CPB larval and adult stages. A total of 27 PCR positive transgenic plants were obtained in both Agria and Lady Olympia transformed with different DS-1 and DS-2 gene constructs.

Transgenic plants showed high toxicity to CPB larvae and adults. Early 100 % mortality of each larval instars was recorded in DS-1AP1 and DS-1LOP1 transgenic plants of Agria and Lady Olympia respectively. These results indicate that *cry3A* gene should have toxic activity to CPB in all stages. The biotoxicity of genes took a longer time in adult stage than it did in larval stages which could be related with the fact that adults were found more reluctant to feed on transgenic plants as compared to the larval instars, where 100 % mortality was recorded after 10 days of incubation. However no egg laying data were recorded for CPB reared on transgenic potato plants as compared ton on transgenic control plants. The data regarding foliage consumption showed that very low leaf consumption area was recorded in all transgenic plants of both Agria and Lady Olympia

as compared to their control plants. The adult CPB consumed more foliage as compared to larval stages. All these promising results of mortality and leaf consumption data indicate that these transgenic plants exhibit significant toxicity to CPB.

Complete life history parameters of CPB were studied in detail on both commercial potato cultivars Agria and Lady Olympia and their transgenic plants. Different population parameters were studied with individually reared method as well as in group reared methods. In both the methods, all the tested insects reared on the transgenic plants died in a short period of time. In the non-transgenic plant, significantly lower survival rate, fecundity, TPOP and adult longevity were recorded in the Agria control plants as compared to Lady Olympia Control plants. By comparing, the life history traits and other parameters, it is obvious that potato cultivar Lady Olympia seems to be a better host than Agria with high population parameters.

These results indicated that CPB resistance to insecticides (imidacloprid) can be controlled with transgenic potato plants transformed with DS-1 and DS-2 plasmid constructs. However, before wide adaptation of this technique in control of CPB, biosafety tests, ecotoxicology test as well as all other required tests for registering these plant lines are needed. Finding of the current study may ultimately result in higher productivity and reduction in potato yield losses.

Future studies, testing of the most promising potato plants in greenhouse and field conditions against susceptible and laboratory resistant CPB colonies as well as the field resistant colony could help to explore full potential of these transgenic plants in control of CPB. Additionally, Lethal Time 50 (LT_{50}) and the mean leaf consumption area needed to produce mortality should be studied for better estimate time scale of events. Studying life table parameters with low gene expressed potato plants could reflect more details about sub lethal effects of the transgenic potato plants.

KAYNAKLAR

Adang, M. J., Brody, M. S., Cardineau, G., Eagan, N., Roush, R. T., Shewmaker, C. K., Jones, A., Oakes, J. V. and McBride, K. E., "The reconstruction and expression of a *Bacillus thuringiensis* cryIIIA gene in protoplasts and potato plants", ***Plant Molecular Biology*** 21(6), 1131-1145, 1993.

Agaisse, H. and Lereclus, D., "How does *Bacillus thuringiensis* produce so much insecticidal crystal protein?", ***Journal of Bacteriology*** 177(21), 27-60, 1995.

Ahammadsahib, K. I., Hollingworth, R. M., Whalon, M. E., Ioannidis, P. M. and Grafius, E. J., "Polysubstrate monooxygenases and other xenobiotic-metabolizing enzymes in susceptible and resistant Colorado potato beetle", ***Pesticide Biochemistry and Physiology*** 49(1), 1-12, 1994.

Ahmed, H. A. A., Onarıcı, S., Bakhsh, A., Akdoğan, G., Karakoç, Ö. C., Özcan, S. F., Aydın, G., Aasım, M., Ünlü, L. and Sancak, C., "Targeted expression of insecticidal hybrid SN19 gene in potato leads to enhanced resistance against Colorado potato beetle (*Leptinotarsa decemlineata* Say) and tomato leafminer (*Tuta absoluta* Meyrick)", ***Plant Biotechnology Reports*** 11(5), 315-329, 2017.

Akköprü, E. P., Atlıhan, R., Okut, H. and Chi, H., "Demographic assessment of plant cultivar resistance to insect pests: a case study of the dusky-veined walnut aphid (Hemiptera: Callaphididae) on five walnut cultivars", ***Journal of Economic Entomology*** 108(2), 378-387, 2015.

Ali, M. I. and Luttrell, R. G., "Susceptibility of bollworm and tobacco budworm (Lepidoptera: Noctuidae) to Cry2Ab2 insecticidal protein", ***Journal of Economic Entomology*** 100(3), 921-931, 2007.

Alkan, M., Gökçe, A. and Kara, K., "Stomach poison activity of some plant extracts on Colorado potato beetle (Coleoptera: Chrysomelidae)", ***Bitki Koruma Bülteni*** 57(3), 305-311,

Alyokhin, A., "Colorado potato beetle management on potatoes: current challenges and future prospects", ***Fruit, Vegetable and Cereal Science and Biotechnology*** 3(1), 10-19, 2009.

Alyokhin, A., Baker, M., Mota-Sanchez, D., Dively, G. and Grafius, E., "Colorado potato beetle resistance to insecticides", ***American Journal of Potato Research*** 85(6), 395-413, 2008.

Alyokhin, A., Mota-Sanchez, D., Baker, M., Snyder, W. E., Menasha, S., Whalon, M., Dively, G. and Moarsi, W. F., "The Red Queen in a potato field: integrated pest management versus chemical dependency in Colorado potato beetle control", ***Pest Management Science*** 71(3), 343-356, 2015.

Alyokhin, A. V. and Ferro, D. N., "Modifications in dispersal and oviposition of Bt-resistant and Bt-susceptible Colorado potato beetles as a result of exposure to *Bacillus thuringiensis* subsp. *tenebrionis* Cry3A toxin", ***Entomologia Experimentalis et Applicata*** 90(1), 93-101, 1999.

Andrews, R. W., Fausr, R., Wabiko, M. H., Roymond, K. C. and Bulla, L. A., "Biotechnology of *Bt*: a critical review", ***Biotechnology*** 6, 163-232, 1987.

Anonymous, Agricultural Structure (Production, Price, Value), ***State Institute of Statistics of Republic of Turkey***, Ankara, 2000.

Anspaugh, D. D., Kennedy, G. G. and Roe, R. M., "Purification and characterization of a resistance-associated esterase from the Colorado potato beetle, *Leptinotarsa decemlineata* (Say)", ***Pesticide Biochemistry and Physiology*** 53(2), 84-96, 1995.

Arai, S. and Abe, K., "Cystatin-based control of insects, with special reference to orycystatin", ***Recombinant Protease Inhibitors in Plants*** 1, 27-42, 2000.

Argentine, J. A., Clark, M. J. and Ferro, D. N., "Genetics and synergism of resistance to azinphosmethyl and permethrin in the Colorado potato beetle (Coleoptera: Chrysomelidae)", ***Journal of Economic Entomology*** 82(3), 698-705, 1989.

Argentine, J. A., Zhu, K. Y., Lee, S. H. and Clark, J. M., "Biochemical mechanisms of azinphosmethyl resistance in isogenic strains of Colorado potato beetle", ***Pesticide Biochemistry and Physiology*** 48(1), 63-78, 1994.

Arpaia, S., Transgenic resistance of eggplants to the Colorado potato beetle, ***Italy***, Arpaia, 1999.

Asghar Fathi, S. A., Fakhr-Taha, Z., Razmjou, J. and Palumbo, J., "Life-history parameters of the Colorado potato beetle, *Leptinotarsa decemlineata*, on seven commercial cultivars of potato, *Solanum tuberosum*", ***Journal of Insect Science*** 13(1), 25-35, 2013.

Atkinson, H. J., Urwin, P. E., Hansen, E. and McPherson, M. J., "Designs for engineered resistance to root-parasitic nematodes", ***Trends in Biotechnology*** 13(9), 369-374, 1995.

Atsumi, S., Miyamoto, K., Yamamoto, K., Narukawa, J., Kawai, S., Sezutsu, H., Kobayashi, I., Uchino, K., Tamura, T. and Mita, K., "Single amino acid mutation in an ATP-binding cassette transporter gene causes resistance to Bt toxin Cry1Ab in the silkworm, Bombyx mori", ***Proceedings of the National Academy of Sciences*** 109(25), 1591-1598, 2012.

