T.C.

ÇANAKKALE ONSEKİZ MART UNIVERSITY

SCHOOL OF GRADUATE STUDIES

DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

EFFECTS OF METHYLATION ON *Plasmopara halstedii* STRESS IN *Helianthus annuus* L.

MASTER OF SCIENCE THESIS

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ÇANAKKALE ONSEKİZ MART ÜNİVERSİTESİ

LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

Nil KURTGÖZ tarafından Doç. Dr. Özge KARAKAŞ METİN yönetiminde [ve Prof. Dr. Mine TÜRKTAŞ ikinci danışmanlığında] hazırlanan ve **05/08/2024** tarihinde aşağıdaki jüri karşısında sunulan "**Effects of Methylation on *Plasmopara halstedii* Stress in *Helianthus annuus* L.**" başlıklı çalışma, Çanakkale Onsekiz Mart Üniversitesi Lisansüstü Eğitim Enstitüsü **Moleküler Biyoloji ve Genetik Anabilim Dalı**'nda **YÜKSEK LİSANS TEZİ** olarak oy birliği ile kabul edilmiştir.

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Tez Savunma Tarihi : 05/08/2024

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ETHICAL STATEMENT

In this thesis study that I prepared following the Thesis Writing Rules of School of Graduate Studies of Çanakkale Onsekiz Mart University; I declare that I have obtained the data information, and documents I presented in the thesis within the framework of academic and ethical rules, I have presented all information, documents, evaluations, and results following scientific ethics and ethical rules, I cited all the works that I used in my thesis study by making appropriate reference, I did not make any changes in the data used and that the study I presented in this thesis is original. Otherwise, I undertake and declare that I accept all loss of rights that may arise against me.

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ABSTRACT

EFFECTS OF METHYLATION ON *Plasmopara halstedii* STRESS IN *Helianthus annuus* L.

Nil KURTGÖZ

Çanakkale Onsekiz Mart University

School of Graduate Studies

Master of Science Thesis in Molecular Biology and Genetic

Advisor: Assoc. Prof. Özge KARAKAŞ METİN

Co-Advisor: Prof. Mine TÜRKTAŞ

05/08/2024, 50

Sunflower (*Helianthus annuus* L.), a member of the Asteraceae family with a diploid chromosome number (n = 17), is an important oilseed crop grown worldwide due to its versatile applications in the food, industrial, and pharmaceutical sectors. Its use as an ornamental plant is also widespread. Sunflower ranks as the fourth most well-known oilseed crop globally. It has been prioritized in genetic and genomic studies due to its economic and ecological significance. Mildew disease in sunflowers, caused by the pathogen *Plasmapora halstedii*, exhibits characteristic symptoms that vary depending on the developmental stage of the plant. Examples include young seedling death, dwarfing, leaf bleaching, and sporulation in infertile flowers. Since its rise as a significant oilseed crop, breeding efforts have incorporated tolerance to diseases like mildew among genotypes. Thirty-seven Pl resistance genes have been identified in sunflowers known to protect against diseases. Genetic control of mildew disease in sunflowers poses challenges due to the pathogen's variability, making it a rigorous area of research. Pathogen infections pose serious threats to plant health and global crop production. Epigenetic processes such as DNA methylation and histone modifications play crucial roles in plant defense responses. Methylation-Sensitive Amplification Polymorphism (MSAP) is a widely used method to assess DNA methylation

changes in plants. It involves digesting DNA with methylation-sensitive restriction enzymes, followed by PCR amplification and gel-based visualization of the amplified products. The primary goal of this study is to profile epigenetic processes, specifically DNA methylation changes using MSAP, in response to pathogen infection in two distinct commercial hybrid sunflower varieties known for their tolerance and susceptibility to mildew disease. Evaluating sunflower genotypes for disease tolerance is crucial for mitigating yield losses and ensuring sustainable use of products.

Keywords: Downy Mildew disease, *Helianthus annuus* L., Methylation Sensitive Amplification Polymorphism (MSAP), Epigenetics, DNA Methylation

ÖZET

Helianthus annuus L. BİTKİSİNDE METİLASYONUN *Plasmopara halstedii* STRESİNE ETKİLERİ

Nil KURTGÖZ

Çanakkale Onsekiz Mart Üniversitesi

Lisansüstü Eğitim Enstitüsü

Moleküler Biyoloji ve Genetik Anabilim Dalı Yüksek Lisans Tezi

Danışman: Doç. Dr. Özge KARAKAŞ METİN

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05/08/2024, 50

Asteraceae familyasının bir üyesi olan ticari hibrit ayçiçeği (*Helianthus annuus* L.) diploit (n = 17), gıda, sanayi ve ilaç sektörlerinde çok yönlü kullanımları nedeniyle dünya çapında yetiştirilen önemli bir yağlı tohum bitkisidir. Ticari hibrit ayçiçeğinin, süs bitkisi olarak da kullanımı yaygındır. Ayçiçeği, ekonomik ve ekolojik öneminden dolayı genetik ve genomik çalışmalarda öncelikli modellerden biri olmuştur. *Helianthus annuus* L., dünyada en bilinen dördüncü yağlı tohum mahsulüdür. Ayçiçeğinde Mildiyö hastalığı, *Plasmapora halstedii* adlı patojenden kaynaklı olup, ayçiçeği hangi gelişimsel aşamasında olduğuna bağlı olarak değişen karakteristik semptomları vardır. İnfertil çiçeklerin üretiminde genç fide ölümü, cüce bitki, yaprak ağarması ve sporülasyon bu semptomlara örnek verilebilir. Ayçiçeği yağlı tohum mahsulü olarak öenm kazandığı dönemden itibaren hastalığa toleranslılık karakterinin genotipler arasında aktarımı ıslah çalışmalarına dâhil edilmiştir. Ayçiçeğini hastalıklara karşı korumak için 37 Pl direnç geni belirlenmiştir. Ayçiçeğinde Mildiyö hastalığının genetik kontrolü, patojenin sürekli değişime uğraması nedeniyle ve zorlu bir araştırma alanıdır. Patojen enfeksiyonları, bitki sağlığı ve küresel mahsul üretimi açısından ciddi bir tehdit oluşturmaktadır. DNA metilasyonu ve histon modifikasyonu gibi epigenetik süreçler bitkinin savunma cevaplarında önemli rol oynar. Metilasyona duyarlı amplifikasyon polimorfizmi (MSAP), bitkilerdeki DNA metilasyon değişikliklerini

değerlendirmek için en çok kullanılan yöntemlerden biridir. Bu yöntem, metilasyona duyarlı restriksiyon enzimleri kullanılarak kesilen ve ardından çeşitli primer kombinasyonları kullanılarak çoğaltılmış DNA'ların PCR ürünlerinin jel tabanlı görselleştirilmesi olarak tanımlanabilir. Bu çalışmada temel amaç Mildiyö hastalığına toleranlı ve hassas olduğunu bildiğimiz iki ayrı ticari hibrit ayçiçeğinin patojenle enfeksiyon sonrası DNA metilasyon değişiklikleri gibi epigenetik süreçleri metilasyona duyarlı amplifikasyon polimorfizmi (MSAP) yöntemi ile profillemektir. Ayçiçeği genotiplerinin hastalığa tolerans yönünden değerlendirilmesi, verim kaybının önüne geçilmesi ve ürünlerin sürdürülebilir kullanımları için çok önemlidir.

Anahtar Kelimeler: Mildiyö Hastalığı, *Helianthus annuus* L., Metilasyona Duyarlı Amplifikasyon Polimorfizmi, Epigenetik, DNA Metilasyonu

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SYMBOLS AND ABBREVIATIONS

%	Percent
µg	Microgram
µl	Microliter
^{o}C	Degrees Celcius
BIA	Benzylimineamine
CTAB	Cetyltrimethylammonium bromide
APS	Ammonium persulfate
bp	Base pair
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylene Diamine Tetra Acetic Acid
FAO	Food and Agriculture Organization of the United Nations
g, G	Gram
M	Molar
mg	Miligram
Min	Minute
MSAP	Methylation Sensitive Amplification Polymorphism
MSL	Methylation Susceptible Loci
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
nm	Nanometer
NML	Non-Methylated Loci
PCR	Polymerase Chain Reaction
PVP	Polyvinylpyrolidone
RNA	Ribonucleic acid
Sec	Second
SSCP	Single-strand conformational polymorphism
Tm	Melting temperature
TAE	Tris-acetate-EDTA
U	Unit

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CHAPTER 1
INTRODUCTION

1.1. Introduction to Sunflower

Common sunflower, scientifically named as *Helianthus annuus* L., is one of the major sources of edible oil in the world. It is diploid (n = 17) and an annual crop. This species can also be used as bird food and as a house and garden ornament. The Greek words “helios” meaning “sun”, and “anthos” meaning “flower” combined to form the name of the genus.

Table 1
Scientific classification of sunflower.

Kingdom	Plantae
Class	Magnoliopsida
Order	Asterales
Family	Asteraceae
Genus	Helianthus
Species	*H. annuus*
Binominal name	*Helianthus annuus* L.

1.2. The History of Sunflower

The origin of the genus *Helianthus* is North America. Before the discovery of North America, the products of the sunflower plant were used as a natural food. According to the statements of many locals, the nutritional value of the plant was known, but they did not know how to extract oil from its grains. According to what was learned from the authorities, a group of locals stated that the stem and roots of the sunflower were used for medicinal purposes, although they knew how to extract oil from their grains, they used it not as a food item, but to lubricate their hair and skin. Sources indicate that sunflower was cultivated as an ornamental plant in Europe until 250 years ago. Currently, sunflower oil accounts for 16-18% of global vegetable oil production. In 1965, sunflower oil production exceeded cotton

oil production, and peanut oil production in 1967 with oil production. Today, sunflower comes in the 4th place in the world in terms of vegetable oil production source, after palm, soybean, and rapeseed.

1.3. The Importance of Sunflower

Sunflower is a valuable oil plant that is cultivated in many countries of the world as in Türkiye and contains high-quality oil in its grains. Today, 39.4% of the oil consumed in human nutrition in our country is derived from sunflowers, among the plants from which oil-producing plants.

