T.R.
NİĞDE ÖMER HALİSDEMİR UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF AGRICULTURAL GENETIC ENGINEERING

OVEREXPRESSION OF JABURETOX 2-Ec in POTATO (*Solanum tuberosum* L.)

MERVE TEKİNSOY

February, 2022

T.R.
NİĞDE ÖMER HALİSDEMİR UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCE
DEPARTMENT OF AGRICULTURAL GENETIC ENGINEERING

OVEREXPRESSION OF JABURETOX 2-Ec in POTATO (*Solanum tuberosum* L.)

MERVE TEKİNSOY

MSc Thesis

Supervisor

Prof. Dr. Mehmet Emin ÇALIŞKAN

February, 2022

The study entitled "**Overexpression of Jaburetox 2-Ec in Potato**" and presented by **Merve TEKİNSOY** under the supervision of **Prof. Dr. Mehmet Emin ÇALIŞKAN** has been accepted as Master thesis by the jury at the Department of Agricultural Genetic Engineerig of Niğde Ömer Halisdemir University, Graduate School of Natural and Applied Sciences.

Head : Prof. Dr. Mehmet Emin ÇALIŞKAN
Niğde Ömer Halisdemir University, Faculty of Agricultural Sciences and Technologies, Department of Agricultural Genetic Engineering

Member : Assoc. Prof. Dr. Ufuk DEMİREL
Niğde Ömer Halisdemir University, Faculty of Agricultural Sciences and Technologies, Department of Agricultural Genetic Engineering

Member : Assoc. Prof. Dr. Kahraman GÜRCAN
Erciyes University, Faculty of Agriculture, Department of Agricultural Biotechnology

CONFIRMATION

This thesis has been found appropriate on this date of …./…./2021 by the jury member above who have been designated by the Board of Directors of Graduate School of Natural and Applied Sciences and has been confirmed with the resolution of Board of Directors on …./…./2021 and has been accepted by the decision numbered ….....

...../...../20...
Prof. Dr. Murat GÖKÇEK
MÜDÜR

THESIS CERTIFICATION

I certify that this thesis has been written by me. I proved that all informations exist in this thesis is scientific and is in accordance with the academic acceptance. Any kind of help and providing of stuffs while conducting thesis research have been acknowledged in thethesis.

Merve TEKİNSOY

SUMMARY

OVEREXPRESSION OF JABURETOX 2-EC IN POTATO (*Solanum tuberosum* L.)

TEKİNSOY, Merve
Niğde Ömer Halisdemir University
Graduate School of Natural and Applied Sciences
Department of Agricultural Genetic Engineering

Supervisor : Prof. Dr. Mehmet Emin ÇALIŞKAN

February 2022, 60 pages

In this study, it was aimed to develop transgenic potato genotypes resistant to *Tuta absoluta* pest by increasing the expression of Jaburetox 2-Ec peptide in potato plant.The gene cassette containing the target peptide sequence under the control of 35S promoter was transferred to potato cultivar Lady Olympia by *Agrobacterium*-mediated gene transfer method. During the experiment, the leaf and internode parts of the plant were used to infect with *Agrobacterium tumefaciens* strain (EHA 105). Neomycin phosphotransferase (*nptII*) gene with 50 mg L-1 concentration was used as a selective marker for transgenic plants and bacteria. As a result of molecular analyzes, it was shown that the T-DNA region containing the target sequence was integrated into the plant genome. Then, the resistance of transgenic potato plants to second, third and fourth instar larvae of *Tuta absoluta* was tested using leaf feeding bioassay method. As a result, mortality rate of larvae fed on transgenic plants ranged from 11% to 33% depending on the larval stage. In addition, it was determined that the weight gain in larvae fed with control plants was higher than the larvae fed with transgenic plants.As a result of this thesis, it was concluded that Jaburetox 2-Ec has an insecticidal activity against *Tuta absoluta* pest, but more research is needed to confirm the initial results.

Keywords: Gene transfer, jaburetox, trangenic plant, insect resistant

ÖZET

PATATESTE (*Solanum tuberosum* L.) JABURETOX 2-EC'NIN İFADESININ ARTIRILMASI

TEKİNSOY, Merve
Niğde Ömer Halisdemir Üniversitesi
Fen Bilimleri Enstitüsü
Tarımsal Genetik Mühendisliği Bölümü

Danışman : Prof. Dr. Mehmet Emin ÇALIŞKAN

Şubat 2022, 60 sayfa

Bu çalışmada patates bitkisinde Jaburetox 2-Ec peptidinin ifadesinin artırılarak *Tuta absoluta* zararlısına karşı dirençli transgenik patates genotiplerinin geliştirilmesi amaçlanmıştır. Bu amaçla, 35S promotoru kontrolündeki hedef peptit dizisini içeren gen kaseti, *Agrobakterium*-aracılı gen transferi yöntemiyle Lady Olympia çeşidine aktarılmıştır. Deney yürütülürken bitkinin yaprak ve interned kısımları kullanılarak Agrobakterium (EHA 105) ile enfeksiyon sağlanmıştır.Transgenik bitki ve bakteri seçici markörü olarak 50 mg L^{-1} konsantrasyonlu Neomisin fosfotransferaz (nptII) geni kullanılmıştır.Yapılan moleküler analizler sonucunda hedef diziyi içeren T-DNA bölgesinin bitki genomuna entegre olduğu gösterilmiştir. Ardından yaprak besleme yöntemi ile transgenik bitkilerin ikinci, üçüncü ve dördüncü evre *Tuta absoluta* larvalarına karşı direnci test edilmiştir. Sonuçta transgenik bitkilerle beslenen larvalarda, larval döneme bağlı olarak 11-33 % arasında ölüm oranı tespit edilmiştir. Ayrıca control bitkilerle beslenen larvalarda ağırlık artışının transgenik bitkilerle beslenen larvalara kıyasla daha yüksek olduğu belirlenmiştir. Bu tez çalışması sonucunda, Jaburetox 2-Ec' nin *Tuta absoluta* zararlısına karşı insektisidal aktiviteye sahip olduğu, ancak bu etkinin doğrulanması açısından daha fazla araştırmaya ihtiyaç olduğu sonucuna varılmıştır.

Anahtar Sözcükler: Gen transferi, jaburetox, trangenik bitki, böcek direnci

ACKNOWLEDGEMENT

I would like to thank to Assoc. Prof. Dr. Allah BAKHSH who helped and guided me to improve and conduct my thesis. Additioanly, pBIN61 expression vector carrying Jaburetox 2-Ec was developed in Prof. Claudio Ratti Lab at Department of Agricultural Sciences, University of Bologna, Italy by Dr. Allah Bakhsh.

I am very grateful to my supervisor Prof. Dr. Mehmet Emin ÇALIŞKAN who supportedand guided me during my Master as well as provided opportunities to complete my degree.

I cordially thank to committee members, Assoc. Prof. Dr. Kahraman GÜRCAN and Assoc. Prof. Dr. Ufuk DEMİREL who helped to improve my thesis with their suggestions.

I would like to special thanks to Rabia Busenaz KAYA, Dudu BOYVAT and Büşra KARADUMAN who always support me as moral in each condition. This period was easier with their kind friendship.

I thank to Muneeb Hassan HASHMI who collected and maintained *Tuta absoluta* insects and guided me during insect analysis.

I really appreciate to AGE lab members; Ress.Asst. Caner YAVUZ, Binnur YEŞİL, Faisal SAEED and Beyza YÜKSEL for their encouragements.They were like my second family.

I dedicate this thesis to my father Ahmet TEKİNSOY, my motherHatice TEKİNSOY, my brothers Emre TEKİNSOY and Enes TEKİNSOY who always support and believe me during my entire educational and social life.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

SYMBOLS AND ABBREVIATION

Symbols	Description
%	Percentage
µ	Micro
µL	Microliter
µM	Micromolar
µmol	Micromole
bp	Basepair
g	Gram
g/L	Gram perliter
h	Hour
L	Liter
M	Molar
mg	Milligram
mg/L	Milligrams perliter
min	Minute
mL	Milliliter
ng	Nanogram
ng/µL	Nanogram permicroliter
^{o}C	Degree celsius
rpm	Revolutions perminute
sec	Second

Abbreviation	Description
2, 4-D	2,4-dichlorophenoxyacetic
BAP	6-Benzylaminopurine
CNTX	Canatoxin
CTAB	Cetly Trimethyl Ammonium Bromide
ddH2O	Double-Distilled Water
DNA	Deoxyribonucleic Acid

EDTA	Etilendiamin Tetra Acetic Acid
FAOSTAT	Food and Agriculture Organization Statistical Databases
GA3	Gibberellic Acid
JBTX	Jaburetox
LB	Luria-Bertani Medium
NAA	1-Naphthaleneacetic Acid
NOS	Nopaline Synthase
nptII	Neomycin Phosphotransferase
PCR	Polymerase Chain Reaction
pH	Potential of Hydrogen
RSM	Regenaration Selection Media
SIM	Shoot Induction Media
TBE	Tris/Borate/EDTA
UV	Ultraviolet

CHAPTER I

INTRODUCTION

Potato (*Solanum tuberosum* L.) is a member of the *Solanaceae* family (Aksoy et al., 2021)**.** The basic chromosome number of potato is n=12 and cultivated potato species can carry diploid, triploid, tetraploid or pentaploid sets of chromosomes (Aksoy et al., 2021). It is estimated that the domestication of potato began almost 6.000 – 10.000 years ago in the vicinity of Peru and Bolivia (Çalışkan, 2020). Potato ranked 4th worldwide after wheat, rice, and maize (corn) (Zaheer and Akhtar, 2016). Today, it is cultivated in nearly all over the world (Haverkort et al., 2009). Top five producer countries can be listed as China, India, Russia, USA and Ukraine (FAOSTAT, 2021). In the world, total production of potatoes were 359,7 tonnes in 2020 and the harvested area was almost 16,494 million hectare (FAOSTAT, 2021). In Turkey, cultivated area was 141,000 hectare and production was 5, 1 million tonnes in 2021 (TÜİK, 2021).

There are numerous insect pests that damage potato such as potato tuber moth, Colorado potato beetle (CPB) etc. (Visser, 2005; Rondon, 2010). On the other hand, *Tuta absoluta* is known as a major pest of tomato however it attacks other *Solanaceous* family members including potato (KanleSatishchandra et al., 2019). The pests and diseases pose a continuous threat to crop plants leading to 37% losses of agricultural production in the world (Gatehouse et al., 1992). If these pests are not controlled, they can completely destroy potatoes. Management of these pests rely on synthetic insecticides but this leads to serious harm to the environment (Vincent et al., 2013).

Usage of insecticides have failed over time because insects have improved resistance against chemicals. Due to this reason new methods have been improved to menage of insects (Balaško et al., 2020). Adoption of GM plants including insect-resistant genes such as *Bacillus thuringiensis* (Bt) toxins can be alternative to manage of insects. As an example, the first GM (genetically modified) potato has been introduced by Monsanto in 1995 that include Bt toxins. This biotech potato has been improved against Colorado potato beetle by using the CryIIIA gene (Halterman et al., 2016; Didoné et al., 2021). Nevertheless, the spreading of the Bt method in the world and usage of inadequate doses lead to improvement of resistance by insects. Due to this reason, a new

entomotoxic protein should be proposed instead of Bt toxin such as urease-derived peptide Jaburetox (Didoné et al., 2021).

Urease is a nickel-dependent metalloenzyme that catalyzes the hydrolysis of urea as ammonia and carbon dioxide. As a result of this reaction, two molecules of ammonia and one molecule of carbon dioxide are provided from urea. Many organisms such as plants, some bacteria, fungi, and invertebrates can synthesize urease (Sirko and Brodzik, 2000).

Ureases have a significant role in germination and seedlings nitrogen metabolism (Polacco and Holland, 1993). Jbure-I, jbure-IIb and jbure-III (canatoxin) were identified in *C. ensiformis*. Studies showed that Jbure-III (canatoxin) is one of the toxic proteins that negatively affect fungi and insects. Namely, canatoxin has fungitoxic and entomotoxic roles besides its ureoliytic roles (Becker-Ritt and Carlini, 2012).

However, canatoxin is low-expressed in organisms and learning of function can be laborious due to its expression level. In order to learn the function of this peptide, the urease-derived peptide, Jaburetox 2-Ec was improved by Mulinari et al. (2007), as a urease derived peptide. This peptide showed insecticidal behavior against insects such as *hemipterans* and *cockroaches.* Besides, Jaburetox 2-Ec reveals significantly negative effects against yeasts and filamentous fungi. In conclusion, Jaburetox 2-Ec can be considered as an alternative way for the management of pest and diseases while improving genetically modified (GM) crops (Sa et al., 2020).

The aim of this study is to improve the Lady Olympia cultivar that produces a new alternative entomotoxic peptide, Jaburetox 2-Ec, against Tutaabsoluta by using the *Agrobacterium*-mediated gene transfer technique. Consequently, the improvement of safe and eco-friendly insect-resistant products is among the goals.

CHAPTER II

LITERATURE REVIEW

2.1 Importance of Potato

Potato (*Solanum tuberosum* L.) is one of the most important crops after rice, wheat and maize for the human diet (Camire et al., 2009). The population of the world is continuously increasing, thus potato is being important crop thanks to its nutritional value (Brown, 2005). Potato tubers include carbohydrates, mineral elements (especially potassium), vitamins (especially C and B vitamins) and protein (Çalışkan, 2014). Therefore, potato consumption provides a good source of energy and other nutrients for human (Zaheer and Akhtar, 2016).

This crop originated from South America and spread to the world and it is cultivated by 160 countries (Camire et al., 2009). In the worldwide, total production of potatoes were 359, 7 tonnes in 2020 and the harvested area was almost 16, 494 million hectar (FAOSTAT, 2021). In recent decades, the top potatoes producers are listed as China and India (Figure 2.1.) **(**Ortiz and Mares, 2017). In Turkey, cultivated area was 1, 4 million decares and production was 5, 1 million tonnes in 2021 (TÜİK, 2021).

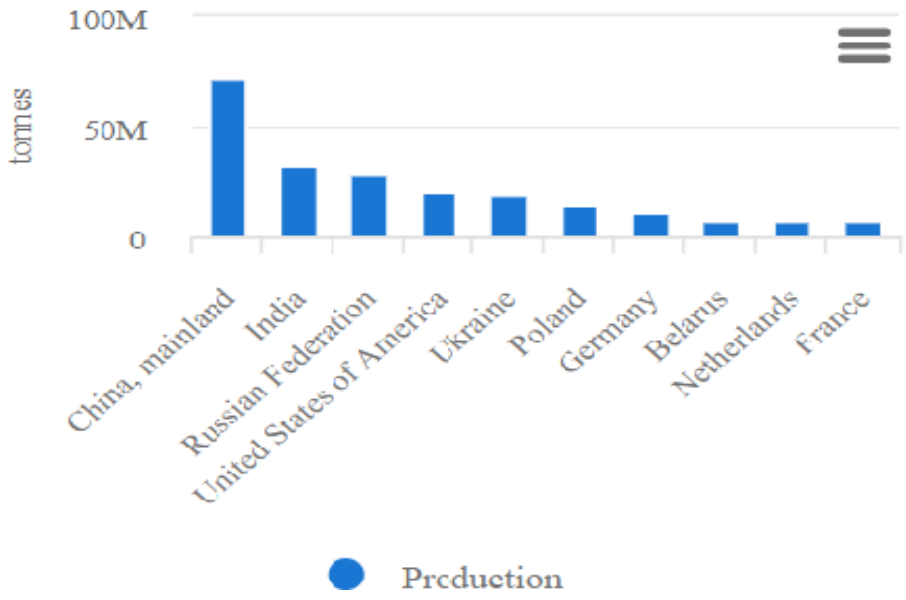

Figure 2.1. Average 1994 – 2020 (FAOSTAT, 2021)

The tetraploid potato is the most widely grown type of this crop. There are also diploid, triploid, and pentaploid species that are cultivated. Pentaploid and triploid species are grown only in the highlands of the Andes, while the diploid potato is the more common variety and used for breeding tetraploid species **(**Watanabe, 2015).

2.2 Biotic Strees Factors Affecting Potato

Biotic and abiotic stresses have a significant effect on crop yield, quality and food security. These stresses affect the physiological, biochemical and molecular structures of plant (Kumar and Verma, 2018). Plants are exposed to continuously changing conditions that are stressful and unfavorable so these conditions affect their growth and development. These unfavorable conditions can be divided into biotic stress (pathogens, herbivores, insects) and abiotic stress (heat, cold, drought, and high amounts of salt or toxic metals like aluminum, arsenate, and cadmium in the soil). The distribution of plants on the earth can be affected by natural conditions such as drought, salt, and temperature. These environmental conditions affect plant production and threaten food security (Jian, 2016).