Azadi, H. and Ho, P., "Genetically modified and organic crops in developing countries: A review of options for food security", ***Biotechnology Advances*** 28(1), 160-168, 2010.

Azzouz, H., Cherqui, A., Campan, E. D. M., Rahbe, Y., Duport, G., Jouanin, L., Kaiser, L. and Giordanengo, P., "Effects of plant protease inhibitors, oryzacystatin I and soybean Bowman–Birk inhibitor, on the aphid *Macrosiphum euphorbiae* (Homoptera, Aphididae) and its parasitoid *Aphelinus abdominalis* (Hymenoptera, Aphelinidae)", ***Journal of insect physiology*** 51(1), 75-86, 2005.

Bakhsh, A., Anayol, E. and Ozcan, S. F., "Comparison of transformation efficiency of five Agrobacterium tumefaciens strains in Nicotiana Tabacum L", ***Emirates Journal of Food and Agriculture*** 26(3), 259-264, 2014.

Bakhsh, A., Shahzad, K. and Husnain, T., "Variation in the spatio-temporal expression of insecticidal genes in cotton", ***Czech Journal of Genetics and Plant Breeding*** 47(1), 1-9, 2011.

Bashir, K., Husnain, T., Fatima, T., Latif, Z., Mehdi, S. A. and Riazuddin, S., "Field evaluation and risk assessment of transgenic indica basmati rice", ***Molecular Breeding*** 13(4), 301-312, 2004.

Bates, S. L., Zhao, J.-Z., Roush, R. T. and Shelton, A. M., "Insect resistance management in GM crops: past, present and future", ***Nature Biotechnology*** 23(1), 57-62, 2005.

Berger, D., "Strategies for transgene pyramiding", ***Transgenic Potatoes for The Benefit of Resource-Poor Farmers in Developing Countries*** 1, 67-74, 2000.

Bizily, S. P., Rugh, C. L. and Meagher, R. B., "Phytodetoxification of hazardous organomercurials by genetically engineered plants", ***Nature Biotechnology*** 18(2), 213-220, 2000.

Boiteau, G., Pelletier, Y., Misener, G. C. and Bernard, G., "Development and evaluation of a plastic trench barrier for protection of potato from walking adult Colorado potato beetles (Coleoptera: Chrysomelidae)", ***Journal of Economic Entomology*** 87(5), 1325-1331, 1994.

Boiteau, G., Picka, J. D. and Watmough, J., "Potato field colonization by low-density populations of Colorado potato beetle as a function of crop rotation distance", ***Journal of Economic Entomology*** 101(5), 1575-1583, 2008.

Bolter, C. J. and Latoszek-Green, M., "Effect of chronic ingestion of the cysteine proteinase inhibitor, E-64, on Colorado potato beetle gut proteinases", ***Entomologia Experimentalis et Applicata*** 83(3), 295-303, 1997.

Bravo, A., Gill, S. S. and Soberon, M., "Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control", ***Toxicon*** 49(4), 423-435, 2007.

Bravo, A., Likitvivatanavong, S., Gill, S. S. and Soberón, M., "*Bacillus thuringiensis*: a story of a successful bioinsecticide", ***Insect Biochemistry and Molecular Biology*** 41(7), 423-431, 2011.

Bravo, A. and Soberón, M., "How to cope with insect resistance to Bt toxins?", ***Trends in Biotechnology*** 26(10), 573-579, 2008.

Brown, C. R., "Antioxidants in potato", ***American Journal of Potato Research*** 82(2), 163-172, 2005.

Burhans, D., Crunch!: A History of the Great American Potato Chip, ***Terrace Books***, New York, USA, 2008.

Cao, J., Zhao, J.-Z., Tang, J., Shelton, A. and Earle, E., "Broccoli plants with pyramided cry1Ac and cry1C Bt genes control diamondback moths resistant to Cry1A and Cry1C proteins", ***Theoretical and Applied Genetics*** 105(2-3), 258-264, 2002.

Carey, J. R., "Insect biodemography", ***Annual Review of Entomology*** 46(1), 79-110, 2001.

Carrière, Y., Crickmore, N. and Tabashnik, B. E., "Optimizing pyramided transgenic Bt crops for sustainable pest management", ***Nature Biotechnology*** 33(2), 161-170, 2015.

Castagnola, A. S. and Jurat-Fuentes, J. L., *Bt* crops: past and future, Chapter: *Bacillus thuringiensis*, In: Biotechnology, ***Springer***, Dordrecht, Netherland, 2012.

Chang, C., Huang, C.-Y., Dai, S.-M., Atlihan, R. and Chi, H., "Genetically engineered ricin suppresses *Bactrocera dorsalis* (Diptera: Tephritidae) based on demographic analysis of group-reared life table", ***Journal of Economic Entomology*** 109(3), 987-992, 2016.

Chen, L., Marmey, P., Taylor, N. J., Brizard, J.-P., Espinoza, C., D'Cruz, P., Huet, H., Zhang, S., de Kochko, A. and Beachy, R. N., "Expression and inheritance of multiple transgenes in rice plants", ***Nature Biotechnology*** 16(11), 1060-1071, 1998.

Chi, H., "Life-table analysis incorporating both sexes and variable development rates among individuals", ***Environmental Entomology*** 17(1), 26-34, 1988.

Chi, H., "Timing of control based on the stage structure of pest populations: a simulation approach", ***Journal of Economic Entomology*** 83(4), 1143-1150, 1990.

Chi, H. and Liu, H., "Two new methods for the study of insect population ecology", ***Bulletin of the Institute of Zoology, Acadmia Sinica*** 24(2), 225-240, 1985a.

Chi, H. and Liu, H., "Two new methods for the study of insect population ecology", ***Bulletin of the Institute of Zoology, Academia Sinica*** 24(2), 225-240, 1985b.

Choudhary, B. and Gaur, K., Biotech cotton in India, 2002 to 2014, ***ISAAA Series of Biotech Crop Profiles***, Ithaca, New York, 2015.

Christou, P., Capell, T., Kohli, A., Gatehouse, J. A. and Gatehouse, A. M. R., "Recent developments and future prospects in insect pest control in transgenic crops", ***Trends in Plant Science*** 11(6), 302-308, 2006.

Cingel, A., Savić, J., Ćosić, T., Zdravković-Korać, S., Momčilović, I., Smigocki, A. and Ninković, S., "Pyramiding rice cystatin OCI and OCII genes in transgenic potato (*Solanum tuberosum* L.) for resistance to Colorado potato beetle (*Leptinotarsa decemlineata* Say)", ***Euphytica*** 198(3), 425-438, 2014.

Clark, J. M., Lee, S. H., Kim, H. J., Yoon, K. S. and Zhang, A., "DNA-based genotyping techniques for the detection of point mutations associated with insecticide resistance in Colorado potato beetle *Leptinotarsa decemlineata*", ***Pest Management Science*** 57(10), 968-974, 2001.

Cloutier, C. and Bauduin, F., "Biological control of the Colorado potato beetle *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) in Quebec by augmentative releases of the two-spotted stinkbug *Perillus bioculatus* (Hemiptera: Pentatomidae)", ***The Canadian Entomologist*** 127(2), 195-212, 1995.

Cohen, J. I., "Poorer nations turn to publicly developed GM crops", ***Nature Biotechnology*** 23(1), 27-34, 2005.

Cohen, M. B., Gould, F. and Bentur, J. S., "Bt rice: practical steps to sustainable use", ***International Rice Research Notes*** 25(2), 4-10, 2000.

Cousins, Y. L., Lyon, B. R. and Llewellyn, D. J., "Transformation of an Australian cotton cultivar: prospects for cotton improvement through genetic engineering", ***Functional Plant Biology*** 18(5), 481-494, 1991.

Dennehy, T. J., Head, G. P., Moar, W., Greenplate, J., Mohan, K. S., Ravi, K. C., Suresh, P. J. and Parimi, S., "Status of PBW resistance to Bollgard cotton in India", ***58th ESA meeting,*** Valencia, Spain, 2010.