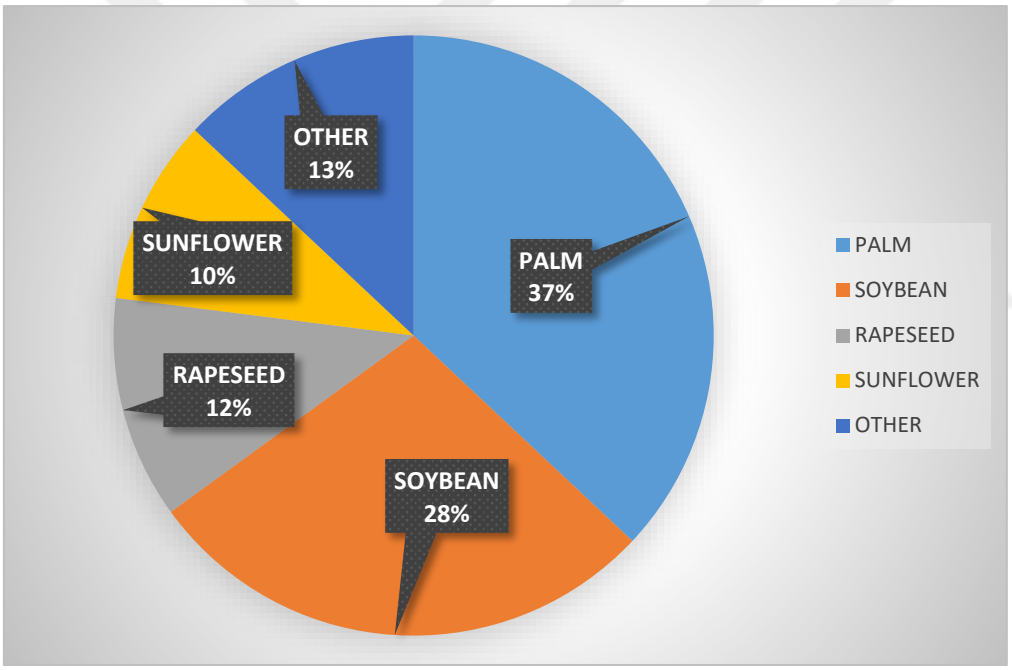

Figure1. The edible oil production of the World (FAO 2022).

According to FAO's data, sunflower ranks fourth in the world in vegetable oil production with 9%. In Türkiye, it ranks first in vegetable oil production with 56.2%. Many types of oilseeds such as cotton, soybean, peanut, and poppy are grown in our country. Sunflower is at the forefront as the oil plant with the highest cultivation area and production. It is the main plant in alternation, especially in the Marmara and Trakya Regions. Sunflower is the most important oilseed plant for our country due to its wide adaptability, ability to grow in both dry and irrigated conditions and suitability for mechanization (Kaya, 2004).

1.4. Why Does Türkiye Import Sunflower Oil?

Sunflower production in Türkiye has been ongoing for approximately 60-70 years. Introduced by Bulgarian immigrants, sunflower cultivation began to rapidly expand after 1950 to meet the oil demand, particularly in the Trakya region. Despite some fluctuations, Türkiye ranked 5th in global oil production according to 1980 statistics. Sunflower imports exceeded the 3 million tons limit for the first time in the 2019/20 season in Türkiye. Sunflower production in the last 20 years, from 2000 to 2020, is 25,54 million tons. Domestic usage amounted to 44.74 million tons. This indicates that 57% of domestic consumption over the past 20 years has been met by production within Türkiye. However, as domestic production has not been sufficient to satisfy local demand, Türkiye has been compelled to import sunflower oil. Therefore, breeding programs and the development of disease-resistant sunflower varieties are crucial for our country.

1.5. Downy Mildew Disease in Sunflower

Downy Mildew in sunflowers is induced by *Plasmopara halstedii* (Farlow) Berlese & de Toni, which is a major disease influencing the production of annual *Helianthus annuus* L.. Pathogen *P. halstedii* was first described by Harvard University botanist Byron D. Halsted in 1876. Mycologists have classified 35 genera and 80 species of *P. halstedii*, which causes downy mildew. Only a few species have a pathogenic effect on sunflowers. A prominent change in metabolism is seen in systemically infected plants. The typical symptom of downy mildew is stunting and a decrease in leaf number (1–3), with a reduction of over 90% in total mass, leaf area, and plant height. Chlorophyll concentration and photosynthetic activity decrease. Stunting is caused by a decrease in the amount of indolacetic acid (IAA). The evaluation of sunflower for downy mildew resistance is simply sowing diseased sunflower seeds into the soil. The most widely used evaluation method is known as inoculation of the whole seedling, 2/3 of the developed seedlings are immersed in the zoospore suspension for a few hours for 2 weeks to develop systemic symptoms. The details of the technique have been carefully studied and refined over and over again. At the

beginning of the endurance studies, the existence of 4 Pl genes and their two known strains were considered to be controllable. Pl_2 genotypes controlled both races, Race 1 and race 2, while Pl_1 genotype controlled only race 1, which was the least virulent race. Pl_3 and Pl_4 were considered to be almost identical to Pl_1 and Pl_{12}, respectively. At least 7 additional genes have been identified in the intervening years. Their races and origins are summarized. (Gulya et al. 1991).

1.6. Epigenetics

DNA, the genetic material, determines the genotype and indirectly the phenotype. The genes, comprising the genotype or DNA sequence, play a crucial role in determining the phenotype—the external structure—by directing the synthesis of numerous enzymes and proteins essential for cellular and organismal function. Epigenetics is the scientific field that investigates changes in gene expression that are not due to alterations in our genetic structure (genotype), but rather, differences in how genes influence the phenotype. The concept of gene expression addresses two fundamental biological questions: how a complex multicellular organism differentiates from a zygote during development, and which molecular mechanisms contribute to the inheritance of phenotypes. (Bock & Lengauer, 2008). Epigenetics focuses on the inheritance and regulation of gene expression that impacts phenotype, rather than on gene sequences themselves. “Epi-”, which means above, above in Greek, briefly means changes made above genetics. This term was first coined by British embryologist Waddington in his 1940 book "Organizers and Genes"; It has been mentioned that environmental conditions can affect gene expression and therefore alter development. While in the early twentieth century, epigenetic theory defined the organism as a product of the reaction between environmental factors, today it is expressed as "concepts related to what genes cannot explain alone" (Pickersgill et al., 2013).

As a result of epigenetic changes, proteins and RNA can regulate gene activities without changing the DNA sequence. Just as genes provide the codes for producing proteins, various chemicals called epigenetic marks provide instructions and determine when and how genes will work, suppressing gene expressions, silencing them, or activating some genes. Another feature of epigenetic mechanisms is that base pair changes are observed with a lower

error rate than mutations in the DNA sequence, suggesting that epigenetic information can be dynamically modified during cellular distinction (Bock & Lengauer, 2008). Various epigenetic mechanisms jointly control the packaging of DNA, thereby determining which genes are reachable for transcription.

These epigenetic mechanisms can be examined under three headings. These are DNA methylations, histone modification, and non-coding RNA regulation. DNA methylation, which occurs as a result of the binding of methyl groups to DNA fragments that directly affect DNA, is the most common of these. It is generally stated that low methylation levels are associated with potential activity and high levels are associated with inactivity (in the form of cessation of protein synthesis), and some methylations occur randomly and others occur in response to certain environmental or developmental stimuli (Jablonka & Lamb, 1998). In simple terms, a hydrogen atom of the cytosine base of DNA is replaced with a methyl group (CH_3). The attachment of methyl groups to gene regions prevents genes from being expressed, effectively silencing them. Consequently, protein production cannot occur from these gene regions. Binding of methyl groups to gene regions prevents the genes from expressing themselves, the genes are turned off or silenced, and as a result, protein production cannot be achieved from this gene region.

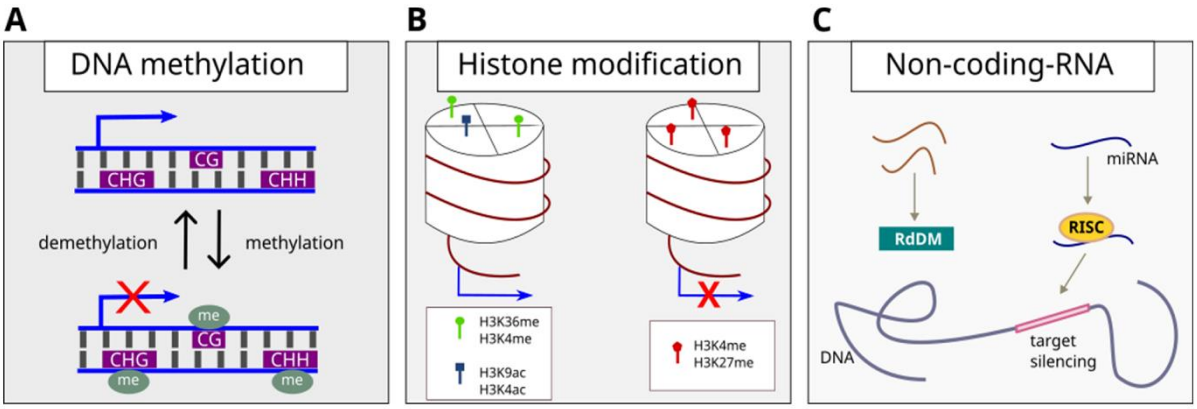

Figure 2. Epigenetic mechanisms (Kurpisz and Pawlowski, 2022).

1.6.1. Histone Modifications

Histones are involved in the packaging of DNA in chromatin. The configuration of histone proteins consists of positively charged amino acids such as lysine and arginine. The negatively charged DNA binds to this structure. Histones are transiently detached from DNA only during DNA replication and remain associated with the DNA during transcription. Unlike DNA, histone proteins undergo modifications that include methylation, acetylation, ubiquitylation, and phosphorylation. These modifications are related to both gene silencing and activation, depending on the nature of the modification and the specific amino acid modification. Some of these methylations are mentioned below (Zhang et al., 2020).