Potato faces almost fifty different viruses in worldwide and some of them can cause major losses. PVY and PLRV are known as the most harmful viruses of potatoes. Both of them can cause tuber losses in single infections and their combination of infections can reach almost 80% losses compared with others. Single infection of PVX causes yield losses of almost 10–40% **(**Kreuze et al., 2019).

Fungal diseases can be listed as early blight, late blight, black scurf, dry rots and these diseases significantly damage to potato. For instance, late blight restricts potato production almost 15 % in worldwide. Late blight occurs first as to pale green lesions edges and tips of leaves. After growing these lesions, they become large brown spots. In addition, early blight can be described as a disease of potato and tomato which affect mature and old plants. Generally, early blight is seen in Africa, Asia, Europe, Australia, and South America. Yield losses of crop is almost 10 to 25 % annually due to early blight (Arora and Khurana, 2006).

On the other hand, there are numerous insect pests that attack to potato by damaging foliage and tubers (Grafius and Douches, 2008). Global losses can reach 16% and if these pests are not controlled, yield losses can be 30 to 70% in locally (Kroschel et al., 2019). Major pests of potato can be listed as; Colorado potato beetle, potato leafhoppers and wireworms etc. (Foster, 2017). Besides, *Tuta absoluta* is known as the major pest of tomato. However, it attacks other members of Solanaceae family such as potato. *Tuta absoluta* can decrease the yield of potatoes by feeding with leaves. Therefore, this harmful insect can be considered as one of the pests of potatoes (Desneux et al., 2010).

2.3 Tomato Leaf Miner (*Tuta absoluta*)

Tuta absoluta (Meyrick) (Lepidoptera: Gelechiidae), is known as a major pest of tomato and it has been introduced in 1917 by Meyrick. This pest is multivoltine species and it can develop easily and spread quickly because of its high reproductive trait (Tropea Garzia et al., 2012). However, it has negative effects on other species in the *Solanaceous* family such as potato, eggplant and tobacco besides tomato (Kanle Satishchandra et al., 2019). Potato is cultivated on open-field and this harmful pest can easily spread to nearby potato crops and cause important damages (Caparros Megido et al., 2013). Tomato leaf miner damage to crops during its larval stage after egg hatching. It builds tunnels within the crop, leaves, and young shoots and causes a significant reduction in yield (Son et al., 2017). The newly released larvae are very rapid and feed with leaves by building tunnels and cause foliar damage due to its mine formation in mesophyll. Females can lay almost 260 eggs and yield losses can reach almost 80-100% (Kroschel et al., 2019; Gebremariam, 2015).

2.4 *Tuta absoluta* Control Methods

Tuta absoluta management is difficult task because of its fast and diverse life cycle. Its adaptibility is easy as well. Management methods of *Tuta absoluta* can be listed as pheromone traps, biopesticides, chemical treatment, biological control and cultural practices (Kroschel et al., 2019).

Pheromones are chemicals released by females to attract males. Sex pheromone-based techniques can be listed as mass trapping and mating disruption. Some trap methods

were developed to use pheromones. A sticky surface is one of the most widely used trap. Additionally, the purpose of the mating disruption is to produce sexual confusion in males by using a synthetic female pheromone.Consequently, this technique prevents pest mating and, decreases the pest population (CaparrosMegido et al., 2013). Cultural control involves crop rotation instead of non-Solanaceous crops. Furthermore, nearby fields also should get rid of these crops because this pest can reinfect again (Illakwahhi and Srivastava, 2017). Biological control can be described as one of the most important alternatives to manage T.*absoluta*because it has already enemies in its place especially parasitoids (Urbaneja at al., 2012). Additionally, *Trichogrammatidae* eggs are also significant enemies of *Tuta absoluta* in the world and drastically reduce the population of tomato leaf miners (Kroschel et al., 2019).Usage of insecticide is adopted against *T. Absoluta.* Insecticides can be divided into three group as microbial, plant extracts and synthetic chemical (Mansour et al., 2018). However, insecticide approaches have the risk because target pests can develop resistance against insecticide (Roditakis et al., 2013).

These studies cause public concerns due to their harmful effect on the environment and human health. Besides, these methods are ineffective due to developing resistance by insects against insecticides although usage of the high amount of doses. Therefore, new tactics are needed.

2.5 Improvement of Effective Methods Against Insect Pests of Potato: Early Attempts and Some Transgenic Approaches

Plant breeding techniques work on increasing yield including environmental adaptability and against biotic stress factors. Basically, plant breeders focus on the increasing yield of agricultural products and creating a natural environment for sustainable agriculture (Charles Brummer et al., 2011). One of the hardest aspects of potato breeding is that describes of best individuals and combined them to obtain F1 progeny with high yield in a short time. Furthermore, other challenges include to development of populations and choosing a parent because genetic purity is significant for breeding **(**Bonierbale, 2019). While conducting potato breeding, different traits exist in two heterozygous tetraploid parents and breeding techniques try to shift desirable traits to offspring. Generally, progeny is checked in the field to get rid of unwanted

traits. But, this approach takes a long time (10-15 years) to eliminate undesirable traits **(**Halterman et al., 2016).

Additionally, the tetrasomic structure of potatoes is a barrier against breeding. In recent times, molecular breedingtechniques has provided rapid introduction of wanted genes to create that have high yield crops.Moreover, genetic engineering approach is a technique to create new genetic variability **(**Kumari et al., 1989). In the early 1980s, the essential approaches of plant genetic engineering has been improved and in the mid-1990s, the first genetically modified crops were adopted as a commercial product. After the adoption of GM crops, this technique has spread rapidly. The aim of the genetic engineering approach is not completely different from conventional breeding. However, genetic engineering allows gene transfer among species thus it is relatively easy method according to previous attempts to breed impossible genes (Qaim, 2009).

Several plant transformation techniques have been introduced for the improvement of GM products. For instance; particle bombardment / biolistics, silicon carbide fiber mediated gene transfer, microinjection, protoplast, transformation, electroporation, lipofection and *Agrobacterium*-mediated transformation (Keshavareddy et al., 2018).

Agrobacterium-mediated transformation is one of the most effective plants genetic engineering techniques. Researchers use this technique for the fast and more specific addition of desirable genes into the host genome. Naturally, *Agrobacterium* inserts its T-DNA into host plant cells and provides to keep stable via its evolved molecular mechanisms into the host genome (Walkerpeach and Velten, 1994). And the expression of T-DNA causes crown gall disease in plant cell (Deblaere et al., 1985). Application of *Agrobacterium*-mediated transformation requires only 6-12 months to insert of specific gene and obtain regeneration of the whole plant**.** Almost 150 kb of DNA can be inserted into the plant genome by allowing to insertion of multiple genes into the genome **(**Halterman et al., 2016).

Beaujean et al. (1998) worked with *Agrobacterium tumefaciens* for the development of an efficient protocol for transgenic potato. Three commercial varieties were used in this study. These can be listed as Desiree, Bintje and KaptahVandel. Internodal segments were used as explants. There were two media for callus induction. The first one

included BM+BAP (1 mg / l)+NAA (0.1 mg/ l)+GA3 (0.1 mg /l) and second one included BM+ZR (0.8 mg /l)+2.4-D (2 mg /l) for callus induction. C58C1Rif was used as an Agrobacterial strain**.** This gene was driven by TR1 promoter and npt II was used as a selectable marker gene.

Ahmed et al. (2017) worked on SN19 gene driven by wound-inducible and CaMV 35S promoter by using *Agrobacterium*-mediated approach in potato. Colorado potato beetle (Coleoptera) and tomato leaf miner (Lepidoptera) was used as target pests. Biotoxicity assays results showed a 100% mortality rate for both insect pests.

Selale et al. (2017) studied on modification of *Bacillus thuringiensis* (Bt) cry1Ac gene by using *Agrobacterium*-mediated transformation in tomatoes. *Tuta absoluta* mortality recorded as 38–100% in transgenic tomato lines. Additionally, gallery structure was decreased to 57–100% for transgenic plants.

Soliman et al. (2017) showed the effect Cry1Ab toxin protein against *Tuta absoluta* in tomato. *Agrobacterium*- mediated transformation approach has been used. pBI121 plasmid included insect resistance gene (cry1Ab) and kanamycin (nptII). Transgenic plants were tested for insect bioassay. The results showed that 100% instars larval mortality after feeding for 4 days.

Lecardonnel et al. (1998) improved a transgenic potato that expreses OCI (Oryzacystatin I) gene against CPB larvae. In order to perform of genetic transformation, *Agrobacterium tumefaciens* approach was used. Transgenic potatoes were improved that contain OCI cysteine protease inhibitors and kanamycin resistance. Results revealed that there is a significant effect of transgenic potato on CPB. According to records, the mortality of insects was almost 53%.

Gatehouse et al. (1998) investigated the function of concanavalin A (ConA) on *Lepidoptera* and *Homoptera* for potato. Construct was driven by CaMV 35S promoter to overexpression of ConA. Bioassay results show that the lectin provides to delay of larval development almost 45%. In addition, larval weights were decreased as well. As a result, there was no important effect on survival rates. However, it had a protective effect on potatoes.

Meiyalaghan et al. (2006) worked on cry1Ca5 and cry1Ba1 genes. Construct included these genes, CaMV 35S promoter and nptII as a selectable marker. This construct was shifted into the potato by using the *Agrobacterium*-mediated approach. Cry1Ca5-transgenic lines results show that there was almost 100% larval mortality on potato tuber moth in the greenhouse experiment while cry1Ba1-transgenic lines results were almost 50 to 100% on potato tuber moth. Eventually, both lines provide additional options to manage of potato tuber moth in potato.

Hussain et al. (2019) worked on (EcR) gene of CPB by using RNA interference technique on Colorado potato beetle. pRNAi-GG vector construct contained partial cDNA of EcR gene and this construct was driven by CaMV 35S promoter. Gene transferred into the potato by using the *Agrobacterium*-mediated approach.Leaf bioassay results show that there was almost 15–80% of CPB mortality. In addition, larval weight was drastically decreased compared to controls.

2.6 Urease and Urease Derived Peptide Jaburetox

2.6.1 Urease

Urease may be defined as a nickel-dependent metalloenzyme that catalyzes the hydrolysis of urea to form ammonia and carbon dioxide. As a result of this reaction that occurs two molecules of ammonia and one molecule of carbon dioxide from urea. Many organisms can synthesize urease such as plants, some bacteria, fungi and invertebrates. Firstly, plant urease was defined by using jack bean (Sirko and Brodzik, 2000).

Two isozymes were described for the soybean with 87% amino acid identity. This can be listed as embryo-specific urease and ubiquitous urease. Developing embryos synthesize the embryo-specific urease and it accumulates in the mature seed. Additionally, it is encoded by the GmEu1gene. Ubiquitous urease is found in all plant tissue and encoded by the GmEu4 gene (Wiebke-Strohm et al., 2012).

Three urease enzymes were defined in C. ensiformis (jackbean); (a) jbure-I, the major classic urease JBURE-I; (b) jbure-IIb, with 86% similarity to the classic urease and (c) jbure-III, or canatoxin (CNTX). The canatoxin is a toxic protein so it has defencerole.It

has been suggested that defense role is associated with both insecticidal and fungicidal properties (Becker-Ritt and Carlini, 2012).

2.6.2 Structure of urease

Studies have shown that plant and bacterial ureases have similar trimeric structures (Figure 2.2.). Polypeptide chains create monomer and their number can change according to the source of urease. Polypeptide chain is single for plant and fungal ureases. However its two subunits or three sorts of polypeptide chains for bacterial ureases.The most common structure of plant ureases is a dimer of trimers. However, dimeric, trimeric, tetrameric plant and fungi ureases have been introduced (Kappaun et al., 2018). In urease structure, one nickel atom binds to two histidine residues (His-246 and His-272), and the second nickel atom binds to three residues, two histidines (His-134 and His-136) and aspartic acid (Asp-360). (Figure 2.3.) Additionally, a carbamate ligand derived from Lys-217 bridges the two nickel ions. Participation of many accessory (or activatory) proteins is necessary while incorporating of nickel atom. These proteins are significant for producing an active urease in both plant and bacteria (Sirko and Brodzik, 2000).

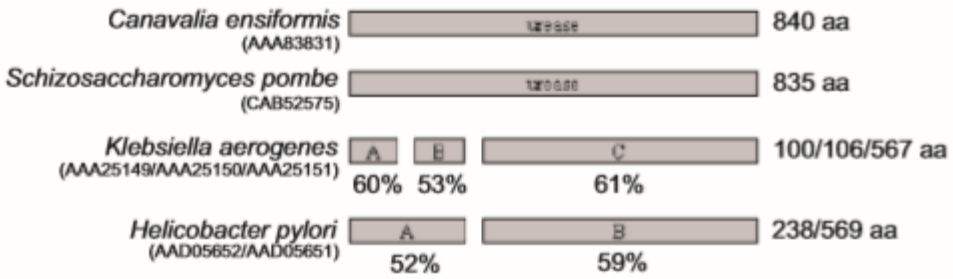

Figure 2.2. Comparision of urease (Sirko and Brodzik, 2000)

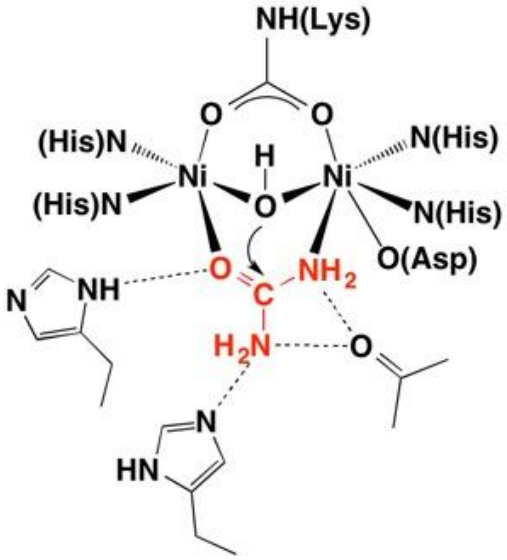

Figure 2.3. Structure of nickel-dependent metalloenzyme (Protopedia, 2020)

2.6.3 Nitrogen bioavalibility of ureases

Urea is originated from plant metabolite (Polacco and Holland, 1993) (Figure 2.4.). Urea is derived from arginine and possibly from the degradation of purines and ureides. Urea including nitrogen should be hydrolyzed by urease. Otherwise, it can not be used by plants. Incorporation of ammonium into organic compounds is performed by glutamine synthetase. The amount of glutamine and glutamine synthetase activity provides to control nitrogen metabolism. Therefore this activity significantly affects plantgrowth (Sirko and Brodzik, 2000).

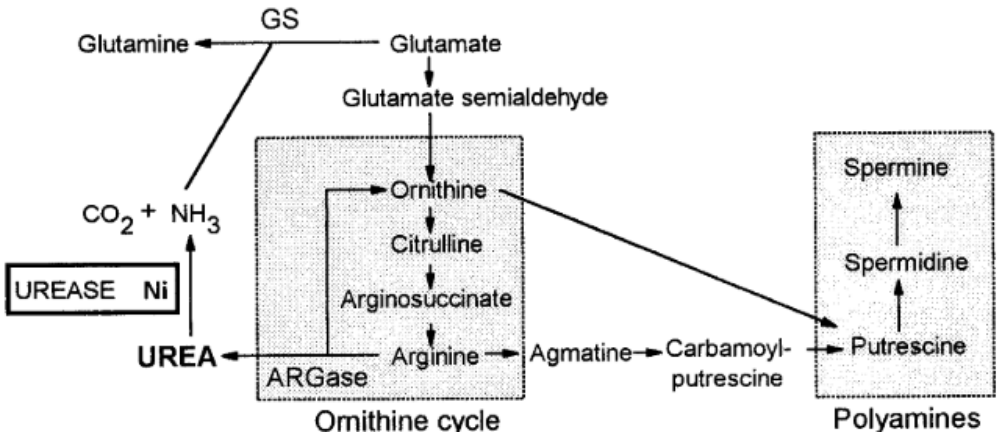

Figure 2.4. Ornithine cycle (Gerendas, 1998)

Urea metabolism in plants include three key enzymes which can be listed as arginase, urease and glutamine synthetase (Sirko and Brodzik, 2000). Firstly, urease provides to the utilization of urea for plants and microorganisms (Kumar, 2015). Additionally, urease has a significant role in germination and in seedlings' nitrogen metabolism. It works coordinately with arginase in the utilization of seed protein reserves during germination (Polacco and Holland, 1993).