Dhurua, S. and Gujar, G.T., "Field-evolved resistance to Bt toxin cry1Ac in the pink bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae), from India", ***Pest Management Science*** 67, 898–903, 2011.

Dively, G. P., Venugopal, P. D. and Finkenbinder, C., "Field-evolved resistance in corn earworm to Cry proteins expressed by transgenic sweet corn", ***PLoS One***, 11(12), e0169115, 2016.

Efron, B. and Tibshirani, R. J., Syntax of referencing in *An introduction to the bootstrap,* **Chapman and Hall**, New York, USA, 1993.

Fabrick, J. A., Mathew, L. G., Tabashnik, B. E. and Li, X., "Insertion of an intact CR1 retrotransposon in a cadherin gene linked with Bt resistance in the pink bollworm, *Pectinophora gossypiella*", ***Insect molecular biology*** 20, 651– 665, 2011.

FAOSTAT., "Statistical data", Food and Agriculture Organization of the United Nations, Rome, 2017.

Farias, J. R., Andow, D. A., Horikoshi, R. J., Sorgatto, R. J., Fresia, P., dos Santos, A. C. and Omoto, C., "Field-evolved resistance to Cry1F maize by *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Brazil", ***Crop Protection*** 64, 150–158, 2014.

Ferre, ´ J., and Van, Rie, J., "Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*", ***Annual Review of Entomology*** 47, 501–533, 2002.

Ferro, D. N., Logan, J. A., Voss, R. H., and Elkinton, J. S., "Colorado potato beetle (Coleoptera, Chrysomelidae) temperature-dependent growth and feeding rates", ***Environmental Entomology*** 14, 343–348, 1985.

Ferro, D. N., Yuan, Q. C., Slocombe, A. and Tuttle, A. F., "Residual activity of insecticides under field conditions for controlling the Colorado potato beetle (Coleoptera, Chrysomelidae)", ***Journal of Economic Entomology*** 86, 511–516, 1993.

Ferry, N., Edwards, M. G., Mulligan, E. A., Emami, K., Petrova, A. S., Frantescu, M., Davison, G. M and Gatehouse, A.M.R., "Engineering resistance to insect pests", In: Christou P, Klee H (eds). Handbook of Plant Biotechnology. Vol. 1. ***John Wiley and Sons, Chichester***, 373-394, 2004.

François, I.E., De Bolle, M.F., Dwyer, G., Goderis, I.J, Woutors, P.F., Verhaert, P.D., Proost, P., Schaaper, W.M., Cammue, B.P. and Broekaert, W.F., "Transgenic expression in Arabidopsis of a polyprotein construct leading to production of two different antimicrobial proteins", Plant Physiology 128(4), 1346-1358, 2002a

Gasser, C.S. and Fraley, R.T., "Transgenic crops", ***Scientific American***, *266*(6), 62-69. 1992.

Gassmann, A.J., Petzold-Maxwell, J.L., Clifton, E.H., Dunbar, M.W., Hoffmann, A.M., Ingber, D.A., and Keweshan, R.S., "Field-evolved resistance by western corn rootworm to multiple *Bacillus thuringiensis* toxins in transgenic maize", ***Proceedings of the National Academy of Sciences***.201317179, 2014.

Gassmann, A.J., Petzold-Maxwell, J. L., Keweshan, R. S., and Dunbar, M. W., "Field-evolved resistance to Bt maize by western corn rootworm", ***PloS one*** 6(7), p.e 22629, 2011.

Gassmann, A.J., Shrestha, R.B., Jakka, S.R., Dunbar, M.W., Clifton, E.H., Paolino, A.R., Ingber, D.A., French, B.W., Masloski, K. ., Dounda, J.W., and St. Clair, C.R., "Evidence

of resistance to Cry34/35Ab1 corn by western corn rootworm (Coleoptera: Chrysomelidae): Root injury in the field and larval survival in plant-based bioassays", ***Journal of economic entomology*** 109(4), 1872-1880, 2016.

Gatehouse, J.A., "Biotechnological prospects for engineering insect-resistant plants" ***Plant Physiology*** 146**,** 881–887, 2008.

Gauthier, N.L., Hofmaster, R.N. and Semel, M., "History of Colorado potato beetle control", In: Lashomb JH, Casagrande R (Eds) Advances in Potato Pest Management, ***Hutchinson Ross Publishing Co.,*** Stroudsburg, PA, 13-33, 1981.

Georghiou, G. P. and Lagunes-Tejeda, A., "The occurrence of resistance to pesticides in arthropods", ***Food and Agriculture Organization of the United Nations***, Rome, 1991.

Giordanengo, P., Pelletier, Y., Vincent, C. (Eds.), "La Pomme de terre, enjeux et opportunités", Cahiers Agric, 17, 329–420. 2008.

Goderis, I.J., De Bolle, M. F., François, I.E., Wouters, P. F., Broekaert, W. F. and Cammue, B. P., "A set of modular plant transformation vectors allowing flexible insertion of up to six expression units", ***Plant molecular biology*** 50(1), 17-27, 2002.

Gökçe, A., Isaacs, R. and Whalon, M.E., "Behavioral response of Colorado potato beetle (*Leptinotarsa decemlineata*) larvae to selected plant extracts", ***Pest Management Science*** 62(11), 1052-1057, 2006.

Gökçe, A., Isaacs, R. and Whalon, M.E., "Dose–response relationships for the antifeedant effects of *Humulus lupulus* extracts against larvae and adults of the Colorado potato beetle", ***Pest management science*** 68(3), 476-481, 2012.

Goodbrod, J.R. and Goff, M.L., "Effects of larval population density on rates of development and interactions between two species of *Chrysomya* (Diptera: Calliphoridae) in laboratory culture", ***Journal of Medical Entomology*** 27:338–343. doi: 10.1093/jmedent/27.3.338, 1990.

Gould, F., “Sustainability of transgenic insecticidal cultivars: integrating pest genetics and ecology”, ***Annual Review of Entomology*** 43:701–726, 1998.

Grafius, E.J. and Bishop, B.A., “Resistance to imidacloprid in Colorado potato beetles from Michigan”, Resistant pest management 8:21–25, 1996.

Grafius, E.J. and Douches, D.S., “The present and future role of insect-resistant genetically modified potato cultivars in IPM”, In integration of insect-resistant genetically modified crops within IPM programs (pp. 195-221). ***Springer Netherlands***, 2008.

Guo, W.C., Wang, Z.A., Luo, X.L., Jin, X., Chang, J., He, J., Tu, E.X., Tian, Y.C., Si, H. J. and Wu, J.H., “Development of selectable marker-free transgenic potato plants expressing *cry3A* against the Colorado potato beetle (*Leptinotarsa decemlineata* Say)”, ***Pest management science*** 72(3), 497-504, 2016.

Guo, Z., Kang, S., Zhu, X., Xia, J. and Wu, Q., “The novel ABC transporter ABCH1 is a potential target for RNAi-based insect pest control and resistance management”, ***Scientific Reports*** 5: 13728, 2015.

Halpin, C. and Ryan, M.D., “Redirecting metabolism by co-ordinate manipulation of multiple genes”, ***Metabolic Engineering in the Post-Genomic*** *Era*, pp.377-408. 2004.

Halpin, C., “Gene stacking in transgenic plants the challenge for 21st century plant biotechnology”, ***Plant Biotechnology journal*** 3:141–155, 2005.

Hameed, A., Tahir, M.N., Asad, S., Bilal, R., Van Eck, J., Jander, G. and Mansoor, S., “RNAi-mediated simultaneous resistance against three RNA viruses in potato”, ***Molecular biotechnology*** *59*(2-3), 73-83, 2017.

Hantas, C., “Investigations on the preference of potato varieties of the larvae and adults of Colorado potato beetle (*Leptinotarsa decemlineata* Say) and their development in Erzincan conditions”, Master thesis, Atatürk University, Erzurum, Turkey (in Turkish with English summary), 1995.

Harcourt, D.G., "Population dynamics of *Leptinotarsa decemlineata* (Say) in eastern Ontario. III Major population processes", ***The Canadian Entomologist*** 103:1049–1061. 1971.