Histone acetylation takes place at the lysine residues of histones. Acetylation at lysine residues loosens the chromatin structure and thereby activates transcription. Deacetylation is associated with transcriptional repression. These processes are governed by histone acetyltransferases and histoneacetylases (Liu et al., 2022).

Histone methylation takes place at the arginine and lysine residues of histones. Reliant on the number of methyl groups added it can be called mono-, di-, trimethylation, and they all have different biological functions. These modifications are carried out by methyltransferases and can turn genes on and off, meaning whether the genes will be expressed (Liu et al., 2022).

Histone phosphorylation reaction takes place by the reversible attaching of phosphate groups to histone deacetylases. Phosphates bind to histone deacetylases via serine, threonine, and tyrosine residues. This reaction is carried out by protein kinases (PK) and protein phosphatases (PP) (Liu et al., 2022).

Ubiquitination of histones has been found to lead to silencing of inherited genes and X chromosome inactivation. A small protein ubiquitin (Ub) can bind to the lysine residues of histones. It causes the formation of a three-step enzymatic reaction. These reactions are conducted by E1, E2, E3 enzymes, and as a result, proteins enter the proteasome pathway and are degraded (Liu et al., 2022).

1.6.2. DNA Methylation

Through DNA methylation changes, plants reorganize their transcriptomes and maintain genome stability to enhance their adaptability to various environments. Alterations in DNA methylation due to environmental stresses can result in a new heritable phenotype. DNA methylation also suppresses noxious genetic elements, benefiting genome stability. DNA methylation is produced by DNA methyl transferases (DNMTs) in CpG islands and at carbon 5 of cytosine. Solely a subgroup of cytosine residues in a genome are methylated by DNA methyltransferases. The level of methylated cytosine differs in each organism, and the distribution of methylated cytosines on DNA is not the same in all species. More than 70% of the C-G base pairs in backbone of DNA are methylated. This methylation varies between organisms and among different tissues within organisms. It has long been thought that methylation profiles may have some functional consequences. In the first studies on this subject, it was determined that there is a relationship between methylation and gene expression. The low level of methylation in the promoter regions of genes correlates with active gene expression. DNMTs are responsible for the *de novo* methylation of DNA and the maintenance of this methylation. Methylation, such as the fifth atom of the cytosine base, typically influences gene expression by modifying proteins that bind to DNA and altering nucleosomes, which are fundamental units for DNA packaging in eukaryotes. DNA methylation, which allows evolutionary adaptation to new conditions without changing the DNA sequence in plants, includes the regulation of gene expression during tissue and organ development, and the response to biotic and abiotic stimuli. Methylation responses to environmental stresses including drought, cold, metals, salt, and developmental changes (seedling, ripening, flowering, fruiting) were studied (Kumar, 2018). Many studies have shown that maturation causes an increase in methylation (hypermethylation) or demethylation (hypomethylation) in reaction to stresses such as salinity, drought, and metal stresses. However, hypomethylation or hypermethylation is not common among organisms or developmental stages. Epigenetic rearrangements include not only responsiveness to ecological signals, but also the expression of other agronomically important traits such as fruit maturation, seed proportions, flowering time, plant magnitude, heterosis, plant height, sex determination, and pathogen resistance (Rehman and Tanti, 2020).

1.6.3. Non-Coding RNAs

Ribonucleic sequences that do not carry any information for the conversion from RNA to protein or can produce very short peptides, but have a regulatory role at the gene expression and protein level, are called non-coding RNAs (ncRNAs). ncRNAs are classified according to their size if they are longer than 200 nucleotides they are referred to as long non-coding RNAs (lncRNAs), and small ncRNAs (sncRNAs) if they are short (Sönmez and Yaylım, 2020).

RNA transcription is under tight control in eukaryotes. Long non-coding RNAs are engaged in the regulation of gene expression. The RNAs in this group are Xist, Tsix, and Linc RNAs (Sönmez and Yaylım, 2020).

Small RNAs (sRNAs) are RNAs consisting of double-stranded RNAs (dsRNA), 19-28 nucleotides (nt) long, non-coding, and whose full functions have not yet been elucidated. Some are thought to consist of parts of mRNAs or intergenic regions of the genome. Their major function is to contribute in gene silencing. Models of small non-coding RNAs are miRNA, siRNA, and piRNA (Yan and Bu, 2021).

1.7. Methylation Sensitive Amplification Polymorphism (MSAP)

Methylation Sensitive Amplification Polymorphism (MSAP) is widely employed to assess DNA methylation alterations in plants. This method is a gel-based depiction of PCR amplicons which are selectively amplified DNA separated using methylation-sensitive restriction enzymes. This method does not rely on a reference genome, making it feasible to assess methylation status across the entire genome. It is an impactful approach for mapping DNA methylation and identifying genes with differential methylation. The stages of the MSAP method are given below:

i) Segmentation of DNA with methyl regions sensitive enzymes,

ii) Separation of DNA fragments with the help of polyacrylamide gel electrophoresis (PAGE) and profiling of bands to detect bands that differ between samples,

iii) Sequencing of divergent bands isolated from the gel.

MSAP method depends on the activity of restriction enzymes (REs) which are methylation sensitive. The restriction enzymes *Msp*I and *Hpa*II are isoschisomers of each other and both can cut the 5'-CCGG-3' sequence. *Hpa*II can cut only non-methylated and hemimethylated CCGG sequences, while *Msp*I can cut unmethylated, hemimethylated, or fully methylated CCGG sequences (Salmon et al. 2008).

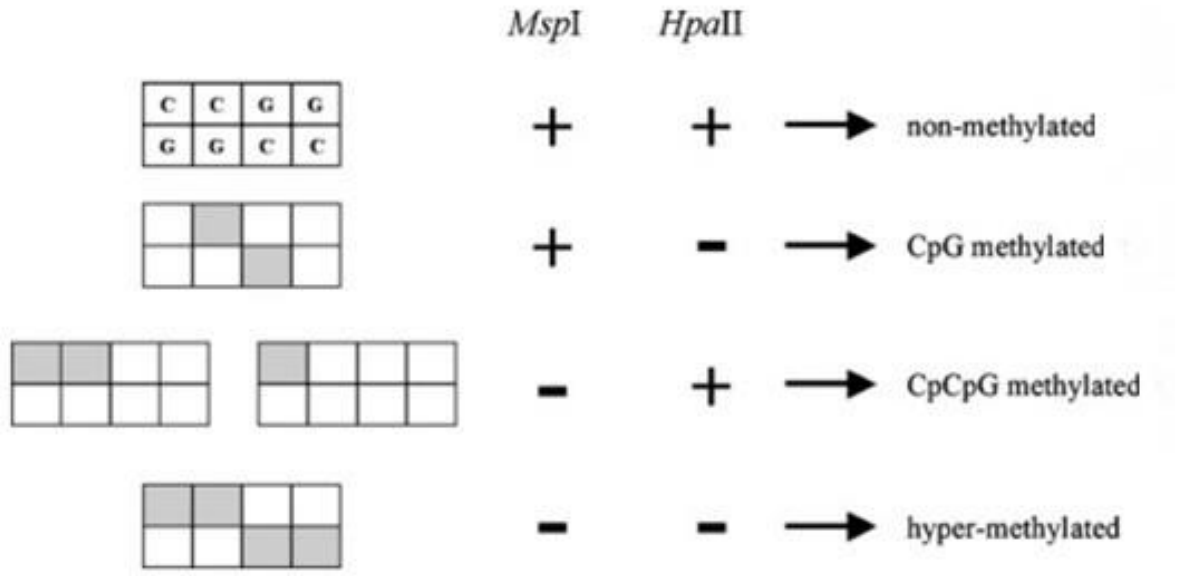

Figure 3. *Msp*I and *Hpa*II sensitivities according to 5'-CCGG methylation status (''+'': enzyme cuts; ''-'': enzyme does not cut) (Salmon et al. 2008)

1.8. Polyacrylamide Gel Electrophoresis (PAGE)

Electrophoretic separation of proteins is a technique that has been used for a prolonged period and has many diverse types. The activities of enzymes can be determined on gels after electrophoretic separation and native gels can be used for this purpose. (Temizkan, 1999), Polyacrylamide, a polymer synthesized from acrylamide and cross-linking agents, is used to create Polyacrylamide Gel Electrophoresis (PAGE) gels, which offer significantly better resolution than agarose gels. Denaturing PAGE can detect single base differences in nucleic acid sequences, while non-denaturing PAGE can identify single base variations within fragments of the same size, such as in single-strand conformational polymorphism (SSCP) analysis. PAGE is particularly useful for analyzing single-stranded nucleic acids, whereas agarose gels are typically used for double-stranded nucleic acids.

PAGE gels are formed through a chemical polymerization reaction catalyzed by ammonium persulfate (10% APS), rather than through a temperature-dependent solubility change as with agarose gels. This polymerization process can take several hours and may occasionally result in regions of the gel that are not fully formed. Acrylamide is a neurotoxin, so gloves must be worn when handling PAGE gels. Because PAGE gels are usually prepared between two glass plates, careful attention is needed to prevent leaks, improperly formed wells, or trapped air bubbles during the preparation process. Glassware plates must be cleaned thoroughly with warm detergent, rinsed with deionized water, and wiped with ethanol after each use. Once tetramethylethylenediamine (TEMED) and Ammonium persulphate (APS) are added to the acrylamide mixture, the mixture must be promptly transferred to the gel assembly to avoid premature polymerization. The mixture is often loaded using syringes with wide-bore needles or Pasteur pipettes. When filling a large PAGE assembly, tapping the glass plates can assist in releasing any trapped air bubbles. Sample combs should also be meticulously cleaned to ensure proper well formation. To ensure complete polymerization, PAGE gels are generally left in the refrigerator for several hours or overnight (Sun et al., 2010).