2.6.4 Jaburetox (JBTX)

Thanks to cathepsin-like enzymes in the digestive system of insects such as *Callosobruchus maculatus, Dysdercusperuvianus and Oncopeltus fasciatus*, Canatoxin leads to entomotoxic effect by releasing up to 10 kDa internal peptide (Pepcanatox) (Sá et al, 2020).

In order to learn the activity of urease-derived peptides can be needed high amount of pepcanatox, therefore the conducting of the study would be diffucult due to few amount of urease. Hence, Jaburetox 2-Ec was produced in E.coli including V5 viral epitope and 6 His-tag N-terminal sequences of pepcanatox. JBU isoform II (JBURE-IIb) was a template for cDNA. The similarity of Jaburetox with plant ureases is 53% to 73% ranging (Figure 2.5.) (Mulinari et al, 2007). Scientists showed that Jaburetox has broad-spectrum insecticidal activity more than canatoxin such as against *Spodoptera* spp.. After that, Jaburetox was obtained containing 93 amino acid residues which are variants of Jaburetox-2Ec only without V5 viral epitope. Although there is no V5 epitope, Jaburetox showed a similar effect on the insect such as its other variants. These insects can be listed as *hemipterans O. fasciatus and cockroaches, Blatella germanica, Phoetalia pallidum, and Nauphoeta cinerea.* Moreover, Jaburetox has an effect on yeasts and filamentous fungi (Sá et al, 2020). According to these properties, Jaburetox would be an alternative for the management of agricultural pests to the improvement of genetically modified crops.

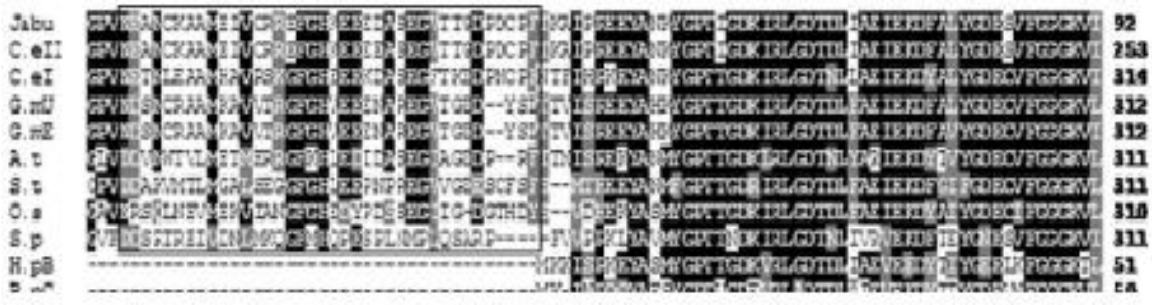

Figure 2.5. Alignment of Jaburetox sequence with plant, fungal and bacterial ureases (Mulinari et al., 2007)

2.6.5 Structure of jaburetox

Molecular modeling of Jaburetox 2-Ec was performed according to the b-hairpin motif at the C-terminal part. Because JBU also contains this motif in its structure. Therefore, it has been suggested that membrane disruptive activity is regulated by this motif (Figure 2.6.) (Kappaun et al, 2018).

Three mutated version was improved for this polypeptide. Actually, the aim of this study is to confirm the significance of the b-hairpin motif for this biological activity. These mutated versions can be listed as one missing the b-hairpin motif, and two additional peptides that suitable to the N-terminal and the C-terminal parts of the recombinant Jaburetox, respectively. The mutated version of Jaburetox without b-hairpin motif showed the same biological activity as wild-type Jaburetox. Moreover, experiments of *O. fasciatus* and *R. prolixus* showed that the source of insecticidal activity is the N-terminal part of Jaburetox. After that, *R. prolixus* was fed with both parts and showed almost the same mortality. Eventually, there are different potencies N- and C- terminal portions of this peptide that affect the neuromuscular activity of cockroach *Phoetalia pallida* nerve-coxal muscle preparation, while both peptides have an equal effect on the inhibiting of fluid secretion in the Malpighian tubules of *R. prolixus* and in disrupting LUV membranes. According to this information, N-terminal part of Jaburetox regulate the entomotoxic activity and C-terminal is also probably related to polypeptide activity by interacting with cell membranes (Lopes et al., 2015). (Figure 2.7.)

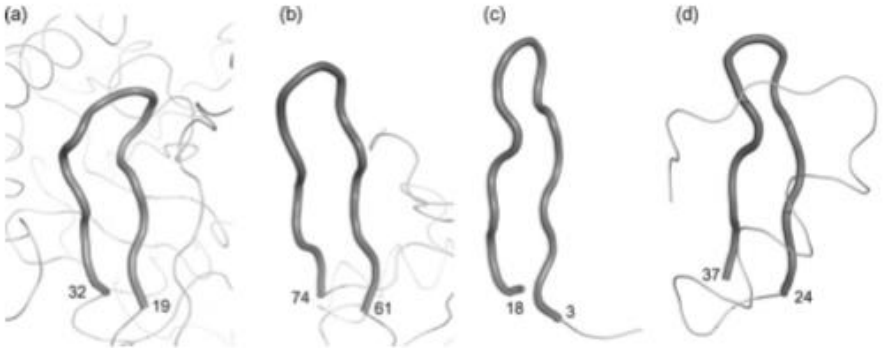

Figure 2.6. Initio modeling of jaburetox-2E (a, b) and comparison to other b-hairpin motifs (c, d) (Mulinari et al., 2007)

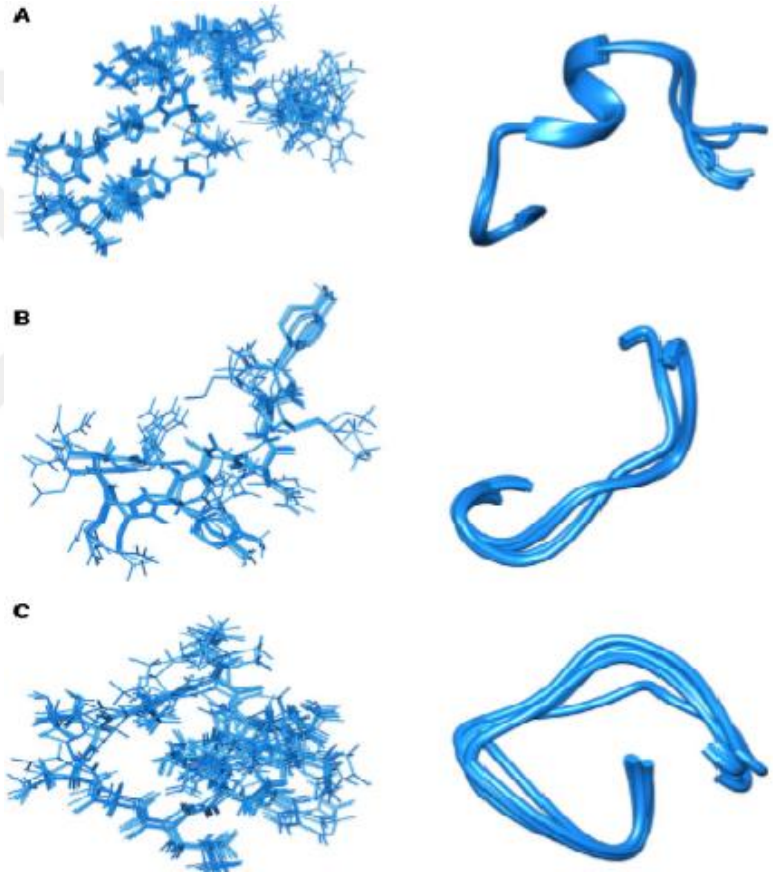

Figure 2.7. NMR modelling of Jaburetox N-terminal a-helix composed of 13 aa (a), two turn like structures located in the middle of the protein (b) and C-terminal portion (c) (Lopes et al., 2015)

2.6.6 Defence mechanism of JBU and derived peptide jaburetox against ınsects

Ureases and derived peptides have biological activity on fungi and insects such as membrane disruption and regulating permeabilization. Jaburetox 2-Ec and Jaburetox

(JBTX) were improved as a recombinant peptide from jack bean urease sequence with 91 amino acids (Becker-Ritt et al., 2017). There is no relationship between the entomotoxic effect and its ureolytic activity (Mulinari et al., 2007). Additionally, the antifungal effect is also independent of its catalytic activity (Postal et al., 2012). The role of ureases-derived peptides is still unclear (Barros et al., 2009). Researchers worked on insects that fed with an artificial diet including Urease or Jaburetox and proposed possible defense mechanisms of this peptide.

In order to investigate the effect of urease on insects, *R.prolixus* is used as a model, it has been suggested that ureases can inhibit the diuresis and disrupt water transport, and also ureases can trigger hemocyte aggregation or affect the phospholipase A2-mediated pathway and eventually it can cause to kill the insect (Stanisçuaski et al., 2009; Defferrari et al., 2014a; Defferrari et al., 2014b). Besides, it has been observed that low-level heart rates and behavioral changes with the effect of JBU in cockroaches. Probably it affects the insect's central nervous system (Carrazoni et al., 2016).

Galvani et al. (2015) worked on the neurotoxic effect of Jaburetox on *Triatoma infestans*. *Triatoma infestans* were fed with Jaburetox-containing artificial diet. Jaburetox binds to UAP (UDP N-acetylglucosamine pyrophosphorylase) as a target in the insect's brain. In addition, JBTX is localized in the insect brain to inhibit of NOS (nitric oxide synthase) activity as well. As a result, Jaburetox negatively affect the nitrergic system of *T.infestans*. Later, scientists revealed that Jaburetox affects the central nervous system of *R. prolixus*. They showed that chitin synthase genes expression pathway can be changed by Jbtx besides NOS and UAP activity **(**Fruttero et al., 2017).

dos Santos et al. (2019) in order to investigate the mechanism of Jbtx, worked on voltage-gated sodium on a cockroach. Finally, Jaburetox causes changes in the behavior of cockroach species (*Nauphoea cinerea*), such as decreasing distance traveled and increasing the standing time. Additionally, it has a cardiotoxic effect on the insect as decreased heart rate. Voltage clamping experiments of JBTX were investigated on *Xenopus laevis* oocytes. Nav 1.1 channels demonstrated an expressive increase in sodium currents. So, it has been shown that Jaburetox has a complex role in behavioral changes starting from the initial activation of the voltage-dependent sodium channels.

Urease and its derived peptides showed that these peptides have entomotoxic and fungitoxic effects on pests and diseases. Therefore these peptides can bu used while improving transgenic plants. Thanks to these information, Wiebke-Strohm et al. (2012), conducted first overexpression of GmEu4 urease study against *Rhizoctonia solani, Phomopsis sp., and Penicillium herguei* in plant. However, they obtained plant with co-suppressed enzymatic activity but results revealed that transgenic plants were more affected than wild-types due to co-suppression. Nevertheless, this study is very important for further studies to improvement of transgenic plant by using urease or urease-derived peptide. Because, informations are very limited about this study.

CHAPTER III

MATERIAL AND METHODS

3.1 Plant Material

In this study, Lady Olympia cultivar was used as plant material. Nodal culture of Lady Olympia was already maintained in (Plant Tissue Culture Lab of the faculty of Agricultural Sciences and Technologies, Nigde Omer Halisdemir University) on Murashige and Skoog (MS0) nutrient medium (Murashige and Skoog, 1962). During the transformation experimentinternodes and leaves were used as explants. Growth conditions of donor plants were 25 ºC and 16/8 h of light/dark photoperiod for cultures in growth chambers.

Table 3.1.MS media components

Components	Amount
Sucrose(DuchefaBiochemie, CAS number 57-50-1)	30 gr
PlantAgar (DuchefaBiochemie, Cat. No. M0222.0050)	8 gr
MS Salt (DuchefaBiochemie, Cat. No. M0222.0050)	4.4 gr

Figure 3.1. *in vitro* plants for transformation experiments

3.1.1 Bacteria and vectormaterial

pBIN61 expression vector carrying Jaburetox 2 Ec was developed by Dr. Allah Bakhsh in Prof. Claudio Ratti Lab at Department of Agricultural Sciences, University of Bologna, Italy. Jaburetox 2-Ec was amplified from its source plasmid (pET21b) using Pfu DNA Polymerase (Thermoscientific Cat. No. EP0501) and cloned in *Sma*I pre-digested pBIN61 vector by blunt-end cloning. The vector was confirmed using colony PCR, restriction analysis and sequencing and was pBIN-JBTX. This construct is available in plant transformation laboratory, Department of Agricultural Genetic Engineering, Faculty of Sciences and Technologies, Nigde Omer Halisdemir University, Turkey.

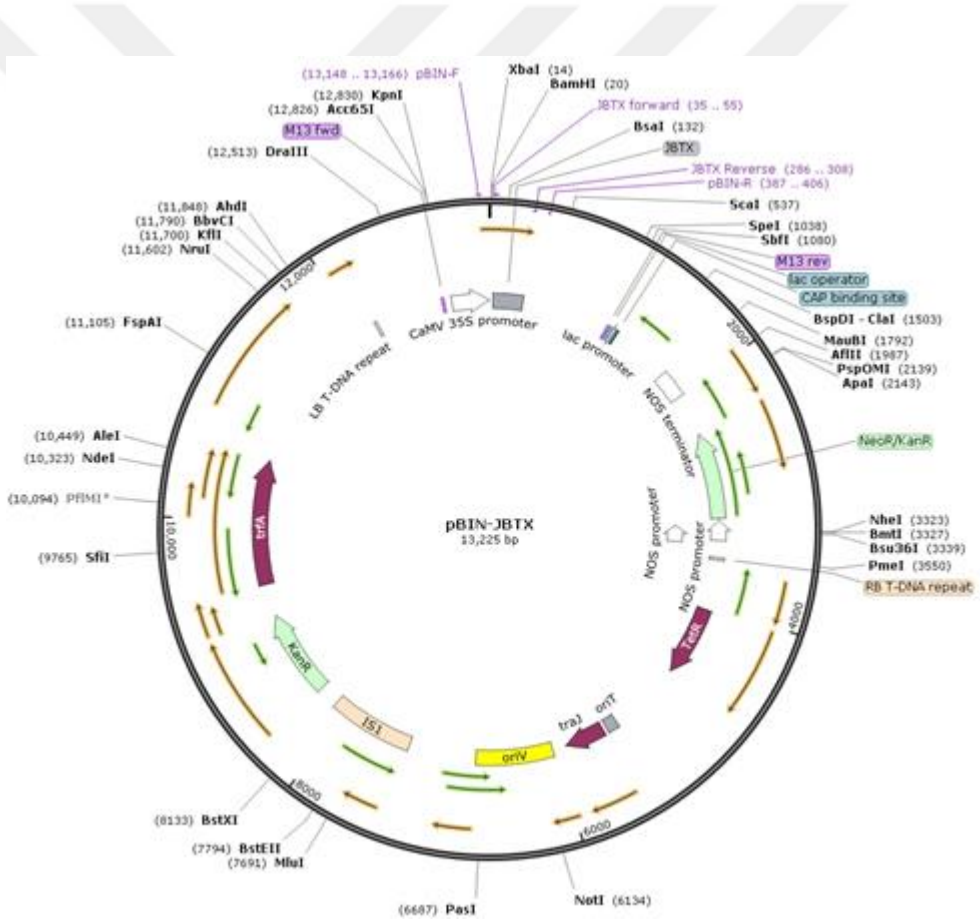

Figure 3.2. Schematic images of expression vector developed by cloning peptide (Jaburetox 2 Ec) in pBIN61. The vector JBTX gene is under the control of 35S promoter and nos terminator. The vector contains kanamycin that used as a plant selectable marker

3.2 Agrobacterium-Mediated Transformation

3.2.1 Bacterial ınoculation for transformation

The glycerol stock which contains target constructs (10 µl) were taken from -80 ºC and prepared Luria-Bertani broth (LB broth) media including 10 µl kanamycin. Bacterial culture was grown in thermos-shaker at 28 ºC with 220 rpm for two days. Streaking plates were prepared including with kanamycin 50 mg L^{-1} and incubated at 28 ºC for two days. Single colony were chosen from streaking plate and put into 10 ml of Luria-Bertani broth (LB broth) withmentioned above antibiotic and incubated at 28 ºC for 48 hours.