Hare, J.D., "Ecology and management of the Colorado potato beetle", *Annual Review of Entomology* 35:81-100, 1990.

Harris, C.R. and Turnbull, S.A., "Contact toxicity of some pyrethroids insecticides, alone and in combination with piperonyl butoxide, to insecticide-susceptible and pyrethroid-resistant strains of the Colorado potato beetle (Coleoptera: Chrysomelidae)", ***The Canadian Entomologist*** 118: 1173–1176, 1986.

Has, A., "Investigations on the Bio-ecology, and Especially Host-plant Relationships of the Colorado Potato Beetle (*Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) in the Conditions of Central Anatolia", Ankara Plant Protection Research Institute, Ankara. 194 pp (in Turkish with English summary). 1992.

Hernández, Rodrigo. and Ferré., "Hernández CS, Rodrigo A, Ferré J. Lyophilization of lepidopteran midguts: a preserving method for *Bacillus thuringiensis* toxin binding studies", ***Journal of Invertebrate Pathology*** 85:182–187. 2004.

Herrnstadt, G., Soares, R.W., Edward, L. and Edwards, D., "A new strain of *Bacillus thuringiensis* with activity against coleopteran insects", ***BioTechnology*** 4: 305-308. 1986.

Hines, M.E., Osuala, C.I. and Nielsen, S.S., "Isolation and partial characterization of a soybean cystatin cysteine proteinase inhibitor of coleopteran digestive proteolytic activity", ***Journal of Agricultural and Food Chemistry*** 39:1515-1520, 1991.

Hofte, H. and Whitely, H.R., "Insecticidal crystal protein of *Bacillus thuringenesis*", ***Microbiological reviews*** 53: 242-255, 1989.

Hoy, C.W. and Head, G., "Correlation between behavioral and physiological responses to transgenic potatoes containing *Bacillus thuringiensis* delta-endotoxin in *Leptinotarsa*

decemlineata (Coleoptera: Chrysomelidae)", ***Journal of Agricultural Entomology*** 88: 480–486, 1995.

Huang, H.W., Chi, H. and Smith, C.L., "Linking demography and consumption of *Henosepilachna vigintioctopunctata* (Coleoptera: Coccinellidae) fed on *Solanum photeinocarpum*: with a new method to project the uncertainty of population growth and consumption", ***Journal of economic entomology*** 111:1–9, 2018.

Huang, Y.B. and Chi, H., "Age-stage, two-sex life tables *of Bactrocera cucurbitae* (Coquillett) (Diptera: Tephritidae) with a discussion on the problem of applying female age specific life tables to insect populations", ***Insect science*** 19, 263– 273, 2012.

Huang, Y.B. and Chi, H., "The age-stage, two-sex life table with an offspring sex ratio dependent on female age", ***Journal of Agriculture and Forestry*** 60(4), 337–345, 2011.

Hutchison, W.D., Burkness, E.C., Mitchell, P.D., Moon, R.D., Leslie, T.W., Fleischer, S. J., Abrahamson, M., Hamilton, K.L., Steffey, K.L., Gray, M.E. and Hellmich, R.L., "Areawide suppression of European corn borer with *Bt* maize reaps savings to non-*Bt* maize growers", ***Science*** 330(6001), 222-225, 2010.

ILSI., "An evaluation of insect resistance management in *Bt* field corn: A science-based framework for risk assessment and risk management", ILSI Press, Washington, D.C., 78 pp, 1998.

Ioannidis, P.M., Grafius, E. and Whalon, M.E., "Patterns of insecticide resistance to azinphosmethyl, carbofuran, and permethrin in the Colorado potato beetle (Coleoptera, Chrysomelidae)", ***Journal of Economic Entomology*** 84:1417–1423, 1991.

Ioannidis, P.M., Grafius, E., Wierenga, J.M., Whalon, M.E. and Hollingworth R.M., "Selection, inheritance and characterization of carbofuran resistance in the Colorado potato beetle (Coleoptera, Chrysomelidae)", ***Pesticide Science*** 35: 215–222, 1992.

ISAAA "Global Status of Commercialized Biotech/GM Crops in 2017: Biotech Crop Adoption Surges as Economic Benefits Accumulate in 22 Years", ISAAA Brief No. 53. ISAAA: Ithaca, NY, 2017.

İşler, N., "Patates Tarımının Mevcut Durumu", Web: www.mku.edu.tr/getblogfile.php?keyid=274, (Erişim:27.06.2015), 2012.

Jacques, R.L., "The Potato Beetles" ***E. J. Brill, Leiden***, 144 , 1988.

Jacques, R.L., "Colorado Potato Beetle, *Leptinotarsa decemlineata* (Say) and False Potato Beetle, *Leptinotarsa juncta* (Germar) (Insecta: Coleoptera: Chrysomelidae)", University of Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, EDIS. 2003.

Kaplanoglu, Emine., "Multi-gene resistance to neonicotinoids in the Colorado potato beetle, *Leptinotarsa decemlineata*" *Electronic Thesis and Dissertation Repository* 3953. http://ir.lib.uwo.ca/etd/3953, 2016.

Kart, M.Ç.Ö., Abay, C.F., Güngör, S. and Özer, Z., "Seed supply and seed preferences of potato farmers: Nigde central and Izmir odemis provinces", ***Seed*** 17(2), 2017.

Kepenekci, İ., Tülek, A., Alkan, M. and Hazır, S., 2013. Biological control potential of native entomopathogenic nematodes against the potato tuber moth, *Phthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae) in Turkey", ***Pakistan Journal of Zoology*** 45(5), 1415-1422, 2013.

Key, S., Ma, J.K. and Drake, P.M., "Genetically modified plants and human health" ***Journal of the Royal Society of Medicine*** 101(6), 290-298, 2008.

Khajuria, C., Buschman, L.L., Chen, M.S., Siegfried, B.D. and Zhu, K.Y., "Identification of a novel aminopeptidase P-like gene (OnAPP) possibly involved in Bt toxicity and resistance in a major corn pest (*Ostrinia nubilalis*)", ***PLoS One*** *6*(8), e23983, 2011.

Kim, H.J. and Clark. J.M., "Evaluation of resistant point mutations in recombinant acetylcholinesterase of Colorado potato beetle", Abstracts of Papers of the ***American Chemical Society*** 224: U103–U103, 2002.

Kim, H.J., Dunn, J.B., Yoon, K.S. and Clark, J.M., "Target site insensitivity and mutational analysis of acetylcholinesterase from a carbofuran-resistant population of

Colorado potato beetle, *Leptinotarsa decemlineata* (Say)", ***Pesticide Biochemistry and Physiology*** 84: 165–179, 2006.

Kim, H.J., Hawthorne, D.J., Peters, T., Dively, G.P. and Clark, J.M., "Application of DNA-based genotyping techniques for the detection of kdr-like pyrethroid resistance in field populations of Colorado beetle", ***Pesticide Biochemistry and Physiology*** 81: 85–96, 2005.

Kivan, M., "Some observations on *Perillus biowlatus* (F.) (Heteroptera: Pentatomidae) a new record for the entomofauna of Turkey Tükiye böcek faunasi icin yeni bir kayit, *Perillus bloculatus* (F.) (Heteroptera: Pentatomidae) üzerinde bazı gözlemler", ***Turkish Journal of Entomology*** 28(2), 2004.

Kos, M., van Loon, J.J., Dicke, M. and Vet, L.E., "Transgenic plants as vital components of integrated pest management", ***Trends in Biotechnology*** 27: 621–627, 2009.

Koštál, V., "Eco-physiological phases of insect diapause", ***Journal of Insect Physiology*** 52: 113-127. 93, 2005.

Kostic, M., Stankovic, S. and Kuzevski, J., "Role of AChE in Colorado potato beetle (*Leptinotarsa decemlineata* Say) resistance to carbamates and organophosphates", **In *Insecticides Resistance***. InTech, 2016.

Krishnan, N., Kodrik, D., Turanli, F. and Sehnal, F., "Stage specific distribution of oxidative radicals and antioxidant enzymes in the midgut of *Leptinotarsa decemlineata*", ***Journal of Insect Physiology*** 53: 67–74, 2007.