CHAPTER 2
PREVIOUS STUDIES

2.1. Detection of *Plasmopara halstedii* in Sunflowers

Martinez et al. performed a molecular test for precise amplification of the *P. halstedii* PHA 42 marker in DNA from full seed specimens including DNA from both the pathogen and the host in 2021. In this study, the presence of pathogen in asymptomatic sunflowers next to naturally infected sunflowers in the field was tested with molecular markers. Diagnostic marker found one of 21 asymptomatic plants that contained the pathogen (Martinez et al., 2021).

In 2019, Singh published a research paper and in this study, he developed an algorithm that can recognize and classify leaf diseases in sunflowers. Leaf diseases can be seen with the naked eye in sunflowers, but it is very difficult to make this observation and classify the diseases in large fields. For this reason, the study serves the purpose of facilitating classification. The photographed sunflower leaves are recognized and classified by the Particle swarm optimization algorithm. The average classification accuracy of the proposed algorithm is 98% (Singh, 2019).

In 2013, Grasse et al. proved the hypovirulant effect of *Plasmopara halstedii* virus (PhV), an isolate of the pathogen *Plasmopara halstedii* oomycete that causes downy mildew in sunflowers (Grasse et al., 2013).

2.2. Resistance to Downy Mildew in Sunflowers

In 2008, Nandeeshkumar et al. observed that resistance to downy mildew was induced in sunflowers treated with chitosan. The results show increased regulation of defense protein gene expression in sunflowers treated with chitosan (Nandeeshkumar et al., 2008).

In 2018, Yonet et al. designed a CRISPR/Cas9 system for biotic and abiotic stress resistance in sunflowers. The management of if the plasmid is transferred for gene editing and whether gene editing is efficient is discussed (Yonet et al., 2018).

2.3. DNA Methylation in Plant Responses

The potato tuber turns green when exposed to light and toxic substances such as solanine can accumulate. In 2022 Xiong et al. investigated the impact of exposure to light on DNA methylation using MSAP throughout this greening process. The results confirmed that changes in DNA methylation were dependent on light exposure (Xiong, 2022).

It was known that nitric oxide can reduce cold damage in peach fruit during cold preservation. In 2022, Guo et al. investigated the impact of nitric oxide on DNA methylation in cold-stressed peaches. It has been indicated that nitric oxide may increase the cold tolerance of after harvest peaches by facilitating DNA methylation (Guo et al., 2022).

CHAPTER 3
MATERIAL METHOD

3.1. Plant Material

This thesis utilized two commercially available genotypes of sunflowers (*Helianthus annus* L.) These included sensitive genotypes, both infected and uninfected with Downy Mildew, and resistant genotypes, similarly infected or uninfected with Downy Mildew. Commercial sunflower genotypes were obtained from the Trakya Agricultural Research Institute, Edirne. Name of genotypes, characteristics, and the number of individuals of each genotype are shown in Table 2.

Table 2

Commercial sunflower (*Helianthus annuus* L.) genotypes used in this study.

Name of Genotypes	**Characteristics**	**Number of Individuals**
IMI-044B	Downy Mildew infected Sensitive	3
SUN 2259-CL	Downy Mildew infected Resistant	3
IMI-044B	Uninfected Sensitive	3
SUN 2259-CL	Uninfected Resistant	3

Sunflower seeds were planted at the Trakya Agricultural Research Institute and germination was observed after 2 days. They prepared a solution by removing the spores on dried leaves from last summer that were waiting at -80^{o}C and kept the germinated seeds in that solution for 4 hours at 15 ^{o}C. Germinated seeds infected in this way were planted in plastic cups to continue growing. Environmental conditions were set at 26 ^{o}C and 65% humidity. It was observed that after 5 days the plants started to grow. At this stage, the plastic cups were bagged and covered, and the environmental conditions were changed to 65% humidity at 15^{o}C, 12 hours at night and 12 hours daylight. When packaged, the humidity level can reach up to 95%. Plant material collected 2 days after this procedure.

The plant's young leaves, suitable for DNA isolation, were preferred during the collection of the material. After the cut leaves were cleaned with alcohol, they were wrapped in aluminum foil and frozen with liquid nitrogen (-196°C). They were stored in liquid nitrogen and then transported to the deep freezer (-80°C).

3.2. DNA Isolation

Genomic DNA isolation was performed with the CTAB (Cetyltrimethylammonium bromide) method (Doyle & Doyle 1987). The protocol was implemented as follows.

Samples kept at -80 °C are weighed approximately 70-80 mg and placed in tubes. Two ceramic beads are added into the tubes and the beads broke down by rotating them in the bead ruptor device at speed 5 for 20 seconds. 450 µl CTAB buffer (Cetyltrimethyl Ammonium Bromide) buffer [0.1 M Tris (pH: 8), 1.4 M NaCl, 0.02 M EDTA, and 2 g 2% CTAB] and 0.2% β -mercaptoethanol is added to the tubes and each sample is kept at 60°C. 8 mg of PVP (polyvinylpyrrolidone) is added into the tubes and incubated at 60 °C for 25 minutes. (Samples are turned upside down every 5 minutes.) Samples taken from the incubation are left to cool until they reach room temperature. 750 µl chloroform:octanol is added and samples are turned upside down 20-25 times. Samples are centrifuged at 13,000 rpm for 15 minutes. The supernatant (volume V) is then transferred to a new tube. 5M sodium chloride, 0.5 times V_1 (V_2), is added to it. Cold 95% EtOH, twice V_2, is added and incubated at -80 °C for 1 hour. Samples taken from -80 °C are kept at room temperature for 5 minutes and centrifuged at 13,000 rpm for 10 minutes and the supernatant is carefully removed. While 1000 µl of 75% EtOH is added, the pellet is washed and centrifuged at 13,000 rpm for 10 minutes. The supernatant is removed and the tubes are left to dry overnight with their caps open. DNA is dissolved by adding nuclease-free water to the dried DNA samples. RNase A enzyme is added and incubated in a 37 °C water bath for 20 minutes and the DNA isolation protocol is completed. Then the isolated DNA samples were run on 1% agarose gel electrophoresis.

3.3. Methylation Sensitive Amplification Polymorphism

3.3.1 Cleavage with Restriction Enzymes

First of all, for each sample from the isolated DNAs, a mixture was prepared by adding 1000 ng/μl DNA, 5 U *Eco*RI (Thermo Scientific), 10 U *Msp*I (Thermo Scientific), 2X Tango Buffer (Thermo Scientific) and 13.5 μl ddH2O in 25 μl volume. The prepared mixture was incubated at 37 °C 3 hours and 65 °C 20 minutes. In a separate tube, another mixture was prepared by adding 1000 ng/μl DNA, 5 U *Eco*RI (Thermo Scientific), 20 U *Hpa*II (Thermo Scientific), 2X Tango Buffer (Thermo Scientific) and 12.5 μl ddH_2O in 25 μl volume. The prepared mixture was incubated for 3 hours at 37 °C and finally for 20 minutes at 65 °C. After that cleaved DNA samples were visualized on agarose gel electrophoresis to see a smear image.

3.3.2. Adapter Binding (Ligation)

Appropriate adapter sites for methylation-sensitive *Hpa*II/*Msp*I and methylation in-sensitive *Eco*RI were attached to the ends of DNA fragments to facilitate DNA amplification. For this, 60 U *Hpa*II (Thermo Scientific) linker, 6 U *Eco*RI (Thermo Scientific) linker, 1 U T4 DNA Ligase (Fermentas), 1X T4 Ligase Buffer (Fermentas), 10 μl restriction mix and 3.4 μl water (ddH_2O) are added. It was taken into a tube with a total volume of 25 μl. The prepared mixture was incubated at 23 °C for 4.5 hours and at 60 °C for 10 minutes and stored for PCR at -20 °C. Adapter sequences used in the reactions are shown in Table 3.

Table 3

Adapter sequences.

Adapters	**Base Sequence 5'-3'**	**Tm (℃)**
***EcoR*1 adapter 5**	CTCGTAGACTGCGTACC	55
***EcoR*1 adapter 3**	AATTGGTACGCAGTCTAC	51
***Hpa*II/*Msp*I adapter 5**	GATCATGAGTCCTGCT	49
***HpaII/ MspI* adapter 3**	CGAGCAGGACTCATGA	52

3.3.3. Pre-Selective Polymerase Chain Reaction (PCR)

For PCR reaction, 5 μl from 1/10 diluted DNA (ligation product) samples of cultivars, 1X PCR Buffer (Invitrogen), 10 mM Mg, 10 mM dNTP (Thermo Scientific), 1.5 U Taq DNA (Invitrogen), 10 $pmol$ linker *EcoR*I, 10 $pmol$ linker *Hpa*II, 9.3 μl water (ddH_2O) was put into the tube, with a total volume of 20 μl. The linker was prepared as follows: 100 $pmol$ *EcoR*1 5'+ 100 $pmol$ *EcoR*1 3' and 100 $pmol$ *Hpa*II 5'+ 100 $pmol$ *Hpa*II 3'.

The PCR protocol was performed at 94 °C for 1 minute denaturation step, at 94 °C for 30 seconds, at 51 °C for 30 seconds, at 72 °C for 1 minute for 30 cycles, at 72 °C for 10 minutes.

3.3.4. Selective Polymerase Chain Reaction (PCR)

For PCR reaction, 1/10 diluted DNA samples of the cultivars with ligation product 5 μl, 1X PCR Buffer (Invitrogen), 10mM Mg, 10 mM dNTP (Thermo Scientific), 1.5 U Taq DNA (Invitrogen), 10 $pmol$ F, 10 $pmol$ R, 9.3 μl water (ddH_2O) was put into the tube, with a total volume of 20 μl. PCR protocol 5 minutes at 94 °C, 30 seconds at 94 °C, 1 minute at 65 °C (by decreasing 0.7 °C in each cycle), 12 cycles at 72 °C for 1 minute, followed by 94 °C 23 cycles of 30 seconds at C, 1 minute at 56 °C, 1 minute at 72 °C, and finally 5 minutes at 72 °C. 3 μl 1% agarose gel was loaded from the pre-amplification mixture and images were taken. Primers used in PCR reactions are shown in Table 4.