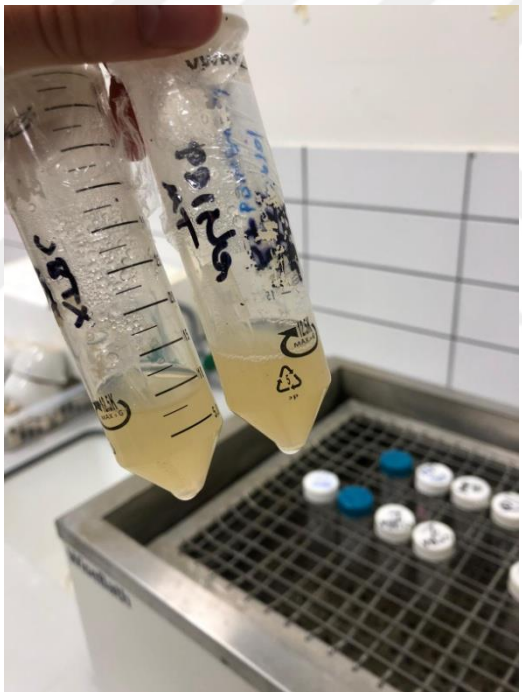

Figure 3.3. Overnight grown *Agrobacterium* (EHA 105) for inoculation

3.2.2 Co-cultivation

Leaves and internodes (4-6 mm) were used as explants. Inoculation was performed with *Agrobacterium* strain (O.D 0.6) including target construct for 15 min with 20 mL of Luria-Bertani broth(LB broth) without antibiotic.After that explants were shifted on co-cultivation media shown in (Table 3.2) for two days. Petri plates were put in growth

chambers with following conditions, at 16/8-hour day / night, 47 µmol / m2 / s light intensity and 25 ± 2 ° C temperature.

Table 3.2. Co-cultivation media components

Components	Amount
Sucrose(DuchefaBiochemie, CAS number 57-50-1)	30 gr
PlantAgar (DuchefaBiochemie, Cat. No. M0222.0050)	8 gr
MS Salt (DuchefaBiochemie, Cat. No. M0222.0050)	4.4 gr
Acetosyringone (Sigma Aldrich, CAS number 2478-38-8)	100 uM

3.2.3 Washing explants with broad-spectrum antibiotics and callus ınduction

Before that, optimization of callus induction media was conducted for this thesis research (Table 3.3) and Regeneration Selection Media (RSM) 1 which gives the best results were used for experiments. In order to rinse of explants, 1 ml of broad-spectrum antibiotic (sulcid) were used. Explants were taken from co-cultivation media and washed with water including with 1 ml of broad spectrum antibiotic (Sulcid) for 5-10 minutes. After washing, explants were shifted onto filter paper to dry. Then dried explants were transferred on regeneration selection media. Callus induction media were carried out for almost one month in growth chamber with following conditions at 16/8-hour day / night, 47 µmol / m2 / s light intensity and 25 ± 2 ° C temperature.

Table 3.3. Regenaration selection media components

Components of RSM 1	Amount
Sucrose (DuchefaBiochemie, CAS number 57-50-1)	30 gr
PlantAgar(DuchefaBiochemie, Cat. No. M0222.0050)	8 gr
MS Salt (DuchefaBiochemie, Cat. No. M0222.0050)	4.4 gr
BAP (DuchefaBiochemie, Cat. No. 1214-39-7)	2 mg/L
NAA (GOLDBIO, Cat. No. Z-105-50)	0,2 mg/L
2,4-D (DuchefaBiochemie, Cat. No. 94-75-7)	2 mg/L
Kinetin (DuchefaBiochemie, Cat. No. 525-79-1)	0,2 mg/L
GA_3 (MerckKGaA, Cat. No. 77-06-5)	0,2 mg/L
Kanamaycin (DuchefaBiochemie, Cat. No. 94-75-7)	50 mg/L
Sulcid	300 mg/L
Components of RSM 2	Amount
Sucrose (DuchefaBiochemie, CAS number 57-50-1)	30 gr
PlantAgar(DuchefaBiochemie, Cat. No. M0222.0050)	8 gr
MS Salt (DuchefaBiochemie, Cat. No. M0222.0050)	4.4 gr
BAP (DuchefaBiochemie, Cat. No. 1214-39-7)	2 mg/L
NAA (GOLDBIO, Cat. No. Z-105-50)	0,2 mg/L
2,4-D (DuchefaBiochemie, Cat. No. 94-75-7)	1 mg/L
Kinetin (DuchefaBiochemie, Cat. No. 525-79-1)	0,1 mg/L
GA_3 (MerckKGaA, Cat. No. 77-06-5)	0,1 mg/L
Kanamaycin (DuchefaBiochemie, Cat. No. 94-75-7)	50 mg/L
Sulcid	300 mg/L

Figure 3.4. Washing explants with broadspectrum antibiotic

3.2.4 Calculation of callusinduction

Calculation of callus induction and shoot regeneration were performed according to this below formula and obtained average number (Sahoo et al., 2011).

Callus induction rate (%) = (Total number of calli / Total number of explants) × 100% (3.1)

3.2.5 Shoot and root induction

Shoot induction media were optimized and components are shown in (Table 3.4). Well-developed calli were chosen and shifted into optimized shoot induction media (SIM 3) for two months. After that 1-2 cm length of shoots were cut and shifted into root media shown in (Table 3.5).

Table 3.4. Shoot induction media components

Components of SIM 1	**Amount**
Sucrose (DuchefaBiochemie, CAS number 57-50-1)	30 gr
PlantAgar (DuchefaBiochemie, Cat. No. M0222.0050)	8 gr
MS Salt (DuchefaBiochemie, Cat. No. M0222.0050)	4.4 gr
BAP(DuchefaBiochemie, Cat. No. 1214-39-7)	2 mg/L
NAA(GOLDBIO, Cat. No. Z-105-50)	0,2 mg/L
Kinetin (DuchefaBiochemie, Cat. No. 525-79-1)	0,2 mg/L
GA_3 (MerckKGaA, Cat. No. 77-06-5)	0,2 mg/L
Kanamaycin (DuchefaBiochemie, Cat. No. 94-75-7)	25 mg/L
Sulcid	200 mg/L
Components of SIM 2	**Amount**
Sucrose (DuchefaBiochemie, CAS number 57-50-1)	30 gr
PlantAgar (DuchefaBiochemie, Cat. No. M0222.0050)	8 gr
MS Salt (DuchefaBiochemie, Cat. No. M0222.0050)	4.4 gr
BAP(DuchefaBiochemie, Cat. No. 1214-39-7)	2 mg/L
NAA(GOLDBIO, Cat. No. Z-105-50)	0,2 mg/L
GA3(MerckKGaA, Cat. No. 77-06-5)	0,2 mg/L
Kanamycin (DuchefaBiochemie, Cat. No. 94-75-7)	25 mg/L
Sulcid	200 mg/L
Components of SIM 3	**Amount**
Sucrose (DuchefaBiochemie, CAS number 57-50-1)	30 gr
PlantAgar (DuchefaBiochemie, Cat. No. M0222.0050)	8 gr
MS Salt (DuchefaBiochemie, Cat. No. M0222.0050)	4.4 gr
BAP (DuchefaBiochemie, Cat. No. 1214-39-7)	2 mg/L
NAA (GOLDBIO, Cat. No. Z-105-50)	0,2 mg/L
Kanamaycin (DuchefaBiochemie, Cat. No. 94-75-7)	50 mg/L
Sulcid	200 mg/L

Table 3.5. Root induction media components

Components	Amount
Sucrose (DuchefaBiochemie, CAS number 57-50-1)	30 gr
PlantAgar (DuchefaBiochemie, Cat. No. M0222.0050)	8 gr
MS Salt (DuchefaBiochemie, Cat. No. M0222.0050)	4.4 gr
IBA (Merc, CAS number 133-32-4)	0.2 mg/L
Sulcid	100 mg/L

3.2.6 Transfer of transgenic plant stopots and acclimatization

The putative transgenic plants were transferred into pots containing mixture of perlite peat and vermiculite (1:1:1 v/v/v) respectiveley. In order to acclimization, pots were put into growth chamber having 25 ± 2°C constant temperature, light/dark photoperiod of 16/8 hours, 47 µmol m^{-2} s^{-1} irradiance and 58 W fluorescent tubes/lights special conditions.

3.3 Confirmation of Gene Integration

The putative transgenic plants were analysed with PCR technique to check integration of target gene in plant. The details of confirmation studies were explain in below;

3.3.1 DNA extraction and PCR analysis

The leaves of Lady Olympia were collected and put into liquid nitrogen. Extraction was perfomed by CTAB method.

Table 3.6. CTAB extraction buffer

Components	For 500 ml	Final Concentaration
1M TrispH 8	50 ml	100 mM
0.5 M EDTA pH 8	20 ml	20mM
4M NACl	175 ml	1,4 M
CTAB	10 gr	2%
PVP 40	10 gr	2%
$Na_2S_2O_5$	500 mg	0,10%

Samples were collected from putative transgenic plants and put into 2mL eppendorf with tungsten carbide beads and samples were grinded by using Tissue Lyser machine (TissueLyser II, Qiagen) for 2 minutes until provide homogenezation. CTAB extraction buffer were added on grinded samples as 900 microlitre. After adding solution, each samples were well mixed by using vortex. Eppendorf tubes were left on incubator for 1 hour at 65 degree. While doing incubation they were mixed by vortex for 10 seconds per 10 minutes. After incubation samples were put at room temperature for 5 minutes.Chloroform-isoamyl alcohol mix (24 : 1) was added on samples as 900 microlitre and mixture was mixed by eversioning for 15 minutes.Samples were centrifuged by centrifuge machine at 14.000 rpm as 15 minutes.Almost 700 microlitre supernatant were shifted into fresh and sterile 1.5 eppendorf tubes without mixing with lower phase.Cold % 100 Isopropanol were added onto samples as 500 microlitre and samples were mixed by eversioning for 15 minutes.Centrifuge was done for 3 minutes at 10.000 rpm and liquid phase was poured .After then 1 ml of 76 % ethanol which include 10 mM NaAc was added onto samples and mixed for 15 minutes as explained before.Pellets were precipitated by centrifuge at 7.000 rpm for 3 minutes and upper supernatant was discarded.After centrifugation tubes were left as opened at room temperature for drying.Dried DNA samples were dissolved into 50 microlitre distilled water and stored at -20 degree.

After genomic DNA extraction, In order to check presence of DNA samples, 5 µL of the DNA observed under UV light. Additionaly, DNA concentrations were checked by spectrophotometer. DNA samples were diluted to 20 ng/µL concentrations and used as PCR template for the confirmation of the putative transgenic plants. PCR was performed with two different primers as 35S and gene specific (JBTX). PCR reaction was conducted in a 20 µL reaction mastermix containing 60 ng of genomic DNA, forward and reverse primers 50 pM, dNTPs 200 µM, 10X PCR Buffer and Taq Polymerase 1 unit. PCR profile were set as: 1 cycle denaturation at 95ºC for 5 minutes, 95ºC for 30 seconds, 55ºC (Annealing) for 30 seconds and 72ºC (extension) for 1 minutes (35 times) and 72ºC (final extention) for 10 minutes for 1 cycle by using SensoQuestLabcyclermachine.Gel electrophoresis was used to observe PCR results. Gel was prepared as 1% using agarose and 0.5x TBE buffer including ethidium bromide 0.5-1ug/mL. Gel was run at 90 V for 1 hour.

Table 3.7. Primer sequences

Primer Name	5' -> 3' Sequence
JBTX-F:	GGTCCAGTTAATGAAGCCAAT
JBTX-R:	TAACTTTTCCACCTCCAAAAACA
35 S -F:	AGGAAACAGCTATGACCATG
35 S -R:	GAACTTCCTTATATAGAGGAAGG

3.4 Confirmation of Gene Expression in Putative Transgenic Plant

In order to check of gene expression level, Real-Time PCR technique was used.

3.4.1 RNA isolation

Total RNA were extracted from of potato leaves using Trizol method. Samples were grinded in liquid nitrogen by using mortar and pestle and shifted into 2 ml eppendorf. Trizol reagent was added as 1 ml onto sample and this mixture were vortexed and left in room temperature for 10 mins. Samples were centrifuged at 14.000 rpm and 4 °C for 10 minutes. Supernatants were transffered into new eppendorf and 200 µl chloroform was added on sample.Samples were eversioning by hands for 15 seconds. Samples were centrifuged at 14,000 rpm at 4 °C for 15 min. Supernatants were taken into a new eppendorf and 500 µl of cold isopropanol alcohol was added onto supernatants and incubated at room temperature for 10 minutes. Samples were centrifuged at 14.000 rpm at 4 °C for 15 minutes and upper phase were poured. Ethanol was added on samples as 75% in order to wash the RNAs. The samples were centrifuged at 4 ºC at 10.000 rpm for 10 minutes. Supernatant was removed from pellets and pellets were dried at room temperatures. Drying pellets were dissolved in 50 µl of DEPC water.

3.4.2 Removal of genomic DNA

Genomic DNA was removed from RNA samples. In order to check RNA concentrations, % 1 agorose gel electrophoresis was conducted and quality of RNA confirmed with nanodrop machine as well. This protocol was performed by using Thermo Scientific cDNA synthesis kit (K1612) protocol including RNA (1000ng/µl), 10x Reaction Buffer (with $MgCl_2$) (1µl), DNase I, RNase free (1-1U), Nuclease free water (1µl). Ingresientswere put into RNase free tubes and incubated at 37 °C for 30

minutes. EDTA (1 µL 50 mM) was added and incubated at 65°C for 10 minutes. RNA quality was checked by using agorose gel electrophoresis technique and nanodrop machine respectively.

3.4.3 First strand cDNA synthesis

RNA samples were converted to cDNA by using cDNA Reverse Transcription Kit (Cat. No.1622). Template RNA 1000 ng/µLand Oligo $(dT)_{18}$ primers 1µL were mixed and add nuclease-free water to 12 µL. Mixture was gently mixed and incubated at 65 ºC for 5 minutes in termocycler machine. After incubation 5X raction buffer 4 µL, Ribolock RNase Inhibitor (20 U/µL) 1 µL , 10mM dNTP Mix and RevertAid M-MuLV RT (200 U /µL) 1 µL to 20 µL added on mixture and incubated at 42 ºC for 60 minutes and 70 ºC for 5 minutes in termalcycler machine.

3.4.4 Real time PCR analysis

In order to show the expression of the Jaburetox 2-Ec, qRT-PCR analysis was performed. QJBTX primers and 18S rRNA primers were used for qRT-PCR reaction. Firstly, QJBTX primer's annealing temperature was optimized. Annealing temperatures of the primers described as 55 ºC. Primer Sequence were showed at Table BIO-RAD, iTaq Universal SYBR Green Supermix (Cat No:172-5121) was used in qRT-PCR reaction.qRT-PCR contents included total Syber green master mix (2X), F Primer (1 µM), R Primer (1 µM), RNase-free water and diluted cDNA as template (1:10). qRTPCR temperature cycle was set up as 95°C for 15 min, 40 cycles at 95°C for 10 sec, 55°C for 15 sec, 72°C for 20 sec and the melting curve analysis at 70°C to 99°C with an increment of 1.0 °C/min.

Table 3.8. Real time PCR primer sequences

Primers	Sequences	Product size(bp)	Tm ºC
JBTX-F:	5'-GGTCCAGTTAATGAAGCCAAT3'	270 bp	55 ºC
JBTX-R:	5'-TAACTTTTCCACCTCCAAAAACA3'	270 bp	55 ºC
18S rRNA-F	5'-GGGCATTCGTATTTCATAGTCAGAG-3'	101bp	55 ºC
18S rRNA-R	5'-CGGTTCTTGATTAATGAAAACATCCT-3'	101bp	55 ºC

The 18S rRNA gene was used for normalization because considered that it is the most stable gene for qRT-PCR analysis (Lin et al., 2010), thanks to its less variance in expression than β-actin and GAPDH. All real time PCR samples were performed as duplicates.Rotor-Gene Q (QIAGEN) device and instrument software were used for gene expression analysis. The fold change of gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

3.5 Calculation of Transformation Efficiency

Transformation efficiency calculations for transgenic plants of Lady Olympia were done after completed required analyses. Calculation formula is shown in below (Sahoo et al., 2011).

Transformation efficiency (%) = (Total number of plants expressing Jaburetox 2-Ec / Total number of calli inoculated)× 100% (3.2)

3.6 Leaf Feeding Bioassay

Leaf feeding bioassay was performed to check the resistance of transgenic plants against *Tuta absoluta. Tuta absoluta* insects were collected from Mersin province in August 2021 and their lifetime were maintained in a growth chamber (temperature 28±1, humidity 60±5 and 16/8 h day/night period) in the Department of Agricultural Genetic Engineering, Faculty of Sciences and Technologies, Nigde Omer Halisdemir University, Turkey. Primary transformant plant leaves were put in petri plates on fitler paper and pre-starved 2nd, 3rd and 4th instar larvae were released on plant. After fivedays of feding, data were collected whether the transgenic plants expressed Jaburetox 2-Ec against the insects in comparison with control.Mortality rate of *Tuta absoluta* larvae were collected following to the formula as below (Henderson and Tilton, 1952).