Kruger, M., Van Rensburg, J.B.J. and Van den Berg, J., "Resistance to Bt maize in *Busseola fusca* (Lepidoptera: Noctuidae) from Vaalharts, South Africa", ***Environmental Entomology*** 40(2), pp.477-483, 2011.

Kuriwada, T., Kumano, N., Shiromoto, K. and Haraguchi, D., "High population density and egg cannibalism reduces the efficiency of mass-rearing in *Euscepes postfasciatus* (Coleoptera: Curculionidae)", ***Florida Entomologist*** 92(2), 221-228, 2009.

Kurt, Ş., Uysal, A., Kara, M., Soylu, S. and Soylu, E.M., "Natural infection of potato by *Sclerotinia sclerotiorum* causing stem rot disease in Turkey", ***Australasian Plant Disease Notes*** 12(1), 39, 2017.

Lashomb, J.H. and Ng, Y.S., "Colonization by the Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) in rotated and non- rotated potato fields", ***Environmental Entomology*** 13, 1352-1356, 1984.

Lashomb, J.H. and Ng, Y.S., "Colonization by Colorado potato beetles, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae), in rotated and nonrotated potato fields", *Environmental Entomology* 13, no. 5 (1984): 1352-1356. 1984.

Lee, S. and Clark, J.M., "Antibody capture immunoassay for the detection of permethrin carboxylesterase in Colorado potato beetle, *Leptinotarsa decemlineata* Say", ***Pesticide Biochemistry and Physiology*** 64:66–75, 1999.

Lee, S. and Clark, J.M., "Permethrin carboxylesterase functions as nonspecific sequestration proteins in the hemolymph of Colorado potato beetle", ***Pesticide Biochemistry and Physiology*** 62:51–63, 1998.

Lee, S. and Clark, J.M., "Purification and characterization of multiple-charged forms of permethrin carboxylesterase(s) from the hemolymph of resistant Colorado potato beetle", ***Pesticide Biochemistry and Physiology*** 60:31–47, 1998.

Lee, S. and Clark, J.M., "Tissue distribution and biochemical characterization of carboxylesterases associated with permethrin resistance in a near isogenic strain of Colorado potato beetle", ***Pesticide Biochemistry and Physiology*** 56:208–219, 1996.

Lee, S.H., Dunn, J.B., Clark, J.M. and Soderlund, D.M., "Molecular analysis of kdr-like resistance in a permethrin-resistant strain of Colorado potato beetle", ***Pesticide Biochemistry and Physiology*** 63:63–75, 1999.

Leo, F.D., Volpicella, M., Licciulli, F., Liuni, S., Gallerani, R. and Ceci, L.R., "PLANT-PIs: a database for plant protease inhibitors and their genes", ***Nucleic acids research***, *30*(1), 347-348. 2002.

Leslie, P.H., “On the use of matrices in certain population mathematics”, ***Biometrika*** 33, 183-212, 1945.

Lewis, E.G., “On the generation and growth of a population”, ***Sankhya*** 6: 93-96, 1942.

Li, W., Yang, Y., Xie, W., Wu, Q., Xu, B., Wang, S., Zhu, X., Wang, S. and Zhang, Y., “Effects of temperature on the age-stage, two-sex life table of *Bradysia odoriphaga* (Diptera: Sciaridae)”, ***Journal of economic entomology*** 108 (1): 126-134, 2015.

Liang, C, Brookhart, G., Feng, G.H., Reeck, G.R. and Kramer, K.J., “Inhibition of Digestive Proteinases of Stored Grain Coleoptera by *Oryza cystatin*, a Cysteine Proteinase-Inhibitor from Rice Seed”, ***FEBS Letters*** 278(2): 139-142. 1991.

Lotka, A.J., “Relation between birth rates and death rates”, ***Science*** 26(653), 21-22, 1907.

Luttrell, R.G., Ali, I., Allen, K.C., Young, S., Szalanski, A., Williams, K., Lorenz, G., Parker Jr. and Blanco, C., “Resistance to Bt in Arkansas populations of cotton bollworm”, ***Proceedings, Beltwide Cotton Conferences***, 5–9 January 2004, San Antonio, TX, National Cotton Council of America, Memphis, TN. 1373–1383. 2004

Lyytinen, A., Lindstrom, L., Mappes, J., Julkunen-Tiitto, R., Fasulati, S. R. and Tiilikkala, K., “Variability in host plant chemistry: behavioral responses and life-history parameters of the Colorado potato beetle (*Leptinotarsa decemlineata*)”, ***Chemecology*** 17: 51–56, 2007.

Manyangarirwa, W., Turnbull, M., McCutcheon, G.S. and Smith, JP., “Gene pyramiding as a Bt resistance management strategy: How sustainable is this strategy”, ***African Journal of Biotechnology*** 5(10): 81-785.2006.

Maqbool, S.B., Riazuddin, S., Loc, N.T., Gatehouse, A.M., Gatehouse, J.A. and Christou, P., “Expression of multiple insecticidal genes confers broad resistance against a range of different rice pests”, ***Molecular Breeding*** 7(1), 85-93, 2011.

McCormac, A.C., Fowler, M.R., Chen, D.F. and Elliott, M.C., “Efficient co-transformation of Nicotiana tabacum by two independent T-DNAs, the effect of T-DNA

size and implications for genetic separation", ***Transgenic Research*** 10(2), pp.143-155, 2001.

McGaughey, W.H. and Whalon, M.E., "Managing insect resistance to *Bacillus thuringiensis* toxins", ***Science-New York Then Washington***, 258, 1451-1451, 1992.

McGraw-Hill, C., "Statistix 8.1 (Analytical Software, Tallahassee, Florida)", ***Maurice/Thomas text***, 2008.

Mehrotra, M., Singh, A.K., Sanyal, I., Altosaar, I. and Amla, D.V., "Pyramiding of modified *cry1Ab* and *cry1Ac* genes of *Bacillus thuringiensis* in transgenic chickpea (*Cicer arietinum* L.) for improved resistance to pod borer insect *Helicoverpa armigera*", ***Euphytica*** 182(1), 87, 2011.

Mi, X., Ji, X., Yang, J., Liang, L., Si, H., Wu, J., Zhang, N. and Wang, D., "Transgenic potato plants expressing cry3A gene confer resistance to Colorado potato beetle", ***Comptes rendus biologies*** 338(7), 443-450, 2015.

Michaud, J.P., "Recombinant Protease Inhibitors in Plants" Austin: ***Landes Bioscience***. 2001.

Monnerat, R., Martins, E., Macedo, C., Queiroz, P., Praça, L., Soares, C.M., Moreira, H., Grisi, I., Silva, J., Soberon, M. and Bravo, A., "Evidence of field-evolved resistance of *Spodoptera frugiperda* to Bt corn expressing c*ry1F* in Brazil that is still sensitive to modified Bt toxins", ***PLoS one*** 10(4), e0119544, 2015.

Morin, S., Biggs, R.W., Sisterson, M.S., Shriver, L., Ellers-Kirk, C., Higginson, D., Holley, D., Gahan, L.J., Heckel, D.G., Carriere, Y. and Dennehy, T.J., "Three cadherin alleles associated with resistance to *Bacillus thuringiensis* in pink bollworm", ***Proceedings of the National Academy of Sciences***, 100(9), 5004-5009, 2003.

Mota-Sanchez, D., "Resistance and metabolism of imidacloprid in Colorado Potato Beetle, *Leptinotarsa decemlineta* Say (Coleoptera: Chrysomelidae)", ***Entomology*** 122. East Lansing: Michigan State University, 2003.

Mota-Sanchez, D., Hollingworth, R.M., Grafius, E.J. and Moyer, D.D., "Resistance and cross-resistance to neonicotinoid insecticides and spinosad in the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae)", ***Pest management science*** 62: 30–37, 2006.

Munib, M., Wani, A.R., Khan, S.H. and Abbas, A., "Pest Complex of Potato (*Solanum tuberosum* L.) with Special Reference to Management of White Grub in Northern Districts of Kashmir Valley", ***Journal of Agricultural Science*** 16;8(3):151, 2016.