Table 4

Primer sequences.

Primers	**Base Sequence 5'-3'**	**Tm (℃**
EcoR1 +1 primer	GACTGCGTACCAATTCA	50
EcoR1 +2 primer a	GACTGCGTACCAATTCAG	54
EcoR1 +2 primer b	GACTGCGTACCAATTCAT	51
EcoR1 +2 primer c	GACTGCGTACCAATTCAC	54
HM + 1 primer	ATCATGAGTCCTGCTCGGT	57
HM + 3 primer a	ATCATGAGTCCTGCTCGGTAA	58
HM + 3 primer c	ATCATGAGTCCTGCTCGGTTC	60
E_d	GACTGCGTACCAATTCAAC	55
E_e	GACTGCGTACCAATTCACG	57
E_f	GACTGCGTACCAATTCACT	55
H_d	ATCATGAGTCCTGCTCGGTAG	60
H_e	ATCATGAGTCCTGCTCGGGTGG	62

3.3.5. Polyacrylamide Gel Electrophoresis (PAGE)

Firstly, to prepare polyacrylamide gel, by adding 6 ml 40% (19:1) Acrylamide/Bis Solution (BIO-RAD), 0.1% APS (Ammonium persulfate) 0.01X TEMED, 1X TAE (Tris-acetate-EDTA) Polyacrylamide gel with 6% concentration was prepared. The prepared gel was optimized to be performed on an adjustable vertical gel electrophoresis instrument (The Cleaver Scientific Ltd.) using a 19.4 cm x 18.5 cm (W x B) gel and 1.0 mm thickness. The prepared gel cassette was placed in the gel pouring chamber. To prevent the gel from leaking, 5% agar solution was applied around the apparatus. The prepared mixture was loaded into the gel cassette and a 12-tooth comb was placed. It was waited for 40 minutes for the gel to polymerize. The combs were then removed. 1x TAE (Tris-acetate-EDTA) buffer is filled into the tank. The cassette containing the polymerized gels was placed in the electrophoresis device.

DNA fragments were separated using the polyacrylamide gel electrophoresis (PAGE) method. The products obtained as a result of the PCR reaction were loaded into the polyacrylamide gel with 6% concentration by centrifugation by adding 3 μl Loading Dye (Thermo Scientific) to the 20 μl PCR product before being run on the polyacrylamide gel and adjusted to flow 200 V. The electrophoresis process was completed by running for about 2 hours.

The bands formed after electrophoretic separation were analyzed using ethidium bromide staining. As a result of the analysis, images of the gel were taken under UV light using the UVP PhotoDoc-It Imaging System model imaging device.

3.4. Scoring

The analysis file was prepared by giving 0 and 1 values according to the scoring method to the bands obtained as a result of the imaging. After the DNA fragments obtained in the analyses were separated using the polyacrylamide gel electrophoresis (PAGE) method, the bands were profiled and the bands that differed between the samples were determined.

MSAP scores were arranged as a binary matrix and transformed to a distance matrix with the aid of PhylTools (Revell, 2012). The jaccard index was used as a similarity measure.

The genetic tree was generated by means of the neighbor-joining method of Saitou and Nei (Saitou and Nei, 1987) and the software Phylip v.3.69 (Felsenstein, 1989). To assess the reliability of the tree, a bootstrap analysis using 1000 replicates (Felsenstein, 1985) was applied. To find the distinctions according to epigenetic similarities within the dataset, a principal coordinate analysis (PCoA) was implemented using NTSYS-pc v.2.1 (Rohlf, 2000). Samples are shown as points on a 3D plot.

CHAPTER 4
RESEARCH FINDINGS

4.1. Collecting Plant Material

Leaves were sampled with a sterile scalpel for genomic DNA isolation from plant varieties that were germinated and grown under suitable environmental conditions. Tissue samples taken from the varieties were stored at -80°C. Images of growing plants are given in Figure 4.

Figure 4. Sensitive and resistant sunflower varieties obtained from Trakya Agricultural Research Institute.

4.2. Genomic DNA Isolation

DNA was isolated from tissues taken from leaves of sunflower varieties. To asses the quality of the isolated DNA samples, the samples were subjected to agarose gel electrophoresis. The concentrations of the DNA samples were measured with a Nanodrop (ND-2100C, Thermo) spectrophotometer device, and the concentrations and purity of the samples were established by Absorbance (A) 260/280 and 260/230 values. The results obtained are given in Figure 5 and Table 5. The NanoDrop emits a wide spectrum of light (usually in the UV-Vis range) and this light passes through the sample. The light interacts with the molecules in the sample as it passes through the sample. Some wavelengths are absorbed by the sample, while others pass through the sample (Grasse et al., 2015).

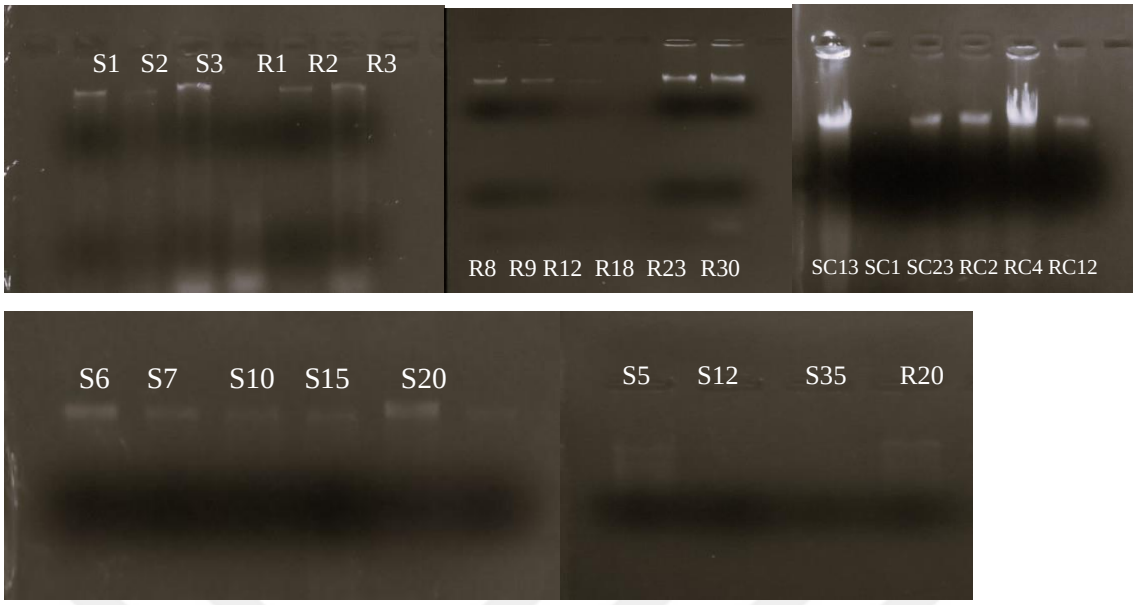

Figure 5. DNA isolation results viewed with 1% agarose gel. (S: Sensitive, R: Resistant, SC: Sensitive Control, RC: Resistant Control).

Table 5

DNA concentrations and purity levels of tissues of sunflower varieties.

Sample Number	**DNA Concentration (ng/µl)**	**A260/A280**	**A260/230**
S3	600	1,988	1,512
R9	667	1,896	1,276
SC23	443	1,863	1,226
RC12	228	1,861	1,243
S6	306	1,849	1,246
R23	634	1,961	1,602
RC2	235	1,843	1,116
SK13	384	1,887	1,354
RC4	234	1,843	1,116
SC15	157	1,656	0,658
R18	334	1,868	1,217
S20	164	1,703	0,76

4.3. MSAP Method

MSAP is a prevalent method for DNA methylation in different plant species due to its low cost and reliability (Chwialkowska et al. 2017). DNA was first cleaved with the methylation-insensitive *Eco*RI, the methylation-sensitive restriction enzyme *Hpa*II, and a methylation-sensitive isoschizomer of *Msp*I. After that, the cleaved DNA was visualized on agarose gel electrophoresis (Figures 6, 7, 8, 9, 10).

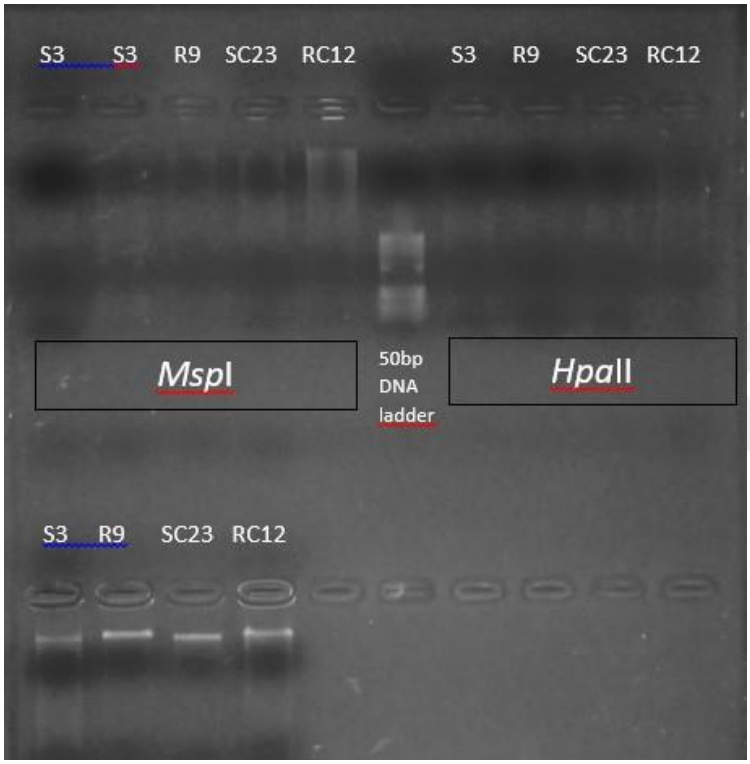

Figure 6. Agarose gel electrophoresis image of DNA cleavage with restriction enzymes.