%Mortality = (1-(Ta * Cb/ Tb * Ca)) x 100 (3.3)

Ta=Insect Population After Treatment
Tb= Insect Population Before Treatment

Ca=Insect Population in Control After Treatment

Cb=Insect Population in Control Before Treatment

3.6.1 Analysis of fold change in weight

Entomotoxic effect of the transgenic plants on *Tuta absoluta* larvae (2nd, 3rd and 4th) was analyzed. Larvae of *Tuta absoluta* were released on transgenic leaves including with non-transgenic plants as control after measuring initial weight of them. Petri plates were put in the growth chamber with temperature 28±1, humidity 60±5 and 16/8 h day/night period. Plates were covered with strech to avoid the escape of larvae. After five days of feeding larvae were recollected and weighed again. Weight of larvae was recorded by using following formula

Fold change = (Final weight-initial weight)/ Initial weight (3.4)

3.7 Statistical Analyses

All statistical analyses were conducted with JMP Statistical Package program. After two-way ANOVA ($p < 0.05$), Tukey's multiple comparison test was performed to determine the significance of variance. Additionally, square root transformation was used while conducting Anova of the weight of larvae (Bek and Efe, 1989).

CHAPTER IV

RESULTS

4.1 Agrobacterium Mediated Transformation

4.1.1 Optimization of ınfection time

To increase the success of infection, inoculation time was optimized. EHA 105 strain including JBTX was used as 1 ml. After optimization, explants were brownish with high infection time but15 minutes infection time was optimum to get better results.

Table 4.1.Optimization of infection time

		Cultured Explants		Initiation of Callus	
Inoculate	**Time (min)**	**Internodes**	**Leaves**	**Internodes**	**Leaves**
EHA 105-Jaburetox 2-Ec	45 min	30	15	0	0
EHA 105-Jaburetox 2-Ec	45 min	30	15	0	0
EHA 105-Jaburetox 2-Ec	45 min	30	15	0	0
EHA 105-Jaburetox 2-Ec	15 min	30	15	28	11
EHA 105-Jaburetox 2-Ec	15 min	30	15	30	10
EHA 105-Jaburetox 2-Ec	15 min	30	15	27	12

Table 4.2. Variance analysis table of infection time

Source	DF Num	MS Num	SS	F Ratio
Replication	2	0,08333	0,16667	1
Time	1	1160,33	1160,33	13924**
Explant	1	225,333	225,333	142,3158*
Time*Explant	1	225,333	225,333	142,3158*
Error	2	0,08333	0,16667	0,0526

(Sum of Squares (SS), Mean Square (MS), Degrees of Freedom (DF))
**p≤ 0.01,*p≤ 0.05

Table 4.3. Tukey post-hoc test of infection time

Time – Explant	Group	Mean
15 min – Internode	A	28,33333
15 min – Leaf	B	11
45 min – Internode	C	0
45 min – Leaf	C	0

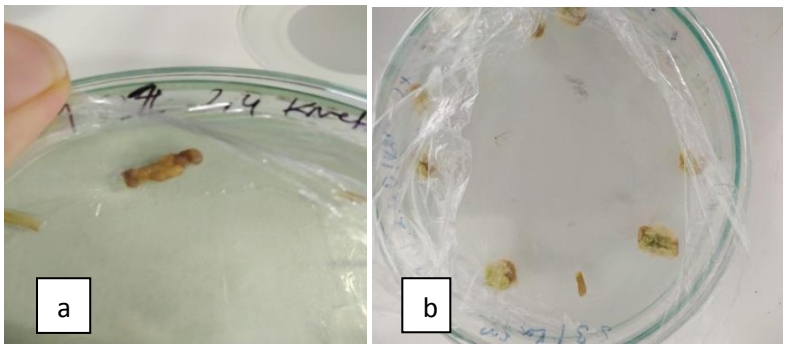

Figure 4.1. Inoculation of explants with 45 min (a) and inoculation of explants with 15 min (b)

4.1.2 Callus induction, shoot and root formation from potato plant

In order to obtain callus, leaves and internodes were cut from potato plants and infected with *Agrobacterium tumefaciens* and put on co-cultivation media for two days. After co-cultivation, explants were washed with sulcid and shifted on RSM (Regeneration Selection Media). After 3 or 4 weeks later callus induction was observed. Almost 1000 explants were used and 161 callus were obtained. Well developed- calli were shifted onto shoot induction media. Approximately 50 callus were put on shoot induction media that was optimized. Finally, two shoots were obtained. When shoots reached almost 2-3 cm length, it was cut and transferred on root media. After rooting period, putative transgenic plants were transferred into soil.

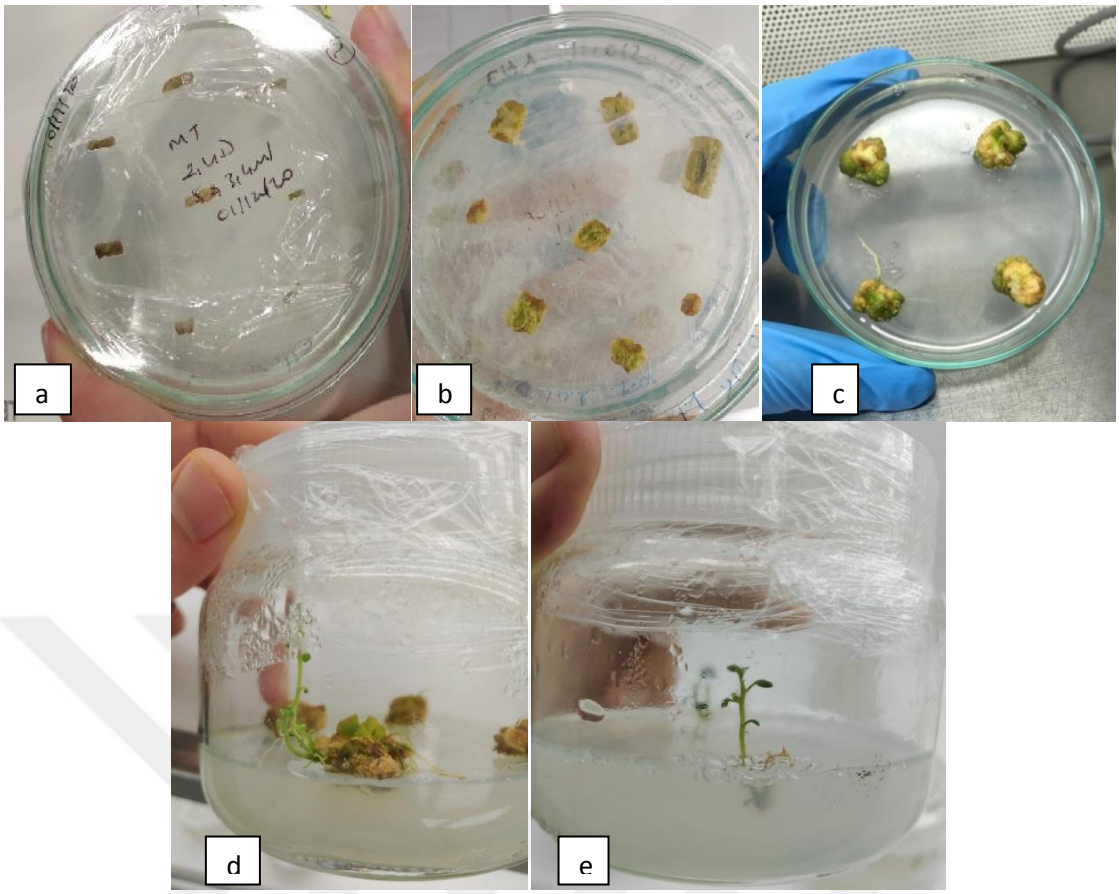

Figure 4.2.. Images show different steps in genetic transformatic of potato (a), explants on RSM media (b), inductionof callus after 3-4 weeks later (c), transfer of callus on shoot induction media (d) and transgenic shoots which are reached 2-3 cm length were shifted onto root induction media (e) root induction

4.1.2.1 Optimization of RSM media

Optimization of media contents took long time. While doing optimization high amount of explants were died. Two sorts of RSM media were tried for callus induction optimization media as RSM 1 and RSM 2. While observing callus formation from RSM 1 (Table 4.2) ,there were no results with RSM 2 media.

Table 4.4. Results of RSM 1

	Internodes	**Leaves**
Number Of Explants	600	400
Callus Formation	153	8

Table 4.5. Variance analysis table of RSM

Source	DF Num	MS Num	SS	F Ratio
Replication	2	1,75	3,5	21*
Explant	1	2002,08	2002,08	24025**
Media	1	2436,75	2436,75	2658,273**
Explant*Media	1	2002,08	2002,08	2184,091**
Error	2	0,08333	0,16667	0,0909

(Sum of Squares (SS), Mean Square (MS), Degrees of Freedom (DF)) **p≤ 0.01,*p≤ 0.05

Table 4.6. Tukey post-hoc test of RSM

Explant - Media	Group	Mean
Internod - RSM 1	A	54,33333
Leaf - RSM 1	B	2,66667
Leaf - RSM 2	B	0
Internod - RSM 2	B	-1,78E-15

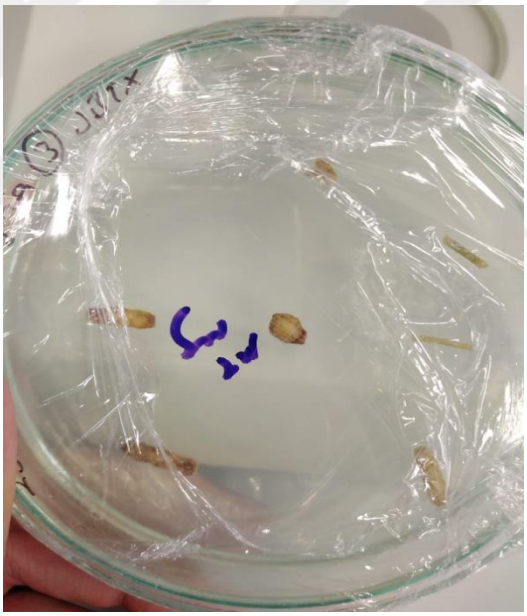

Figure 4.3. Brownish explants for RSM 2 media

4.1.2.2 Optimization of shoot ınduction media

In order to optimization of shoot induction media (SIM), three different kind of media were prepared as SIM1, SIM 2, SIM3. Shoot formation was obtained from two internodes in SIM 3 media. However any results obtained from other media.

Table 4.7. Variance analysis table of SIM

Source	DF Num	MS Num	SS	F Ratio
Replication	2	0,05556	0,11111	1
Explant	1	0,22222	0,22222	4
Media	2	0,22222	0,44444	4
Explant*Media	2	0,22222	0,44444	4
Error	2	0,05556	0,11111	1

(Sum of Squares (SS), Mean Square (MS), Degrees of Freedom (DF)) $p > 0.05$

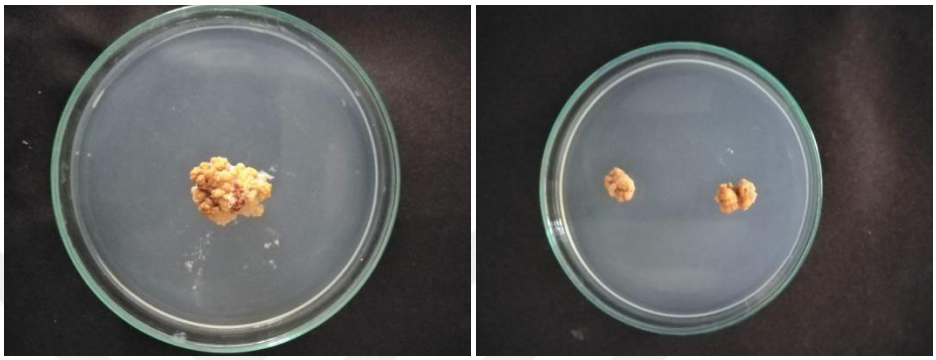

Figure 4.4. Brownish callus after two months later (SIM 1 and SIM 2)

4.2 Acclimization

A total of 7 putative transgenic plants were transferred from 2 lines, one of them 3 and one of them 4 respectively in pots, and all plants were acclimatized in a growth chamber with (25 ± 2°C, light/dark photoperiod of 16/8 hours, 47 µmol m-2 s -1 irradiance and 58 W fluorescent tubes/lights) special conditions. Phenotypic appearance (leaves, stems, etc.) of transgenic plants were different than control plants. Their leaves were thicker and smaller than wild types.

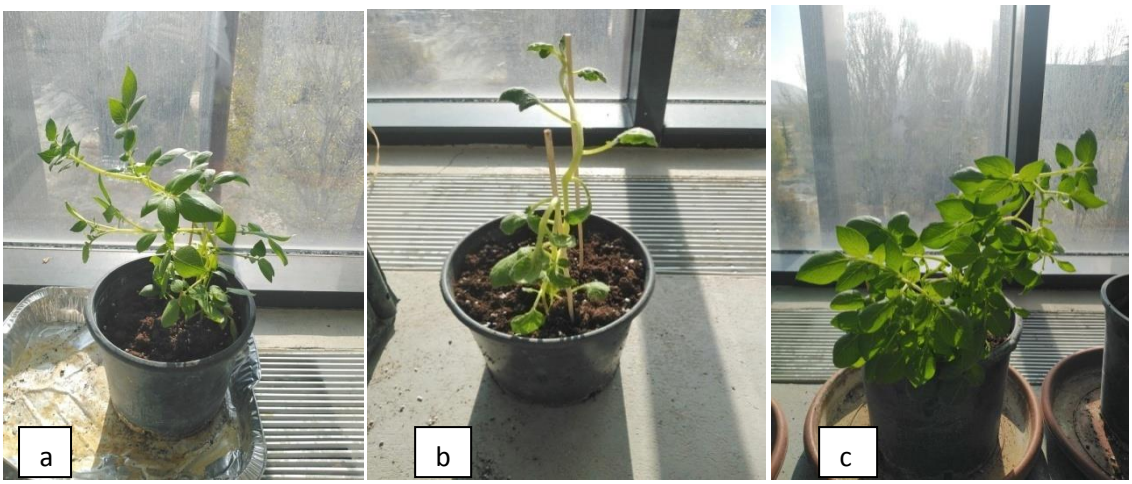

Figure 4.5. Putative transgenic plants 1 and 2 respectively (a, b) and non-transgenic plant (c)

4.3 Transformation Data

The data of transformed plants of Lady Olympia is shown in (Figure 4.6) below. Totally, 1000 explants were used for this study and obtained 161 callus formation from leaves and internodes as 8 and 153, respectiveley. While doing optimization, some calli were died and 50 callus were used for shoot induction media. Consequently two shoots were obtained from SIM 3 media. Callus induction rate was calculated as 2 and 25, 5 % for leaves and internodes respectively. Total callus induction rate was calculated as 16, 1 % .

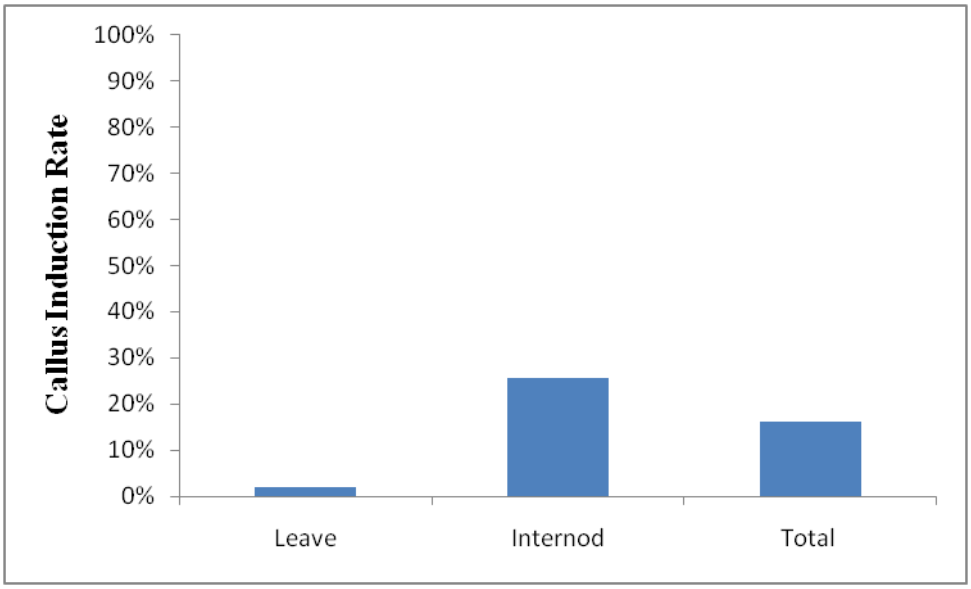

Figure 4.6. Callus induction rates from explants and total transformation data

According to results, callus induction rate was low for leaf explant compared to internodes.