Murashige, T. and Skoog F. "A revised medium for rapid growth and bio assays with tobacco tissue cultures". ***Physiologiae Plantarium*** 15: 473-497, 1962.

Naimov, S., Dukiandjiev, S. and De Maagd, R.A., "A hybrid *Bacillus thuringiensis* delta-endotoxin gives resistance against a coleopteran and a lepidopteran pest in transgenic potato", ***Plant Biotechnology Journal*** 1(1), 51-57, 2003.

Naimov, S., Weemen-Hendriks, M., Dukiandjiev, S. and de Maagd, R.A., "*Bacillus thuringiensis* delta-endotoxin Cry1 hybrid proteins with increased activity against the Colorado potato beetle", ***Applied and environmental microbiology***, *67*(11), 5328-5330. 2001.

Naimov, S., Zahmanova, G., Boncehva, R., Kostova, M., Minkov, I., Dukiandjiev, S. and De Maagd, R., "Expression of synthetic SN 19 hybrid delta-endotoxin encoding gene in transgenic potato", ***Biotechnology & Biotechnological Equipment*** 20(3), 38-41, 2006.

Ocelotl, J., Sánchez, J., Arroyo, R., García-Gómez, B.I., Gómez, I., Unnithan, G.C., Tabashnik, B.E., Bravo, A. and Soberón, M., "Binding and oligomerization of modified and native Bt toxins in resistant and susceptible Pink Bollworm", ***PloS One*** 3;10(12): e0144086, 2015.

Oerke, E.C., "Crop losses to pests", ***Journal of Agricultural Science***, 144, 31-43, 2006.

Oppert, B., Morgan, T.D., Culbertson, C. and Kramer, K.J., "Dietary mixtures of cysteine and serine proteinase inhibitors exhibit synergistic toxicity toward the red flour beetle, *Tribolium castaneum*", Comparative Biochemistry and Physiology Part C: ***Comparative Pharmacology,*** *105*(3), 379-385, 1993.

Örmeci Kart, M.Ç., Abay, C.F., Güngör, S. and Özer, Z., "Seed supply and seed preferences of potato farmers: Nigde Central and Izmir Odemis provinces", Scientific Papers: ***Management, Economic Engineering in Agriculture & Rural Development*** 17(2), 2017.

Perlak, F.J., Stone, T.B., Muskopf, Y.M., Petersen, L.J., Parker, G.B., McPherson, S.A., Wyman, J., Love, S., Reed, G., Biever, D. and Fischhoff, D.A., "Genetically improved potatoes: protection from damage by Colorado potato beetles", ***Plant molecular biology*** 22(2),313-321, 1993.

Rahbé, Y., Deraison, C., Bonadé-Bottino, M., Girard, C., Nardon, C. and Jouanin, L., "Effects of the cysteine protease inhibitor *Oryza cystatin* (OC-I) on different aphids and reduced performance of *Myzus persicae* on OC-I expressing transgenic oilseed rape", ***Plant science*** 164(4), 441-450, 2003.

Rao, A.Q., Bakhsh, A., Kiani, S., Shahzad, K., Shahid, A.A., Husnain, T. and Riazuddin, S., "The myth of plant transformation", ***Biotechnology advances*** 27(6), 753-763, 2009.

Ribeiro, A.P.O., Pereira, E.J.G., Galvan, T.L., Picando, M.C., Picoli, E.A.T., da Silva, D.J.H., Fári, M.G. and Otoni, W.C., "Effect of eggplant transformed with *Oryza cystatin* gene on *Myzus persicae* and *Macrosiphum euphorbiae",* ***Journal of Applied Entomolology***.;130:84–90. doi: 10.1111/j.1439-0418.2005.01021. x. 2006.

Rivnay, E., "External morphology of the Colorado potato beetle [*Leptinotarsa decemlineata* (Say)]", ***Journal of the New York Entomological Society*** 36: 125–145, 1928.

Rose, R.L. and Brindley, W.A., "An evaluation of the role of oxidative-enzymes in Colorado potato beetle resistance to carbamate insecticides", ***Pesticide Biochemistry and Physiology*** 23:74–84, 1985.

Roush, R.T. and Tingey, W.M., "Strategies for the management of insect resistance to synthetic and microbial insecticides", ***Advances in Potato Pest Biology and Management*** pp.237-254, 1994.

Roush, R.T., "Two–toxin strategies for management of insecticidal transgenic crops: can pyramiding succeed where pesticide mixtures have not?.", *Philosophical Transactions of the Royal Society of London B:* ***Biological Sciences***, *353*(1376), 1777-1786. 1998.

Salim, M., Gökçe, A., Naqqash, M.N., Bakhsh, A., "An overview of biological control of economically important lepidopteron pests with parasitoids", ***Journal of Entomology and Zoological studies*** 4(1): 354-362, 2016.

Saljoqi, A.U.R., Salim, M., Khalil, S.K. and Khurshid, I., "Field Application of *Trichogramma chilonis* (Ishii) for the management of Sugarcane Borers", ***Pakistan Journal of Zoology*** 47(3), 2015.

Schlüter, U., Benchabane, M., Munger, A., Kiggundu, A., Vorster, J., Goulet, M.C., Cloutier, C. and Michaud, D., "Recombinant protease inhibitors for herbivore pest control: a multitrophic perspective", ***Journal of experimental botany***, *61*(15), 4169-4183. 2010.

Scott, G. J., Rosegrant, M. W. and Ringler, C., "Global projections for root and tuber crops to the year 202", ***Food Policy*** 25: 561-597, 2000.

Sezen, K., Muratoglu, H., Nalcacioglu, R., Mert, D., Demirbag, Z. and Kati, H., "Highly pathogenic *Bacillus thuringiensis* subsp. *tenebrionis* from European shot-hole borer, Xyleborusdispar (Coleoptera: Scolytidae)", ***New Zealand Journal of Crop and Horticultural Science*** 36:1, 77-84, DOI: 10.1080/01140670809510223, 2008.

Shelton, A.M., Tang, J.D., Roush, R.T., Metz, T.D. and Earle, E.D., "Field tests on managing resistance to Bt-engineered plants", ***Nature Biotechnology*** 18:339–342, 2000.

Shelton, A.M., Zhao, J.Z. and Roush, R.T., "Economic, ecological, food safety, and social consequences of the deployment of *Bt* transgenic plants", ***Annual Review of Entomology*** 47(1), 845-881, 2002.

Siegel, J.P., “Bacteria. In Field Manual of Techniques in Invertebrate Pathology” (Lacey, L.L. and Kaya, H.K. eds), pp.209–230. Dordrecht, Netherlands: ***Kluwer Scientific Publishers,*** 2000.

Silcox, C.A., Ghidiu, G.M. and Forgash, A.J., “Laboratory and field evaluation of piperonyl butoxide as a pyrethroid synergist against the Colorado potato beetle (Coleoptera: Chrysomelidae)”, ***Journal of Economic Entomology*** 78: 1399–1405, 1985.

Smigocki, A.C., Ivic-Haymes, S., Li, H. and Savić, J., “Pest protection conferred by a Beta vulgaris serine proteinase inhibitor gene” ***PloS one***, *8*(2), e57303. 2013.

Smith, J.J., Herzig, V., King, G.F. and Alewood, P., “The insecticidal potential of venom peptides”, ***Cellular and Molecular Life Sciences*** 70(19), pp.3665-3693, 2013.

Smith, J.L., Lepping, M.D., Rule, D.M., Farhan, Y. and Schaafsma, A.W., “Evidence for field-evolved resistance of *Striacosta albicosta* (Lepidoptera: Noctuidae) to Cry1F *Bacillus thuringiensis* protein and transgenic corn hybrids in Ontario, Canada”, ***Journal of economic entomology*** 110(5), 2217-2228, 2017.

Soberon, M., Gill, S.S. and Bravo, A., “Signaling versus punching hole: How do Bacillus thuringiensis toxins kill insect midgut cells?”, ***Cellular and Molecular Life Sciences*** 1;66(8):1337-49, 2009.

Soberón, M., Pardo-López, L., López, I., Gómez, I., Tabashnik, B.E. and Bravo, A., “Engineering modified Bt toxins to counter insect resistance”, ***Science***.318, 1640–1642, 2007.