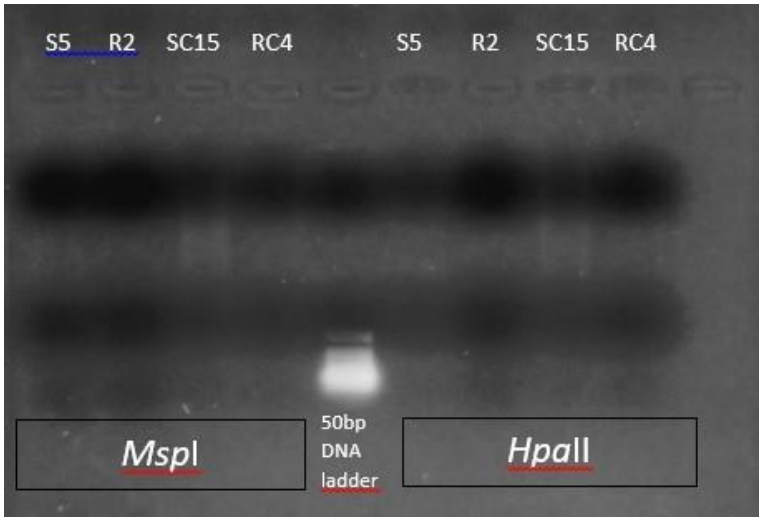

Figure 7. Agarose gel electrophoresis image of DNA cleavage with restriction enzymes.

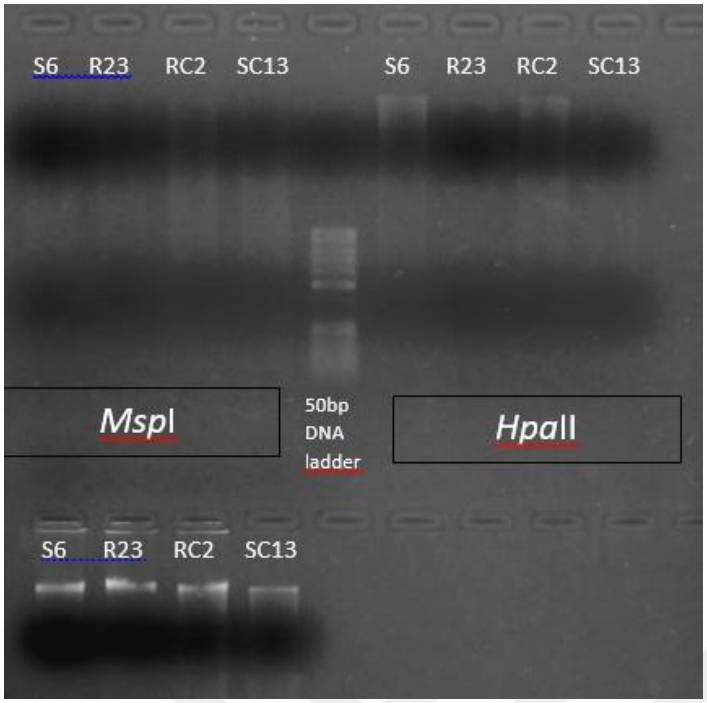

Figure 8. Agarose gel electrophoresis image of DNA cleavage with restriction enzymes.

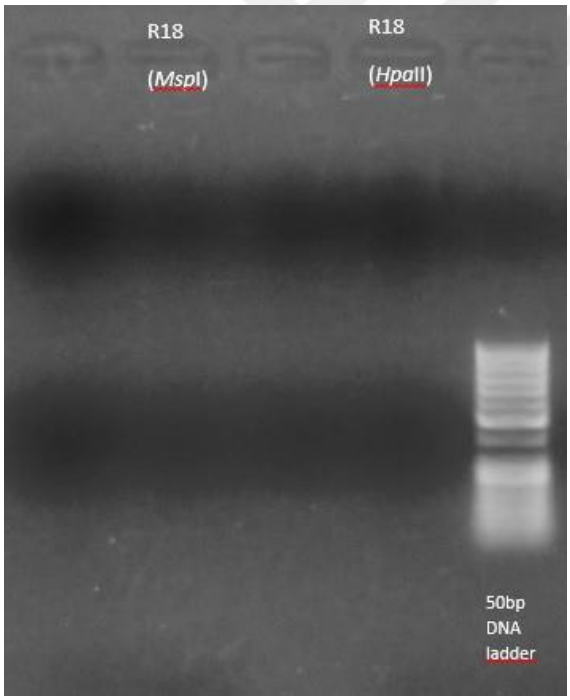

Figure 9. Agarose gel electrophoresis image of DNA cleavage with restriction enzymes.

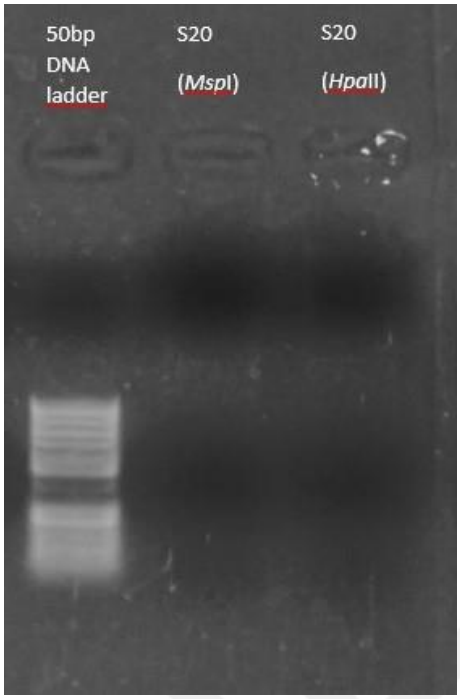

Figure 10. Agarose gel electrophoresis image of DNA cleavage with restriction enzymes.

Complementary specific adapters were ligated to the sticky ends formed as a result of the cleaved DNA, PCR was performed with pre-selective primers, and the band profile was visualized on agarose gel electrophoresis (Figure 11, 12, 13).

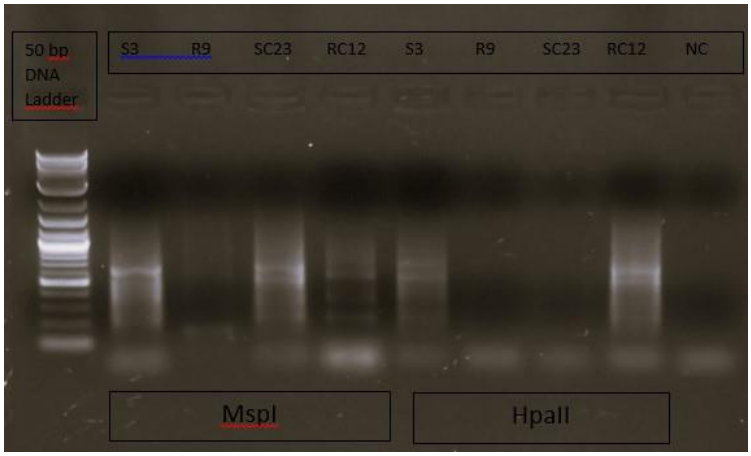

Figure 11. Agarose gel electrophoresis image of pre-selective amplification.

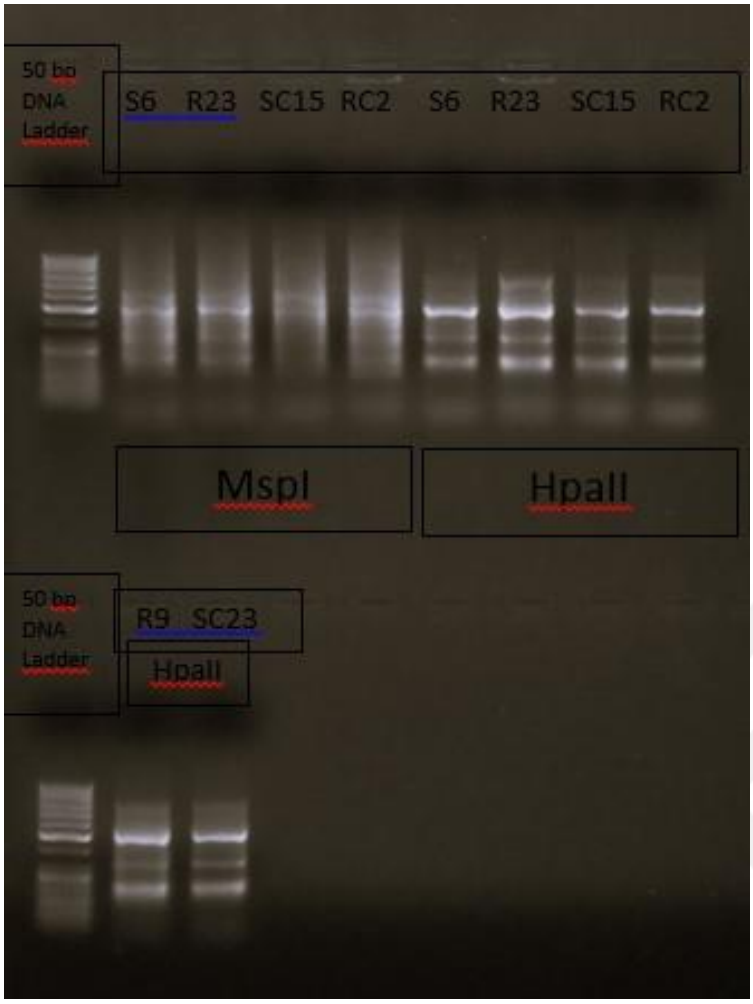

Figure 12. Agarose gel electrophoresis images of pre-selective amplification.