4.4 Confirmation of Putative Transgenic Plants

4.4.1 PCR analysis

In order to confirm of putative transgenic plants, genomic DNA was isolated from samples. Concentration of DNA was checked with Nanodrop and observed on 1% agarose gel electrophoresis. DNA was diluted to optimum concentration for PCR. Putative transgenic plants were confirmed with two different primers as 35S and gene specific (Jaburetox) primers. All target sites were multiplied with PCR and any contamination was not observed in target sites.Primer sequence information has been given in (Table 3.8.).

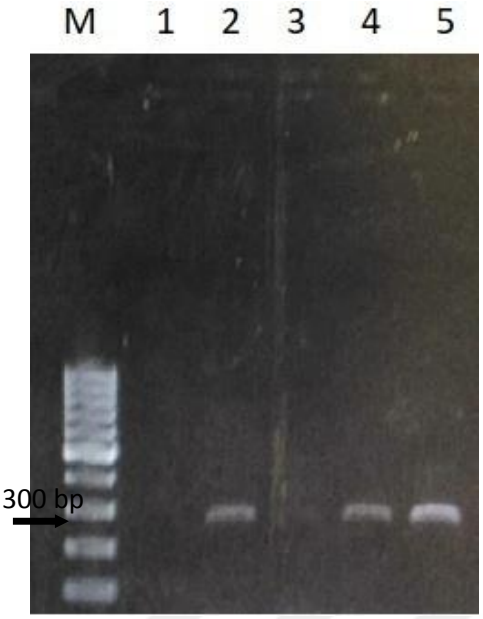

Figure 4.7. 35S sequence control in putative transgenic Lady Olympia plants. (Lane M):100 bp DNA marker, (Lane 4,5): Putative transgenic Lady Olympia, (Lane 3): Mock, (Lane 2): Positive control, (Lane 1): Negative control

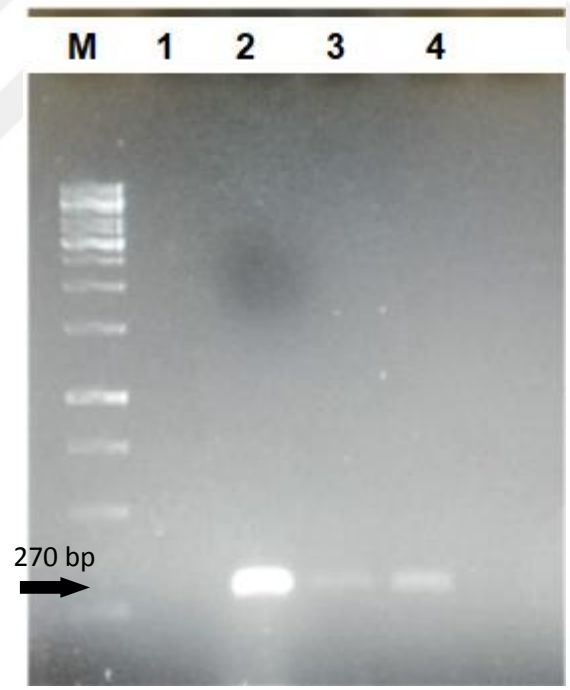

Figure 4.8. Jaburetox 2-Ec specific gene control in putative transgenic plants (M):100 bp DNA marker, (Lane 1): Negative control, (Lane 2): Positive control, (Lane 3,4): Putative transgenic plants

Both plants have been chosen for further analysis because positive bands were obtained from each plant.

4.5 Transformation Efficiency

Transformation efficiency of transgenic plants was calculated as 2% for target construct (EHA 105-JBTX).

4.6 Real – Time PCR

In order to calculate of gene expression levels, qRT-PCR analysis were carried out. Ct values of samples were used while calculating standart deviations and gene expression levels were defined by using Microsoft Excel program according to 2-ΔΔCt method (Livak and Schmittgen, 2001). Two plants were taken as sample according to standartpcr results. However one of them was not transgenic. On the other hand transgenic one has a 14 fold change expression level than control.

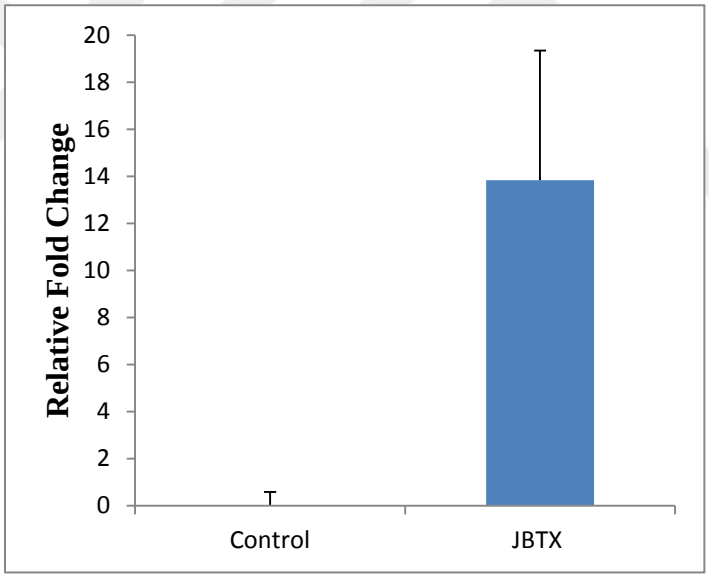

Figure 4.9. Relative fold expression of transgenic plant

4.7 Biotoxicity Assay

In order to show the efficacy of Jaburetox 2-Ec on *Tuta absoluta*, biotoxicity assay was performed. In this reason, feeding of *Tuta absoluta* was carried out with control conditions. Second, third and fourth instar larvae were collected and starved before assay. Two groups were used for experiment as control and transgenic plant. One larvae were released on each leaf. Three replicates were carried out and 9 larvae were used

totally. Petri plates were put in incubator with following conditions (Temperature= 28±1 °C; RH: 60%). Assays were performed for 5 days and data was recorded in every 24 hours as mortality rate and changes of weight.

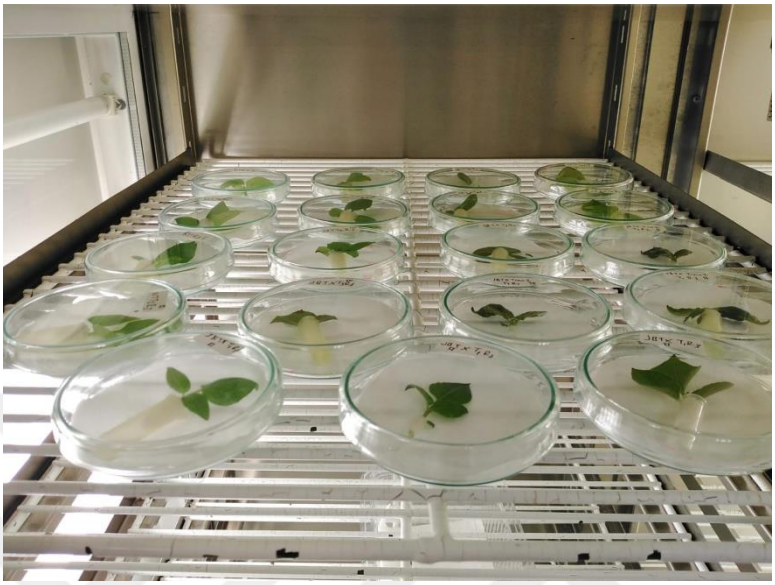

Figure 4.10. Feeding assay

4.7.1 Second ınstar biotoxicity assay

Mortality rates were recorded every 24 hours for each instar larvae.After 24 hours feeding no mortality was observed in each sample. After 48 hours later %33 mortality rate was observed in JBTX transgenic plant. After 72 hours of feding, %11 mortality rate was observed. However, there was no mortality rate in 96 and 120 hours later in all samples.

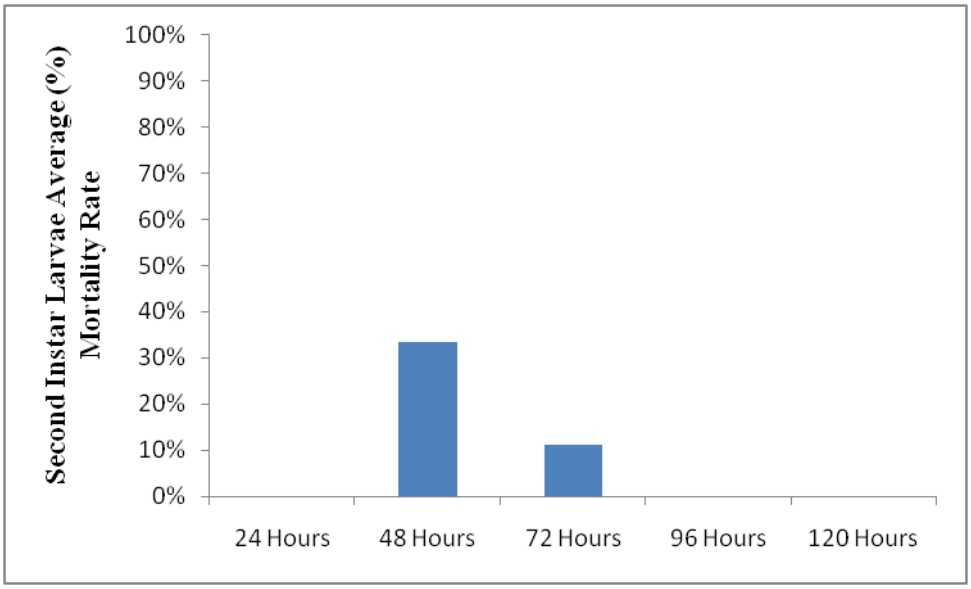

Figure 4.11. Summary of the mortality rates of second instar larvae

Figure 4.12. Second instar larve under microscope: feeded by wild types (a) and transgenic plants (b)

Figure 4.13. Leaf feeding bioassay transgenic plant (a) and wild-type plant (b)

4.7.2 Third ınstar biotoxicity assay

After 24 hours of feding any mortality rate was recorded for transgenic plant. Additionaly, %11 mortality rate was observed after 48 hours of feeding. On the other hand, after 96 hours later % 22 mortality rate was recorded. Finally, %33 mortality rate was observed after 120 hours later.

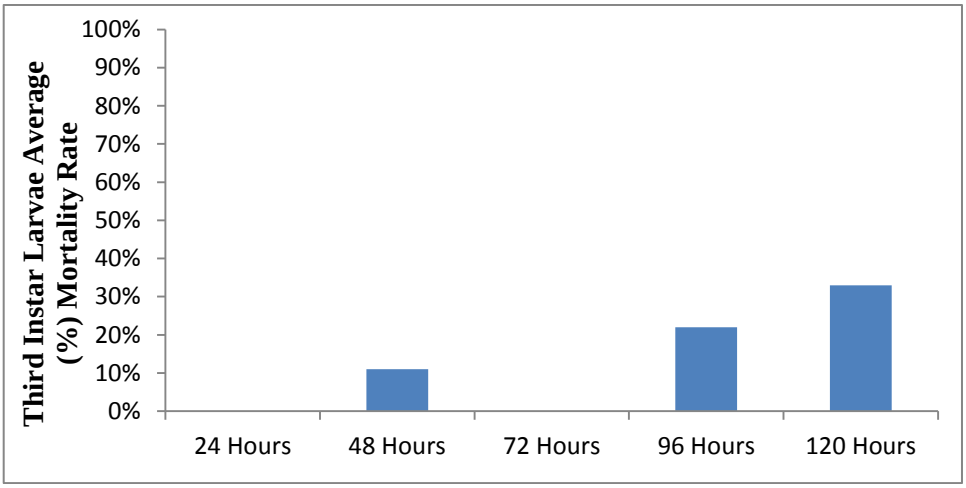

Figure 4.14. Summary of the mortality rates of third instar larvae

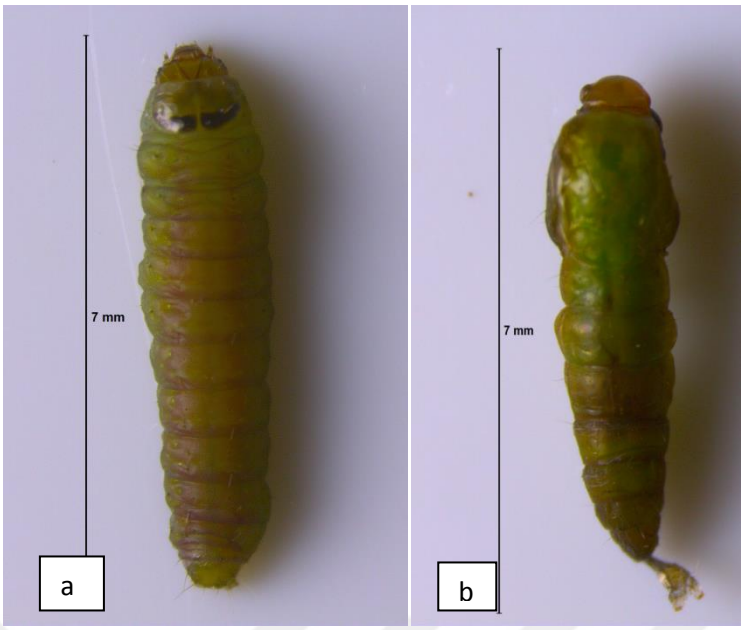

Figure 4.15. Third instar larvae under microscope: feeded by transgenic (a) and wild type (b)

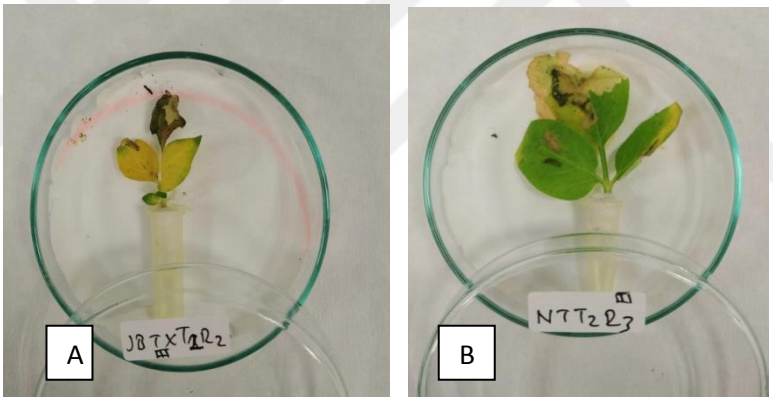

Figure 4.16. Leaf feeding bioassay transgenic plant (a) and wild-type plant (b)

4.7.3 Fourth instar biotoxicity assay

After 24 hours later there was no mortality rate. After 48 and 72 hours later % 22 mortality rate was observed. Additionally, there were %11 mortality rate in 96 hours later.