Soderlund, D.M., Hessney, CW. and Jiang. M.G., “Metabolism of fenvalerate by resistant Colorado potato beetles adults”, Abstracts of Papers of the ***American Chemical Society*** 186: 28– PEST, 1983.

Sohail, M.N., Karimi, S.M., Asad, S., Mansoor, S., Zafar, Y. and Mukhtar, Z., “Development of broad-spectrum insect-resistant tobacco by expression of synthetic cry1Ac and cry2Ab genes”, ***Biotechnology Letter V***. 34:1553–1560, 2012.

Soto, N., Enríquez, G.A., Ferreira, A., Corrada, M., Fuentes, A., Tiel, K. and Pujol, M., "Efficient transformation of potato stems segments from cultivar 120 Désirée using phosphinothricin as selection marker", ***Biotecnología Aplicada*** 24(02), 139-144, 2007.

Stankovic, S., Zabel, A., Kostic, M., Manojlovic, B. and Rajkovic, S., "Colorado potato beetle [*Leptinotarsa decemlineata* (Say)] resistance to organophosphates and carbamates in Serbia", ***Journal of Pest Science*** 77: 11–15, 2004.

Storer, N.P., Babcock, J.M., Schlenz, M., Meade, T., Thompson, G.D., Bing, J.W. and Huckaba, R.M., "Discovery and characterization of field resistance to Bt maize: *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Puerto Rico", ***Journal of economic entomology*** 103(4), 1031-1038, 2010.

Sunilkumar, G., Mohr, L., Lopata-Finch, E., Emani, C. and Rathore, K. S., "Developmental and tissue-specific expression of CaMV 35S promoter in cotton as revealed by GFP", ***Plant molecular biology*** 50(3), 463-479, 2002.

Tabashnik, B.E. and Carrière, Y., "Surge in insect resistance to transgenic crops and prospects for sustainability", ***Nature Biotechnology*** 35(10), p.926. 2017.

Tabashnik, B.E., "ABCs of insect resistance to Bt", ***PLoS genetics*** 11(11), p.e 1005646. 2015.

Tabashnik, B.E., Brevault, T. and Carrière, Y., "Insect resistance to Bt crops: lessons from the first billion acres", ***Nature Biotechnology*** 31, 510–521, 2013.

Tabashnik, B. E., Mota-Sanchez, D., Whalon, M. E., Hollingworth, R. M. and Carrière, Y., "Defining terms for proactive management of resistance to Bt crops and pesticides" ***Journal of economic entomology*** 107, 496–507. 2014.

Tabashnik, B.E., Sisterson, M.S., Ellsworth, P.C., Dennehy, T.J., Antilla L., Liesner, L., Whitlow, M., Staten, R.T., Fabrick, J.A., Unnithan, G.C. and Yelich, A.J., "Sup pressing resistance to Bt cotton with sterile insect releases", ***Nature Biotechnology*** 28, 1304–1307, 2010.

Tanpure, R.S., Barbole, R.S., Dawkar, V.V., Waichal, Y.A., Joshi, R.S., Giri, A.P. and Gupta, V.S., "Improved tolerance against *Helicoverpa armigera* in transgenic tomato over-expressing multi-domain proteinase inhibitor gene from *Capsicum annuum*", ***Physiology and Molecular Biology of Plants*** 23(3), 597-604, 2017.

Tauber, M.J. and Tauber, C.A., "Prolonged dormancy in *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae): a ten-year field study with implications for crop rotation", ***Environmental Entomology*** 31(3), 499-504, 2002.

Tester, M. and Langridge, P., "Breeding technologies to increase crop production in a changing world", ***Science*** 327(5967), 818-822, 2010.

Thiele, G., Theisen, K., Bonierbale, M. and Walker, T., "Targeting the poor and hungry with potato science", ***Potato Journal*** 37: 75-86. 2010.

Thomson, J.M., Lafayette, P.R., Schmidt, M.A. and Parrott, W.A., "Artificial gene-clusters engineered into plants using a vector system based on intron-and intein-encoded endonucleases", ***In Vitro Cellular & Developmental Biology-Plant*** 38(6), .537-542. 2002.

TÜİK, 2015, Türkiye İstatistik Kurumu, Web: ***http://www.tuik.gov.tr/*** UstMenu.do?metod=kategorist (Erişim:20.10.2015).

Urwin, P.E., Atkinson, H.J., Waller, D.A. and McPherson, M.J., "Engineered O*ryza cystatin*-I expressed in transgenic hairy roots confers resistance to *Globodera pallida*", ***Plant Journal*** 8: (1), 121-131. 1995.

Vachon, V., Laprade, R. and Schwartz, J.L., "Current models of the mode of action of *Bacillus thuringiensis* insecticidal crystal proteins: a critical review", ***Journal of Invertebrate Pathology*** 111, 1–12. 2012.

Vaneva-Gancheva, T. and Dimitrov, Y., "Chemical control of the potato tuber moth *Phthorimaea operculella* (Zeller) on tobacco", ***Bulgarian Journal of Agricultural Science*** 19(5), 1003-1008, 2013.

Veale, M.A., Slabbert, M.M. and Van Emmenes, L., "*Agrobacterium*-mediated transformation of potato cv. Mnandi for resistance to the potato tuber moth (*Phthorimaea operculella*)", ***South African journal of botany*** 80, 67-74, 2012.

Vincent, C., Alyokhin, A. and Giordanengo, P., "Potatoes and their Pests–Setting the Stage", ***Insect Pests of Potato*** 1, 2013.

Voss, R.H., Ferro, D.N. and Logan, J.A., "Role of reproductive diapause in the population-dynamics of the Colorado potato beetle (Coleoptera, Chrysomelidae) in western Massachusetts", ***Environmental Entomology*** 17:863–871, 1988.

Wang, L., Ma, Y., Wan, P., Liu, K., Xiao, Y., Wang, J., Cong, S., Xu, D., Wu, K., Fabrick, J. A. and Li, X., "Resistance to *Bacillus thuringiensis* linked with a cadherin transmembrane mutation affecting cellular trafficking in pink bollworm from China", ***Insect Biochemistry and Molecular Biology*** 1;94:28-35, 2018.

Weber, D.C., Ferro, D.N., Buonaccorsi, J. and Hazzard, R.V., "Disrupting spring colonization of Colorado potato beetle to nonrotated potato fields", ***Entomologia experimentalis et applicata*** 73(1), 39-50, 1994.

Weisz, R., Smilowitz, Z. and Christ, B., "Distance rotation and border crops affect Colorado potato beetle (Coleoptera: Chrysomelidae) colonization and population density and early blight (*Alternaria solani*) severity in rotated potato fields", ***Journal of Economic Entomology*** 723-729, 1994.

Wharton, D.R.A., Lola, J.E. and Wharton, M.L., "Growth factors and population density in the American cockroach, *Periplaneta Americana*", ***Journal of Insect Physiology*** *14*(5), 637-653, 1968.

Wierenga, J.M. and Hollingworth, R.M., "Inhibition of altered acetylcholine esterases from insecticide-resistant Colorado potato beetles (Coleoptera: Chrysomelidae)", ***Journal of Economic Entomology*** 86: 673–679, 1993.

Wierenga, J.M. and Hollingworth, R.M., "The role of metabolic enzymes in insecticide-resistant Colorado potato beetles", ***Pesticide science*** 40:259–264, 1994.

Windley, M.J., Herzig, V., Dziemborowicz, S.A., Hardy, M.C., King, G.F. and Nicholson, G. M., "Spider-venom peptides as bioinsecticides", ***Toxins*** 4(3), 191-227. 2012.

Windley, M.J., Herzig, V., Dziemborowicz, S.A., Hardy, M.C., King, G.F. and Nicholson, G.M., "Spider-venom peptides as bioinsecticides", ***Toxins*** 4(3), 191-227, 2012.

Womack, J.S., Randall, J. and Kemp, J.D., "Identification of a signal peptide for oryzacystatin-I", ***Planta*** 210(5), 844-847, 2000.