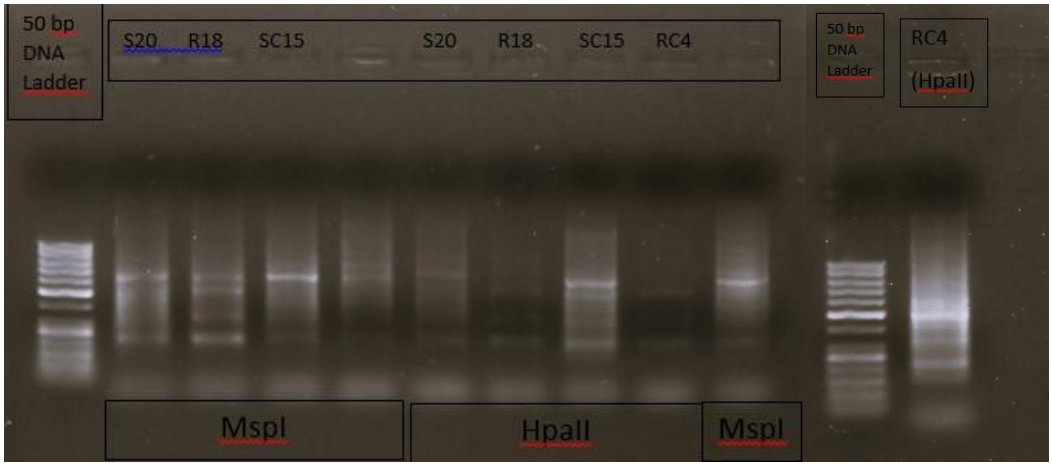

Figure 13. Agarose gel electrophoresis images of pre-selective amplification.

10 primer combinations were used (Table 6) and then two-step PCR amplification was performed: pre-selective PCR and selective PCR. For detailed separation of the fragments obtained from 10 primer combinations, the products obtained as a result of the PCR reaction were separated using the polyacrylamide gel electrophoresis (PAGE) method.

Obtained PAGE images taken under UV light using the UVP PhotoDoc-It Imaging System model imaging device are given below (Figure 14).

Table 6

Forward-reverse primer combinations.

Primer Combinations	**Forward**	**Reverse**
1st combination	E_e	H_d
2nd combination	E_e	H_e
3rd combination	EcoRI +2 primer a	HM + 3 primer a
4th combination	EcoRI +2 primer a	HM + 3 primer c
5th combination	EcoRI +2 primer b	HM + 3 primer a
6th combination	EcoRI +2 primer b	HM + 3 primer c
7th combination	EcoRI +2 primer c	HM + 3 primer a
8th combination	EcoRI +2 primer c	HM + 3 primer c
9th combination	E_f	HM + 3 primer a
10th combination	E_f	HM + 3 primer c

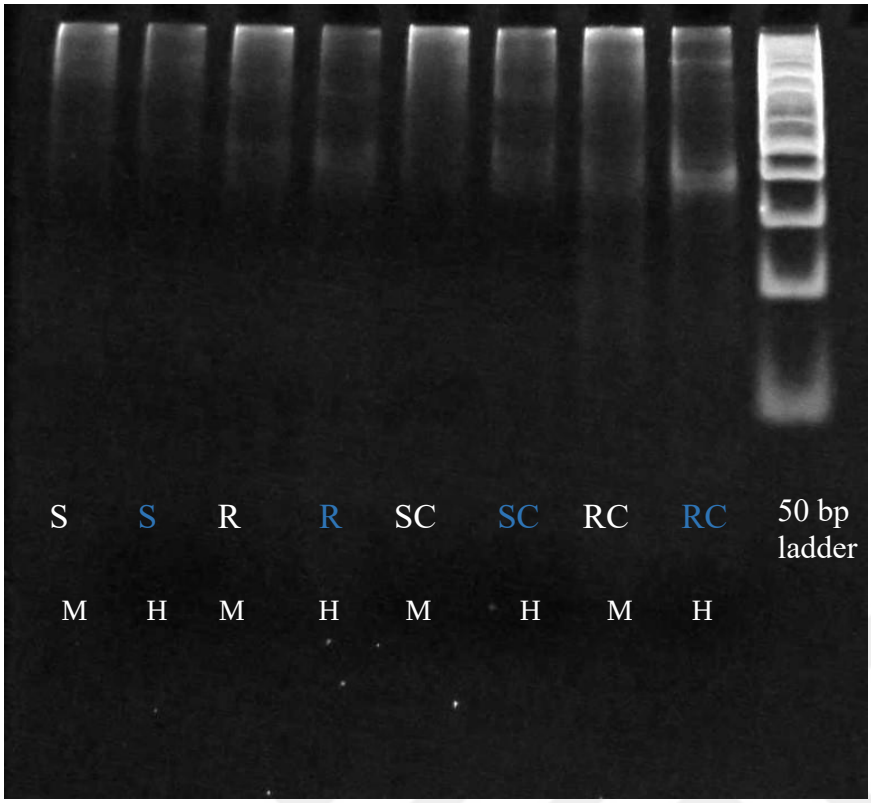

Figure 14. PAGE image of the selective PCR with the 1st combination. (E_e, H_d) (S: Sensitive, R: Resistant, SC: Sensitive Control, RC: Resistant Control, M: *Msp*I H: *Hpa*II. The letters written in blue mean that the sample was cleaved with *Hpa*II and the letters in white were cleaved with *Msp*I.)

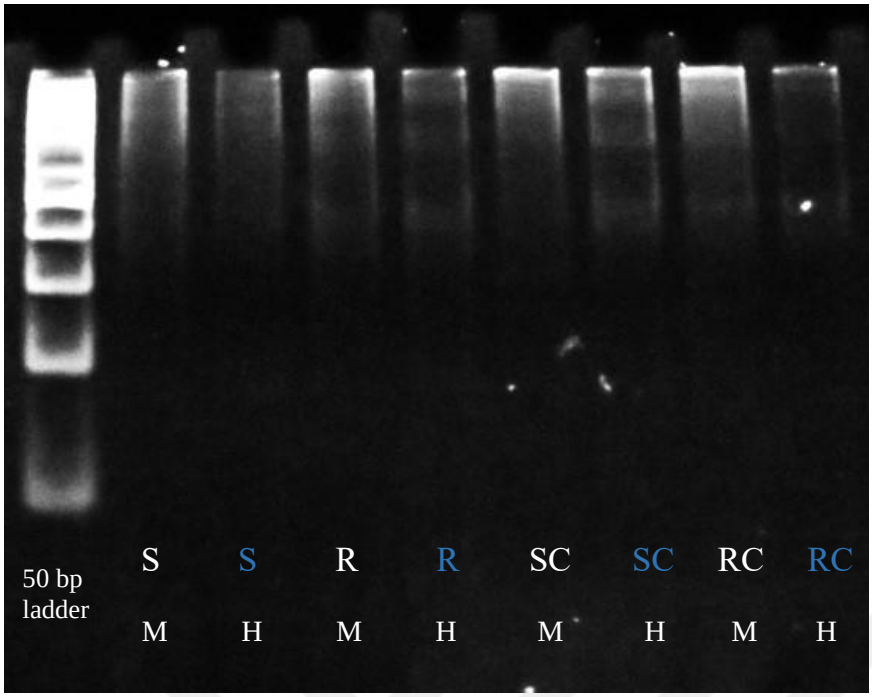

Figure15. PAGE image of the selective PCR with the 2nd combination. (E_e, H_e) (S: Sensitive, R: Resistant, SC: Sensitive Control, RC: Resistant Control, M: *Msp*I H: *Hpa*II) (The letters written in blue mean that the sample was cleaved with *Hpa*II and the letters in white were cleaved with *Msp*I.)

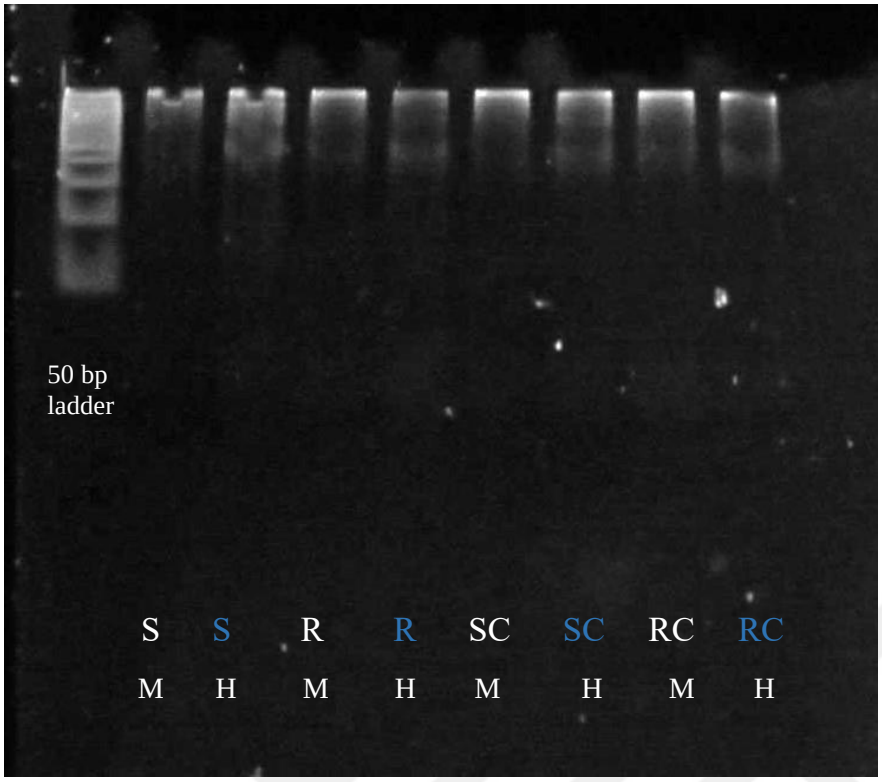

Figure 16. PAGE image of the selective PCR with the 3rd combination. (E+2a, H+3a) (S: Sensitive, R: Resistant, SC: Sensitive Control, RC: Resistant Control, M: *Msp*I H: *Hpa*II) (The letters written in blue mean that the sample was cleaved with *Hpa*II and the letters in white were cleaved with *Msp*I.)