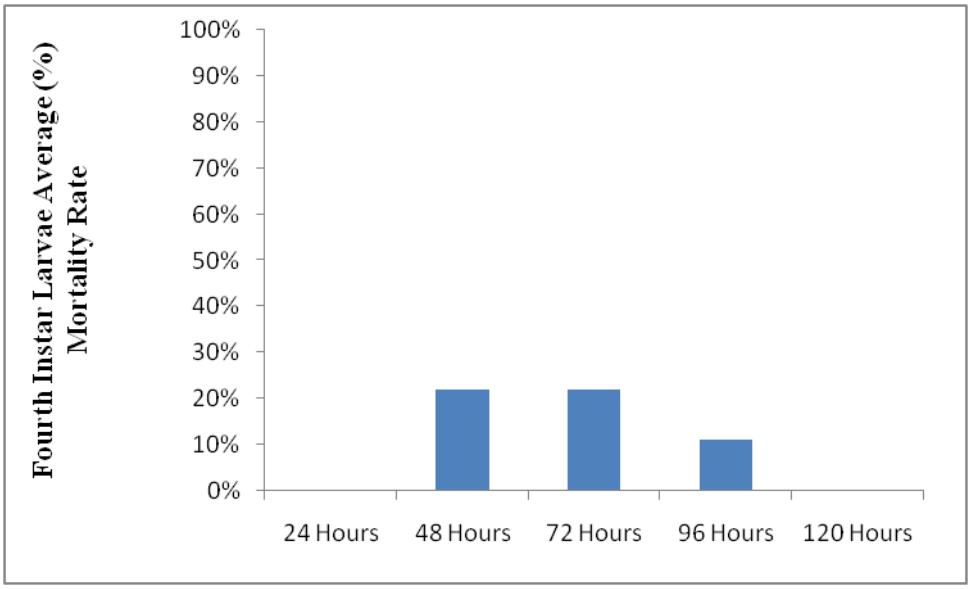

Figure 4.17. Summary of the mortality rates of fourth instar larvae

Figure 4.18. Fourth instar larvae under microscope: feeded by transgenic (a) and wild type (b)

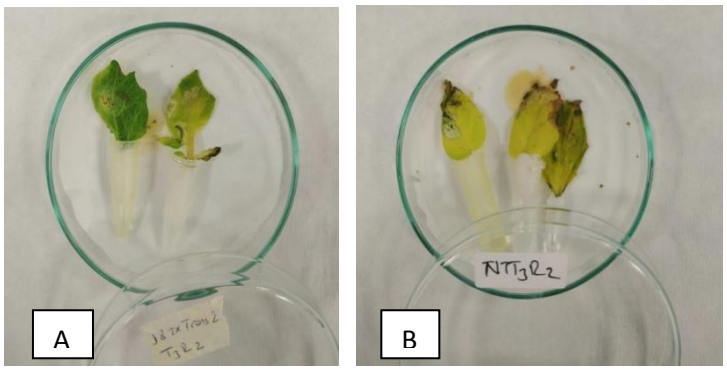

Figure 4.19. Leaf feeding bioassay transgenic plant (a) and wild-type plant (b)

Table 4.8. Variance analysis table of comparison of mortality rates between instars

Source	DF Num	MS Num	SS	F Ratio
Replication	2	0,13794	0,27588	2,9231
Larval Stage	2	0,00726	0,01452	0,1538
Hours	4	0,04235	0,1694	1,4583
Larval Stage*Hours	8	0,04961	0,39688	1,7083
Error	4	0,04719	0,18876	1,625

(Sum of Squares (SS), Mean Square (MS), Degrees of Freedom (DF)) p> 0.05

4.7.4 Fold change in weight of different larval instars

There was significantly differences between transgenic and control plant as weight fold change. In second instar larvae , 1.2 fold change was recorded in transgenic while it was 4.4 in control plant.In case of third instar larval stage, 0.5 fold change was observed and its control was high level as 1.6 compared to transgenic. Likewise in fourth instar larval stage was high with 1,7 compared to transgenic plant as 0,78 .

Table 4.9. Fold change of larvae compared to control samples

Samples	Second Instar	Third Instar	FourthInstar
JBTX	1,2	0,5	0,78
Control	4,4	1,6	1,7

Table 4.10. Variance analysis table of insect weight

Source	DF Num	MS Num	SS	F Ratio
Replication	2	0,00311	0,00621	1,2152
Larval Stage	2	5,64437	11,2887	2208,667**
Treatment	1	13,6939	13,6939	7062,751**
Larval Stage*Treatment	2	2,36954	4,73908	1222,112**
Error	4	0,00256	0,01022	1,3181

(Sum of Squares (SS), Mean Square (MS), Degrees of Freedom (DF)) **p≤ 0.01

Table 4.11. Tukey post-hoc test of insect weight

Larval Stage – Treatment	Group	Mean
Second Instar – Control	A	4,4
Fourth Instar – Control	B	1,69
Third Instar – Control	B	1,6
Second Instar – Transgenic	C	1,21
Fourth Instar – Transgenic	D	0,78
Third Instar - Transgenic	E	0,466667

CHAPTER V

DISCUSSION

In the present study, transgenic potato plants were obtained with overexpression of Jaburetox 2-Ec against *Tuta absoluta*. The mechanism of urease is still not clear (Barros et al., 2009). Thus, Jaburetox was improved from urease to understand entomotoxic effect of urease (Mulinari et al., 2007). Because previous studies showed that urease has an entomotoxic effect on insects.

Using this peptide, insect resistance potatoes were obtained that express high-level Jaburetox 2-Ec under 35S promoter. In this present study, overexpressed transgenic plants caused mortality in *Tuta absoluta* insects compared to control material.

It has been suggested that the Jaburetox peptide can be digested by insects that have cathepsin-based digestion system (Carlini et al., 1997). However, some studies showed that insects that have trypsin-like alkaline serine-proteinases showed susceptibility to Jaburetox. In this study, we choose *Tuta absoluta* which belongs to Lepidoptera to show entomotoxic effect of Jaburetox 2-Ec.

In order to manipulate gene, several methods have been introduced such as, particle bombardment, protoplast, biolistic, and *Agrobacterium*-mediated transformation (Keshavareddy et al., 2018). In our current study, *Agrobacterium*-mediated transformation approach was adopted according to Beaujean et al. (1998), with some changes. We used 1000 explants as internodes and leaves for *Agrobacterium* infection. According to Bakhsh (2020), internode has satisfactory results for transformation studies. Additionally, Lady Olympia is more effective in transformation efficiency among other cultivars. Researchers have shown different transformation eficiencies. Hussain et al. (2019) transferred *EcR* gene in Lady Olymipia and reported 2.8% transformation efficiency. Brunelle et al. (1999) obtained 15% with the Mnandi cultivar. Beaujean et al. (1998) obtained 88.7% transformation efficiency for Desiree cultivar. In our study we obtained 2% transformation efficiency. Consequently, transformation efficiency can be affected by many factors such as explants type, co-cultivation time, and medium contents (Li et al., 2017).

Initially, transformation efficiency related to infection time while conducting *Agrobacterium* transformation was studied in this M.S thesis research.Yadav et al. (2012), has shown that 15 min of infection time gave the best results compared to 10, 20, and 30 min. In our study, we tried 45 and 15 min for optimum infection time. Brownish explants were obtained due to the high level of infection time, especially leaves were very sensitive. Thus, infection time was decreased to 15 min and consequently, best responses were obtained from both internode and leaf explant.

2, 4-D is one of the most effective growth regulators for potato callus induction when used with other cytokinins (Abd Elaleem et al., 2009). Dangol et al. (2020), tried three different kind of media including with 2,4-D hormone but they could not obtain callus induction by using 2,4-D combination. Their results show that combinations of 2 mg L-1 BAP and 2 mg L-1 NAA was the best media for callus induction with 83% transformation efficiency. In our present study optimization of callus induction media took more than two months. High numbers of explants have died. After that we tried combinations of auxin and cytokinin. Consequently, we obtained callus from optimized media supplemented with (BAP (2 mg/l), NAA (0.2 mg/l), GA3 (0.2 mg/l), 2, 4 D (2 mg/l), Kinetin (0.2 mg/l) Kanamaycin (25 mg/l) and Sulcid (200 mg/L).

While doing transformation, selectable markers are used in order to choose transgenic plants. Kanamycin is one of the most widely used selection marker for plant transformation. However one of the most major problem is to escape of shoots from the selection (Estopà et al., 2001). This can be related with the instability of T-DNA or genomic regulations during meisosis. Moreover, some shoots can be chimeric and carry both the transgenic and non-transgenic lines (McHughen and Jordan, 1989). In our study we did not observe gene escape.

Polymerase chain reaction (PCR) is the most widely used GMO detection technique. Because it amplifies specific target DNA sites (Wu et al., 2014). To detection of transgenic plants with PCR , 35S and NOS terminator primers can be used (Oraby et al., 2005).In our current study, we used 35S and gene specific primers in order to check putative transgenic plants and two transgenic plants were obtained finally.

In order to confirm of standard pcr results, Real-Time PCR technique was used as further analysis. Basically, transformation studies can be resulted as multiple gene copies number and detection of these genes can be conducted with southern blot analysis. However, this traditional tecnique is laborouis and time consuming. Instead of this technique, the quantitative Real-Time PCR approach has been improved and this technique easily detects PCR products (Li et al., 2004). In our study, qRT-PCR analyses were conducted to determine expression levels of the Jaburetox gene. As a result of the analysis, one transgenic plant was obtained and other standard PCR positive plant was eliminated. Finally, we obtained significantly high expression from a single transgenic plant compared to the control plant.

Transgenic plants were checked as a gene integration and expression level. After confirmation, leaf bioassays were carried out to show of toxicity trait of trangenic plant against insects by feeding 2nd, 3rd, and 4th instar larvae of *Tuta absoluta*. Data were recorded every 24 hours as mortality rate. At the end of the assay, weight data also were recorded compared to the initial weight. Average mortality rates of each instar larvae were recorded as 11-33%. Didoné et al. (2021), fed insects with an artificial diet including Jaburetox and compared the effect of two different versions of Jaburetox. As a result of this study, they demonstrated that % 25 mortality rate after 4 days later compared with the full version of the peptide. However, after 6 days later this ratio reached % 75 for both version.

CHAPTER VI

CONCLUSIONS

This master thesis was carried out to overexpression of Jaburetox 2-Ec in potato cultivar Lady Olympiaby using *Agrobacterium*-mediated gene transfer approach, and to determine the resistance of transgenic plants against *Tuta absoluta* pest.This is the first study of using *Agrobacterium*-mediated technology against *Tuta absoluta* in potatoes with the help of Jaburetox 2-Ec peptide in the world. The key findings and conclusions from this study are listed below;

- The Jaburetox 2-Ec peptide was successfuly transferred to potato cv. Lady Olympia using *Agrobacterium*-mediated gene transfer approach. A total of 2 PCR positive plants were obtained and transformation efficiency was calculated as 2%. However, according to results of Real-Time PCR, Jaburetox 2-Ec was not expressed in one of the putative transgenic plant.
- The most effective infection time was 15 min for leaf and internode explants.
- Internode explants gave satisfactory transformation efficiency results compared to leaf explants.
- According to the results, the most suitable regeneration selection media was RSM 1 and the suitable shoot induction media was SIM 3.
- The mortality rate of *Tuta absoluta* larvae fed on transgenic plants ranged from 11% to 33% in each instar stage. The highest mortality rate (33%) was determined from the larvae at second and third instar stages.
- The weight gain in larvae fed with control plants was higher than the larvae fed with transgenic plants.
- It was concluded that Jaburetox 2-Ec has insecticidal activity against *Tuta absoluta* pest, but more research is needed to confirm the initial results.

REFERENCES

Abd Elaleem, K.G., Modawi, R.S., and Khalafalla, M.M., "Effect of plant growth regulators on callus induction and plant regeneration in tuber segment culture of potato (Solanum tuberosum L.) cultivar Diamant", ***African Journal of Biotechnology*** 8 (11), 2529–2534, 2009.

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

Aksoy, E., Demirel, U., Bakhsh, A., Zia M.A.B., Naeem, M., Saeed, F., Çalışkan, S., and Çalışkan, M.E., Advances in Plant Breeding Strategies: Vegetable Crops, Jameel M. Al-Khayri., S. Mohan Jain Dennis V. Johnson, ***Springer Nature***, Switzerland, 2021.

Arora, R.K. and Khurana, S.M.P., "Major fungal and bacterial diseases of potato and their management", ***Fruit and Vegetable Diseases*** 1, 189–231, 2006.

Bakhsh, A. "Development of efficient, reproducible and stable agrobacterium-mediated genetic transformation of five potato cultivars", ***Food Technology and Biotechnology*** 58(1), 57–63, 2020.

Balaško, M.K., Mikac, K.M., Bažok, R. and Lemic, D., "Modern techniques in colorado potato beetle (Leptinotarsa decemlineata say) control and resistance management: History review and future perspectives", ***Insects*** 11(9), 1–17, 2020.

Barros, P.R., Stassen, H., Freitas, M.S., Carlini, C.R., Nascimento, M.A.C. and Follmer, C., "Membrane-disruptive properties of the bioinsecticide Jaburetox-2Ec: Implications to the mechanism of the action of insecticidal peptides derived from ureases", ***Biochimica et Biophysica Acta – Proteins and Proteomics*** 1794(12), 1848–1854, 2009.

Beaujean, A., Sangwan, R.S., Lecardonnel, A. and Sangwan-Norreel, B.S., "Agrobacterium-mediated transformation of three economically important potato cultivars using sliced internodal explants: An efficient protocol of transformation", ***Journal of Experimental Botany*** 49(326), 1589–1595, 1998.

Becker-Ritt, A.B. and Carlini, C.R., "Fungitoxic and insecticidal plant polypeptides", ***Biopolymers*** 98(4), 367–384, 2012.

Becker-Ritt, A.B., Portugal, C.S. and Carlini, C.R., "Jaburetox: Update on a urease derived peptide", ***Journal of Venomous Animals and Toxins Including Tropical Diseases*** 23(1), 1–8, 2017.

Bek, Y. ve Efe, E., Araştırma ve Deneme Metodları, ***Çukurova Ziraat Fakültesi***, Adana, 1989.

Bonierbale M.W., Amoros W.R., Salas E. and de Jong W., The potato crop: Its agricultural, nutritional and social contribution to humankind, Hugo Campos and Oscar Ortiz, ***Springer Nature,*** Switzerland, 2019.

Brown, C.R., "Antioxidants in potato", ***American Journal of Potato Research*** 82, 163–172, 2005.

Brunelle, F., Nguyen-Quoc, B., Cloutier, C. and Michaud, D., "Protein hydrolysis by Colorado potato beetle, Leptinotarsa decemlineata, digestive proteases: The catalytic role of cathepsin D", ***Archives of Insect Biochemistry and Physiology*** 42(1), 88–98, 1999.

Çalışkan, M.E., "Türkiye'de patates üretimi ve patates politikamız", ***TÜRKTOB Dergisi*** 10, 18-24, 2014.

Çalışkan, M.E., Patates, ***Tarım Türk Dergisi***, 2020.

Camire, M.E., Kubow, S. and Donnelly, D.J., "Potatoes and human health", ***Critical Reviews in Food Science and Nutrition*** 49(10), 823–840, 2009.

Caparros Megido, R., Haubruge, E. and Verheggen, F.J., "Pheromone-based management strategies to control the tomato leaf miner, Tuta absoluta (Lepidoptera: Gelechiidae)". A review | Synthèse bibliographique: Lesstratégies de lutte phéromonale utilisées pourcontrôler la mineuse de la tomate, Tuta absoluta, ***Lepi. Biotechnology, Agronomy and Society and Environment*** 17(3), 475–482, 2013.

Carlini, C.R., Oliveira, A.E., Azambuja, P., Xavier-Filho, J. and Wells, M.A., "Biological effects of canatoxin in different insect models: evidence for a proteolytic activation of the toxin by insect cathepsinlike enzymes", ***Journal of Economic Entomology*** 90(2), 340–348, 1997.

Carrazoni T., de Avila Heberle M., Perin A.P., Zanatta A.P., Rodrigues P.V., DosSantos F.D., de Almeida C.G., Vaz Breda R., Dos Santos D.S., Pinto P.M., da Costa J.C., Carlini C.R. and Dal Belo C.A., "Central and peripheral neurotoxicity induced by the Jack Bean Urease (JBU) in Nauphoetacinerea cockroaches", ***Toxicology*** 368, 162-171, 2016.

Charles Brummer, E., Barber, W.T., Collier, S.M., Cox, T.S., Johnson, R., Murray, S. C., Olsen, R.T., Pratt, R.C. and Thro, A.M., "Plant breeding for harmony between agriculture and the environment", ***Frontiers in Ecology and the Environment*** 9(10), 561–568, 2011.

Dangol, S. Das, Yel, İ., Çalişkan, M.E., and Bakhsh, A., "Manipulating genome of diploid potato inbredline solanum chacoense M6 using selectable marker gene", ***Turkish Journal of Agriculture and Forestry*** 44(4), 399–407, 2020.

Deblaere R., Bytebier B., De Greve H., Deboeck F., Schell J., Van Montagu M. and Leemans J., "Efficient octopine Ti plasmid-derived vectors for Agrobacterium-mediated gene transfer to plants", ***Nucleic Acids Research*** 13 (13), 1985.

Defferrari, M.S., da Silva, R., Orchard, I., and Carlini, C.R., “Jackbean (Canavalia ensiformis) urease induces eicosanoid-modulated hemocyte aggregation in the Chagas' disease vector Rhodnius prolixus”, ***Toxicon*** 82, 18-2, 2014a.