Womack, J.S., Randall, J., Kemp, J.D., "Identification of a signal peptide for *Oryza cystatin*-I", ***Planta*** 210, 844–847. 2000.

Yang, Z., Chen, H., Tang, W., Hua, H. and Lin, Y., "Development and characterization of transgenic rice expressing two *Bacillus thuringiensis* genes", ***Pest management science*** 67:414–422, 2011.

Yas, B. and Güngör, M.A., "Determination of life table and biology of Colorado potato beetle, *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae), feeding on five different potato varieties in Turkey", ***Applied Entomology and Zoology*** 40(4), pp.589-596.

Ye, X., Al-Babili, S., Kloti, A., Zhang, J., Lucca, P., Beyer, P. and Potrykus, I., "Engineering the provitamin A (b-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm" ***Science*** 287:303–305, 2000.

Yocum, G.D., Rienhart, J.P. and Larson, M.L., "Monitoring diapause development in the Colorado potato beetle, *Leptinotarsa decemlineata*, under field conditions using molecular biomarkers", ***Journal of Insect Physiology*** 57: 645-652, 2011.

Yoon, K.S., Nelson, J.O. and Clark, J.M., "Selective induction of abamectin metabolism by dexamethasone, 3-methylcholanthrene, and phenobarbital in Colorado potato beetle, *Leptinotarsa decemlineata* (Say)", ***Pesticide Biochemistry and Physiology*** 73: 74–86, 2002.

Zhang, X., Candas, M., Griko, N. B., Rose-Young, L. and Bulla, Jr, LA., "Cytotoxicity of *Bacillus thuringiensis* Cry1Ab toxin depends on specific binding of the toxin to the cadherin receptor BT-R 1 expressed in insect cells", ***Cell death and differentiation*** 12(11), 1407, 2005.

Zhang, X., Candas, M., Griko, N.B., Taussig, R. and Bulla, L.A., "A mechanism of cell death involving an adenylyl cyclase/PKA signaling pathway is induced by the Cry1Ab toxin of *Bacillus thuringiensis*", ***Proceedings of the National Academy of Sciences*** 103(26), 9897-9902, 2006.

Zhao, J.Z, Cao, J., Collins, H.L., Bates, S. L., Roush, R.T., Earle, E.D. and Shelton, A.M., "Concurrent use of transgenic plants expressing a single and two *Bacillus thuringiensis* genes speeds insect adaptation to pyramided plants", ***Proceedings of the National Academy of Sciences*** 102:8426–8430, 2005.

Zhao, J.Z., Cao, J., Li, Y., Collins, H.L., Roush, R.T., Earle, E.D. and Shelton, A.M., "Transgenic plants expressing two *Bacillus thuringiensis* toxins delay insect resistance evolution", ***Nature Biotechnology*** 21:1493–1497, 2003.

Zhao, J-Z., Bishop, B. A. and Grafius, E. J., "Inheritance and synergism of resistance to imidacloprid in the Colorado potato beetle (Coleoptera: Chrysomelidae)", ***Journal of economic entomology*** 93:1508–1514, 2000.

Zhao, Y., Botella, M.A., Subramanian, L., Niu, X., Nielsen, S.S., Bressan, R.A. and Hasegawa, P.M., "Two wound-inducible soybean cysteine proteinase inhibitors have greater insect digestive proteinase inhibitory activities than a constitutive homolog" ***Plant Physiology*** 111:1299-1306, 1996.

Zhou, Z., Pang, J., Guo, W., Zhong, N., Tian, Y., Xia, G. and Wu, J., "Evaluation of the resistance of transgenic potato plants expressing various levels of Cry3A against the Colorado potato beetle (*Leptinotarsa decemlineata* Say) in the laboratory and field", ***Pest management science*** 68(12), 1595-1604, 2012.

Zhu, K.Y., Lee, S.H. and Clark, J.M., “A point mutation of acetylcholinesterase associated with azinphosmethyl resistance and reduced fitness in Colorado potato beetle”, ***Pesticide Biochemistry and Physiology*** 55:100–108. [PubMed], 1996.

Zukoff, S.N., Ostlie, K. R., Potter, B., Meihls, L.N., Zukoff, A.L., French, L., Ellersieck, M.R., Wade, F.B. and Hibbard, B.E., “Multiple assays indicate varying levels of cross resistance in Cry3Bb1-selected field populations of the western corn rootworm to mCry3A, eCry3. 1Ab, and Cry34/35Ab1”, ***Journal of Economic Entomology*** 109(3), 1387-1398, 2016.

Appendix-A

SSTE Buffer

10 mM Tris-HCl, pH 8.0

1 M NaCl

1 mM EDTA

0.5% sodium dodecyl sulfate

Use RNase-free water and autoclave before using.

Appendix-B

MS Medium (Murashige and Skoog, 1962) Composition

MS Salts with vitamins	4.4g /L
Sucrose	30g/ L
Plant Agar	7g/ L
pH 5.5-5.7	

Appendix-C

LB (Lauria-Bertani) Agar (1L)

Agar 28 g/ L of deionized water (Distilled water)

Appendix-D

Co cultivation medium

MS Salts with vitamins	4.4g /L
Sucrose	30g/ L
Plant Agar	7g/ L
pH 5.5-5.7	
Acetosyringone 5	0mM/L

Appendix-E

Regeneration Selection Medium

MS Salts with vitamins	4.4g /L
Sucrose	30g/ L
Plant Agar	7g/ L
pH	5.5-5.7
BAP	2mg/ L
NAA	0.2mg/ L
Kinetin	1mg/L
Trans zeatin	2mg/ L
Hygromycin	4mg/ L
Sulcid	500mg/ L

Appendix-F

Shoot Induction Medium

MS Salts with vitamins	4.4g /L
Sucrose	30g/ L
Plant Agar	7g/ L
pH	5.5-5.7
BAP	2mg/ L
NAA	0.2mg/ L
Gibberellic acid (GA3)	0.1mg/ L
Hygromycin	2mg/ L
Sulcid	400mg/ L

Appendix-G

DNA Extraction Buffer

0.2M Tris-HCl pH 8.0

2% PVP

5mM EDTA pH 8.0

0.2% Mercaptoethanol

0.5M Glucose

Appendix-H

TE Buffer (Tris EDTA Buffer)

1mM Tris-HCl, pH 8.0

1mM EDTA, pH 8.0

Appendix-I

Protein Extraction Buffer

0.5M EDTA	0.4mL
1M TrisCl	0.05mL
NH4Cl	26.7mg
5M NaCl	0.15mL
PMSF	2mM
Glycerol	0.5mL
DTT	15mg

Appendix-J

PBS Buffer

Na2HPO4	1.44 g
NaCl	8.00 g
KCl	0.2 g
KH2PO4	0.24 g

Dissolve in 1 liter of distilled water; adjust pH to 7.4 and autoclaved.

Appendix-K

10x SSC (Saline-Sodium Citrate)

Na-Citrate	88.2g
NaCl	175.3g

Adjust pH to 7.0 with NaOH and make up volume to 2 litter with distilled water.

CURRICULUME VITAE

Muhammad SALIM was born on November 06, 1984 in Mardan, Pakistan. He completed his secondary education from Pasban Model School, Shahbaz Garhi in 2001. Afterwards, he joined Aziz Bhatti Shaheed Army College, Mardan, Pakistan and completed his higher secondary education in 2003. Then he was enrolled in The University of Agriculture, Peshawar for his undergraduate studies. He completed his B.Sc. (Hons) from Department of Plant Protection, The University of Agriculture, Peshawar Pakistan in 2008. He completed his M.Sc. (Hons) from the same department in 2010. He was awarded double Gold medals upon successful completion of his M.Sc. (Hons) degree program.

He got admission in Graduate School of Natural and Applied Sciences, Department of Plant Production and Technologies at Niğde Ömer Halisdemir University, Niğde, Turkey in January 2015 to pursue his PhD education under the supervision of Prof. Dr. Ayhan GÖKÇE. During his PhD thesis research, he worked on pyramiding of insecticidal genes in potato to encode resistance against Colorado potato beetle, *Leptinotarsa decemlineata* (say), (Coleoptera: Chrysomelidae). He knows English, Urdu and Pashto languages.