Defferrari, M.S., Lee, D.H., Fernandes, C.L., Orchard, I. and Carlini CR., “A phospholipase A2 gene is linked to Jackbeanurease toxicity in the Chagas' disease vector Rhodnius prolixus”, ***Biochimica et BiophysicaActa*** 1840(1), 396-405, 2014b.

Desneux, N., Wajnberg, E., Wyckuys, K., “Biological invasion of European tomato crops by Tuta absoluta: ecology, geographic expansion and prospects for biological control”, ***J Pestic Sci***. 83, 197–215, 2010.

Didoné, D.A., Lopes, F.C., Souza Martinelli, A.H., Ceccon, C.C., De Silva, M.R., Salvadori, J.R., Carlini, C.R., Shatters, R.G. and Grando, M.F., “Toxicity of the Jaburetox Peptide to the Multi-Host Insect-Pest Helicoverpa armigera (Lepidoptera: Noctuidae) Larvae”, ***Florida Entomologist*** 104(3), 230–238, 2021.

Dos Santos, D.S., Zanatta, A.P., Martinelli, A.H.S., Rosa, M.E., de Oliveira, R.S., Pinto, P.M., Peigneur, S., Tytgat, J., Orchard, I., Lange, A.B., Carlini, C.R. and Dal Belo, C. A., “Jaburetox, a natural insecticide derived from Jack Bean Urease, activates voltage-gated sodium channels to modulate insect behavior”, ***Pesticide Biochemistry and Physiology*** 153(September), 67–76, 2019.

Estopà, M., Marfà, V., Melé, E. and Messeguer, J., “Study of different antibiotic combinations for use in the elimination of Agrobacterium with kanamycin selection in carnation”, ***Plant Cell, Tissue and Organ Culture*** 65(3), 211–220, 2001.

FAOSTAT., “Statistical data.” ***Food and Agriculture Organization of the United Nations***, Rome, 2021.

Foster, R.E., “Vegetable Insects”, ***Purdue University (Extension Entomology),*** 1–6, 2017.

Fruttero, L.L., Moyetta, N.R., Krug, M.S., Broll, V., Grahl, M.V.C., Real-Guerra, R., Stanisçuaski, F. and Carlini, C.R., "Jaburetox affects gene expression and enzyme activities in Rhodnius prolixus, a Chagas' disease vector", ***Acta Tropica*** 168, 54–63, 2017.

Galvani, G.L., Fruttero, L.L., Coronel, M.F., Nowicki, S., Demartini, D.R., Defferrari, M.S., Postal, M., Canavoso, L.E., Carlini, C.R., and Settembrini, B.P., "Effect of the urease-derived peptide Jaburetox on the central nervous system of Triatoma infestans (Insecta: Heteroptera)", ***Biochimica et BiophysicaActa - General Subjects*** 1850(2), 255–262, 2015.

Gatehouse, A.M.R., Davison, G.M., Stewart, J.N., Gatehouse, L.N., Kumar, A., Geoghegan, I.E., Birch, A.N.E. and Gatehouse, J.A., "Concanavalin A inhibits development of tomato moth (Lacanobiaoleracea) and peach-potatoaphid (Myzuspersicae) when expressed in transgenic potato plants", ***Molecular Breeding*** 5(2), 153–165, 1999.

Gatehouse, A.M.R., Hilder, V.A. and Boulter, D., "Potential of plant-derived genes in the genetic manipulation of the crops for insect resistance, in: Biotechnology in Agriculture N° 7: Plant genetic manipulation for crop protection", ***CAB International***, 155-181, 1992.

Gebremariam, G., "Tuta absoluta: a global looming challenge in tomato production, review paper", ***Journal of Biology, Agriculture and Health Care*** 5(14), 57–63, 2015.

Grafius, E.J. and Douches, D.S., The present and future role of insect-resistant genetically modified potato cultivars in IPM, ***Integration of Insect-Resistant Genetically Modified Crops within IPM Programs***, 195–221, 2008.

Halterman, D., Guenthner, J., Collinge, S., Butler, N., and Douches, D., "Biotech potatoes in the 21st century: 20 years since the first biotech potato", ***American Journal of Potato Research*** 93(1), 1–20, 2016.

Haverkort, A.J., Struik, P.C., Visser, R.G.F. and Jacobsen, E., "Applied biotechnology to combat lateblight in potato caused by phytophthora infestans", ***PotatoResearch*** 52(3), 249–264, 2009.

Henderson, C.F. and Tilton, E.W., "Tests with acaricides against the Brown wheat mite", ***J Econ Entomol*** 48, 157-161,1952.

Hussain, T., Aksoy, E., Çalışkan, M.E. and Bakhsh, A., "Transgenic potato lines expressing hairpin RNAi construct of molting-associated EcR gene exhibit enhanced resistance against Colorado potatobeetle (Leptinotarsa decemlineata, Say)", ***Transgenic Research*** 28(1), 151–164, 2019.

Illakwahhi, D.T. and Srivastava, P.B.B.L., "Control and management of Tomato leafminer -Tuta absoluta (Meyrick) (Lepidotera, Gelechiidae.) a review", ***IOSR Journal of Applied Chemistry*** 10 (06), 14–22, 2017.

Jian-Kang Z., "Abiotic stress signaling and responses in plants", ***Cell*** 167 (3), 313–324, 2016.

Kanle Satishchandra, N., Chakravarthy, A.K., Özgökçe, M.S., and Atlihan, R., "Population growth potential of Tuta absoluta (Meyrick) (Lepidoptera: Gelechiidae) on tomato, potato, and eggplant", ***Journal of Applied Entomology*** 143 (5), 518–526, 2019.

Kappaun, K., Piovesan, A.R., Carlini, C.R. and Ligabue-Braun, R., "Ureases: Historical aspects, catalytic, and non-catalytic properties – A review", ***Journal of Advanced Research*** 13, 3–17, 2018.

Keshavareddy, G., Kumar, A. R. V. and Ramu, V., "Methods of plant transformation- a review", ***International Journal of Current Microbiology and Applied Sciences*** 7 (07), 2656–2668, 2018.

Kreuze J.F., Souza-Dias J.A.C, Jeevalatha A., Figueira A.R., Valkonen J.P.T., and Jones R.A.C., The potato crop: Its agricultural, nutritional and social contribution to humankind, Hugo Campos and Oscar Ortiz, ***Springer Nature,*** Switzerland, 2019.

Kroschel J., Mujica N., Okonya J. and Alyokhin A., The potato crop: Its agricultural, nutritional and social contribution to humankind, Hugo Campos and Oscar Ortiz, ***Springer Nature,*** Switzerland, 2019.

Kumar, A. and Verma, J.P., "Does plant—Microbe interaction confer stres tolerance in plants: A review", ***Microbiological Research*** 207, 41–52, 2018.

Kumar, S., "Plant Ureases: Physiological significance, role in agriculture and industrial applications- A review", ***South Asian J. Food Technol. Environment*** 1 (2), 105-115, 2015.

Kumari M., Kumar M. and Solankey S.S., "Breeding Potato for Quality Improvement", Intech, http://dx.doi.org/10.5772/intechopen.71482, 1989.

Lecardonnel, A., Chauvin, L., Jouanin, L., Beaujean, A., Prévost, G. and Sangwan-Norreel, B., "Effects of rice cystatin I expression in transgenic potato on Colorado potato beetle larvae", ***Plant Science*** 140 (1), 71–79, 1999.

Li, S., Cong, Y., Liu, Y., Wang, T., Shuai, Q., Chen, N., Gai, J. and Li, Y., "Optimization of agrobacterium-mediated transformation in soybean", ***Frontiers in Plant Science*** 8 (February), 1–15, 2017.

Li, Z., Hansen, J.L., Liu, Y., Zemetra, R.S. and Berger, P.H., "Using real-time PCR to determine transgene copy number in wheat", ***Plant Molecular Biology Reporter*** 22 (2), 179–188, 2004.

Livak, K.J. and Schmittgen, T.D., "Analysis of relative gene expression data using real time quantitative PCR and the 2 (-Delta Delta C(T)) Method", ***Methods*** 25 (4), 402-8, 2001.

Lopes, F.C., Dobrovolska, O., Real-Guerra, R., Broll, V., Zambelli, B., Musiani, F., Uversky, V.N., Carlini, C.R. and Ciurli, S., "Pliable natural biocide: Jaburetox is an intrinsically disordered insecticidal and fungicidal polypeptide derived from jackbean urease", ***FEBS Journal*** 282 (6), 1043–1064, 2015.

Mansour, R., Brévault, T., Chailleux, A., Cherif, A., Grissa-Lebdi, K., Haddi, K., Mohamed, S.A., Nofemela, R.S., Oke, A., Sylla, S., Tonnang, H.E.Z., Zappalà, L., Kenis, M., Desneux, N. and Biondi, A., "Occurrence, biology, natural enemies and management of Tuta absoluta in Africa", ***Entomologia Generalis*** 38 (2), 83–112, 2018.

McHughen, A. and Jordan, M.C., "Recovery of transgenic plants from "escape" shoots", ***Plant Cell Reports*** 7 (8), 611–614, 1989.

Meiyalaghan, S., Jacobs, J.M.E., Butler, R.C., Wratten, S.D., and Conner, A.J., "Transgenic potato lines expressing cry1Ba1 or cry1Ca5 genes are resistant to potato tuber moth", ***Potato Research*** 49 (3), 203–216, 2006.

Mulinari, F., Stanisçuaski, F., Bertholdo-Vargas, L.R., Postal, M., Oliveira-Neto, O.B., Rigden, D.J., Grossi-de-Sá, M.F. and Carlini, C.R., "Jaburetox-2Ec: An insecticidal peptide derived from an isoform of urease from the plant Canavalia ensiformis", ***Peptides*** 28 (10), 2042–2050, 2007.

Murashige, T. and Skoog, F., "A revised medium for rapid growth and bioassays with tobacco tissue cultures", ***Physiology of Plant*** 15, 473-97, 1962.

Oraby, H.A.S., Hassan, A.A. and Abou Mossallam, A.A., "Screening food products for the presence of CaMV 35S promoter and NOS 3′ terminator", ***Journal of the Science of Food and Agriculture*** 85 (12), 1974–1980, 2005.

Ortiz, O. and Mares, V., **"**The historical, social, and economic importance of the potato crop", ***The Potato Genome*** 1–10, 2017.

Polacco, J.C. and Holland, M.A., "Roles of urease in plant cell", ***International Review of Cytology*** 145, 1993.

Postal, M., Martinelli, A.H.S., Becker-Ritt, A.B., Ligabue-Braun, R., Demartini, D.R., Ribeiro, S.F.F., Pasquali, G., Gomes, V.M. and Carlini, C.R., "Antifungal properties of Canavalia ensiformis urease and derived peptides", ***Peptides*** 38 (1), 22–32, 2012.

Protopedia, https://proteopedia.org/wiki/index.php/Urease, 2021.

Qaim, M., The economics of genetically modified crops, ***The Annual Review of Resource Economics*** 1, 665–93, 2009.

Roditakis, E., Skarmoutsou, C. and Staurakaki, M. "Toxicity of insecticides to populations of tomato borer Tuta absoluta (Meyrick) from Greece", ***Pest Management Science*** 69 (7), 834–840, 2013.

Rondon, S.I., "The potato tuber worm: A literature review of its biology, ecology, and control", ***American Journal of PotatoResearch*** 87, 149–166, 2010.

Sá, C.A., Vieira, L.R., Pereira Almeida Filho, L.C., Real-Guerra, R., Lopes, F.C., Souza, T.M., Vasconcelos, I.M., Staniscuaski, F., Carlini, C.R., Urano Carvalho, A.F. and Farias, D.F., "Risk assessment of the antifungal and insecticidal peptide Jaburetox and its parental protein the Jack bean (Canavalia ensiformis) urease", ***Food and Chemical Toxicology*** 136, 110977, 2020.

Sahoo, K.K., Tripathi, A.K., Pareek, A., Sopory, S.K. and Singla-Pareek, S.L., "An improved protocol for efficient transformation and regeneration of diverse indica rice cultivars", ***Plant Methods*** 7, 49, 2011

Selale, H., Dağlı, F., Mutlu, N., Doğanlar, S. and Frary, A., "Cry1Ac-mediated resistance totomato leaf miner (Tuta absoluta) in tomato", ***Plant Cell, Tissue and Organ Culture*** 131 (1), 65–73, 2017.

Sirko, A. and Brodzik, R., "Plant ureases: Roles and regulation", ***Acta Biochimica Polonica*** 47 (4), 1189–1195, 2000.

Soliman, H.I.A., Abo-El-Hasan, F.M., El-seedy, A.S. and Mabrouk, Y.M., "Agrobacterium-mediated transformation of tomato (Lycopersicon esculentum Mill.) using a synthetic cry1ab gene for enhanced resistance against Tuta absoluta (Meyrick)", ***Journal of Microbiology, Biotechnology and Food Sciences*** 7 (1), 67–74, 2017.

Son, D., Bonzi, S., Somda, I., Bawin, T., Boukraa, S., Verheggen, F., Francis, F., Legreve, A. and Schiffers, B., "First Record of Tuta absoluta (Meyrick, 1917) (Lepidoptera: Gelechiidae) in Burkina Faso", ***African Entomology*** 25 (1), 259–263, 2017.

Stanisçuaski, F., Te Brugge, V., Carlini, C.R. and Orchard, I., "In vitro effect of Canavalia ensiformis urease and the derived peptide Jaburetox-2Ec on Rhodnius prolixus Malpighian tubules", ***Journal of Insect Physiology*** 55, 255–263, 2009.

TropeaGarzia, G., Siscaro, G., Biondi, A., and Zappalà, L., "Tuta absoluta, a South American pest of tomato now in the EPPO region: Biology, distribution and damage", ***EPPO Bulletin*** 42 (2), 205–210, 2012.

TÜİK, "Bitkisel üretim istatistikleri." ***Türkiye İstatistik Kurumu Haber Bülteni***, 37249, 2021.

Urbaneja, A., González-Cabrera, J., Arnó, J. and Gabarra, R., "Prospects for the biological control of Tuta absoluta in tomatoes of the Mediterranean basin", ***Pest Management Science*** 68 (9), 1215–1222, 2012.

Vincent, C., Alyokhin, A. and Giordanengo, P., "Potatoes and their Pests – Setting the Stage", Philippe Giordanengo, Charles Vincent, and Andrei Alyokhin, ***Elsevier***, USA, 2013.

Visser D., "Guide to potato pests and their natural enemies in South Africa", ***Arc-Roode plant Vegetable and Ornamental Plant Institute, Pretoria***, 105, (2005).

Walkerpeach, C.R. and Velten, J., "Agrobacterium-mediated gene transfer to plant cells: cointegrate and binary vector systems", ***Plant Molecular Biology Manual*** 33–51, 1994.

Watanabe, K., "Potato genetics, genomics, and applications", ***Breeding Science*** 65 (1), 53–68, 2015.

Wiebke-Strohm, B., Pasquali, G., Margis-Pinheiro, M., Bencke, M., Bücker-Neto, L., Becker-Ritt, A.B., Martinelli, A.H.S., Rechenmacher, C., Polacco, J.C., Stolf, R., Marcelino, F.C., Abdelnoor, R.V., Homrich, M.S., Del Ponte, E.M., Carlini, C.R., de Carvalho, M.C.C.G. and Bodanese-Zanettini, M.H., "Ubiquitous urease affects soybean susceptibility to fungi", ***Plant Molecular Biology*** 79 (1–2), 75–87, 2012.

Wu, Y., Wang, Y., Li, J., Li, W., Zhang, L., Li, Y., Li, X., Zhu, L. and Wu, G., "Development of a general method for detection and quantification of the P35S promoter based on assessment of existing methods", ***Scientific Reports*** 4, 1–13, 2014.

Yadav, S. K., Katikala, S., Yellisetty, V., Kannepalle, A., Narayana, J. L., Maddi, V., Mandapaka, M., Shanker, A. K., Bandi, V. and Bharadwaja, K. P., "Optimization of Agrobacterium mediated genetic transformation of cotyledonary node explants of Vignaradiata", ***Springer Plus*** 1 (1), 1–8, 2012.

Zaheer, K. and Akhtar, M. H., "Potato Production, Usage, and Nutrition—A Review", ***Critical Reviews in Food Science and Nutrition*** 56 (5), 711–721, 2016.

CURRICULUME VITAE

Merve TEKİNSOY was born on in , . She completed her primary, secondary and high school education in . After high school, she completed her , , . During B.Sc., she went to internship in

. After completing her under graduate education in , she started her master's degree in Jalisco Uni of ce, ment Agri eering .