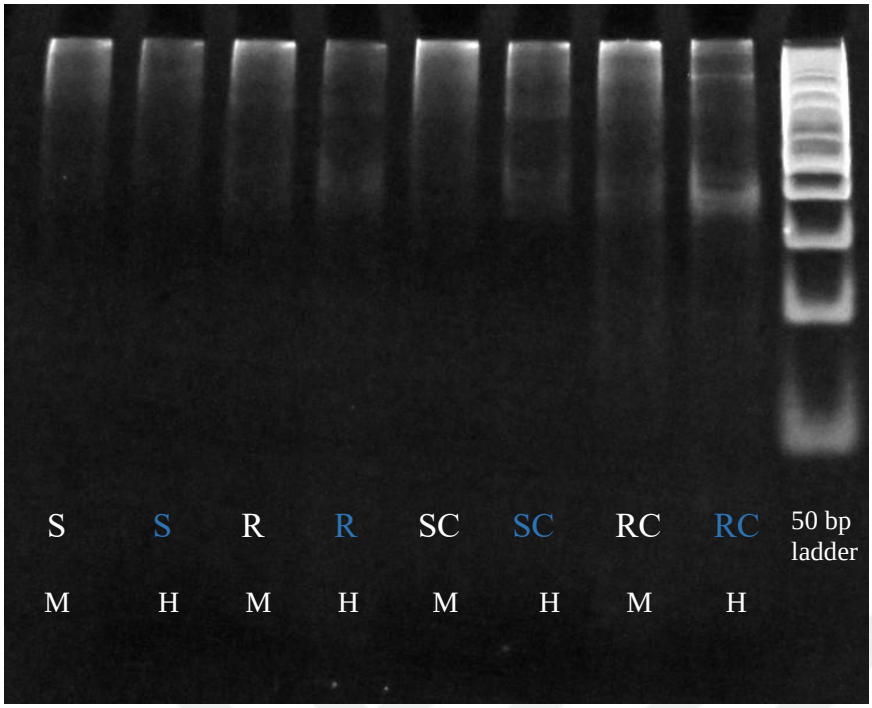

Figure 17. PAGE image of the selective PCR with the 4th combination. (E+2a, H+3c) (S: Sensitive, R: Resistant, SC: Sensitive Control, RC: Resistant Control, M: *Msp*I H: *Hpa*II) (The letters written in blue mean that the sample was cleaved with *Hpa*II and the letters in white were cleaved with *Msp*I.)

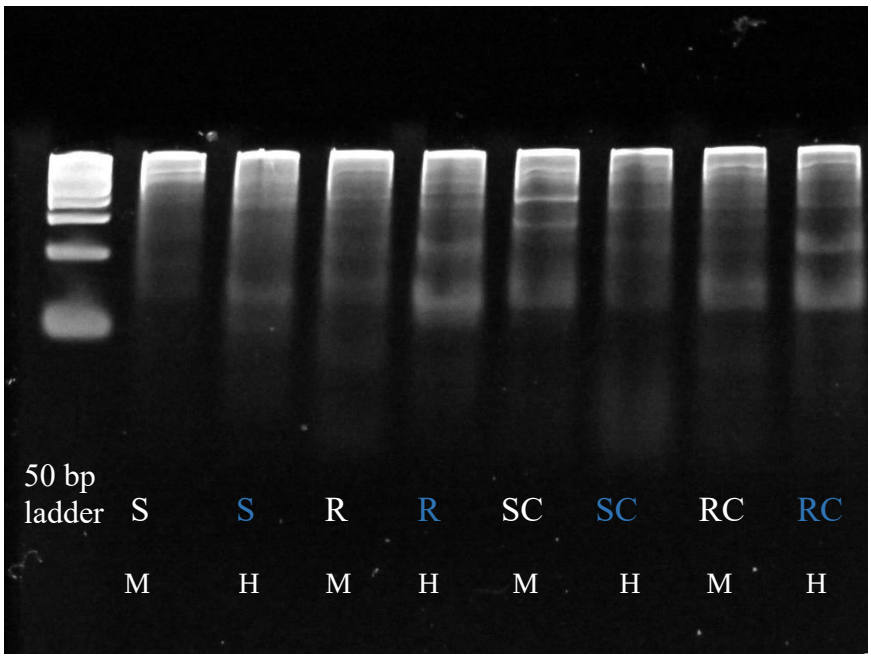

Figure 18. PAGE image of the selective PCR with the 5th combination. (E+2b, H+3a) (S: Sensitive, R: Resistant, SC: Sensitive Control, RC: Resistant Control, M: *Msp*I H: *Hpa*II) (The letters written in blue mean that the sample was cleaved with *Hpa*II and the letters in white were cleaved with *Msp*I.)

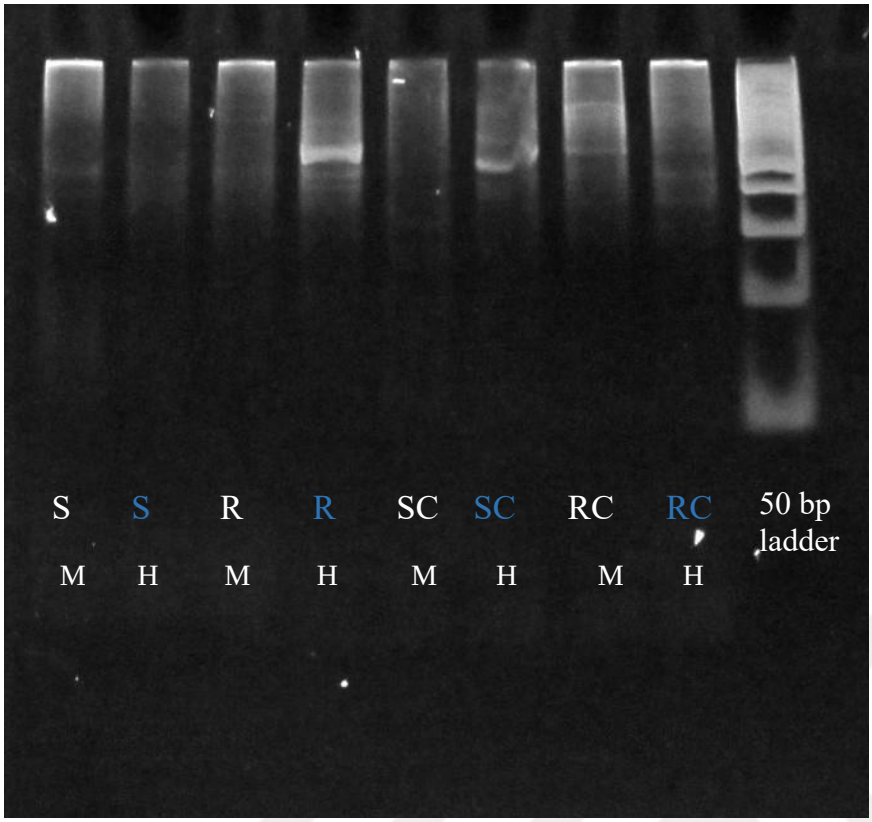

Figure 19. PAGE image of the selective PCR with the 6th combination. (E+2b, H+3c) (S: Sensitive, R: Resistant, SC: Sensitive Control, RC: Resistant Control, M: *Msp*I H: *Hpa*II) (The letters written in blue mean that the sample was cleaved with *Hpa*II and the letters in white were cleaved with *Msp*I.)

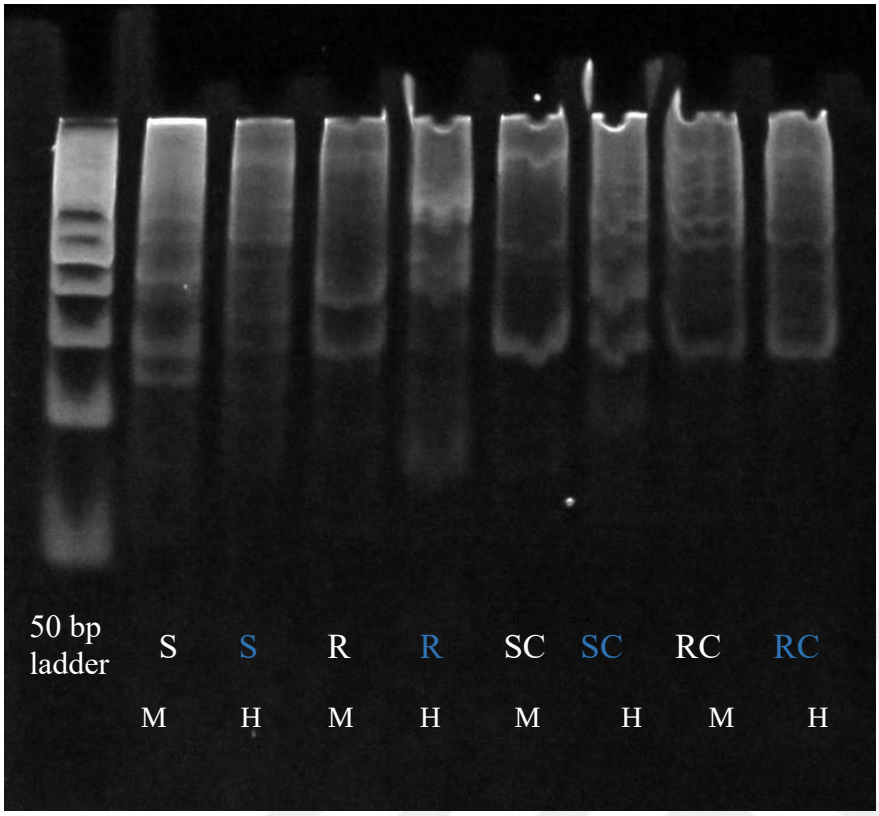

Figure 20. PAGE image of the selective PCR with the 7th combination. (E+2c, H+3a) (S: Sensitive, R: Resistant, SC: Sensitive Control, RC: Resistant Control, M: *Msp*I H: *Hpa*II) (The letters written in blue mean that the sample was cleaved with *Hpa*II and the letters in white were cleaved with *Msp*I.)

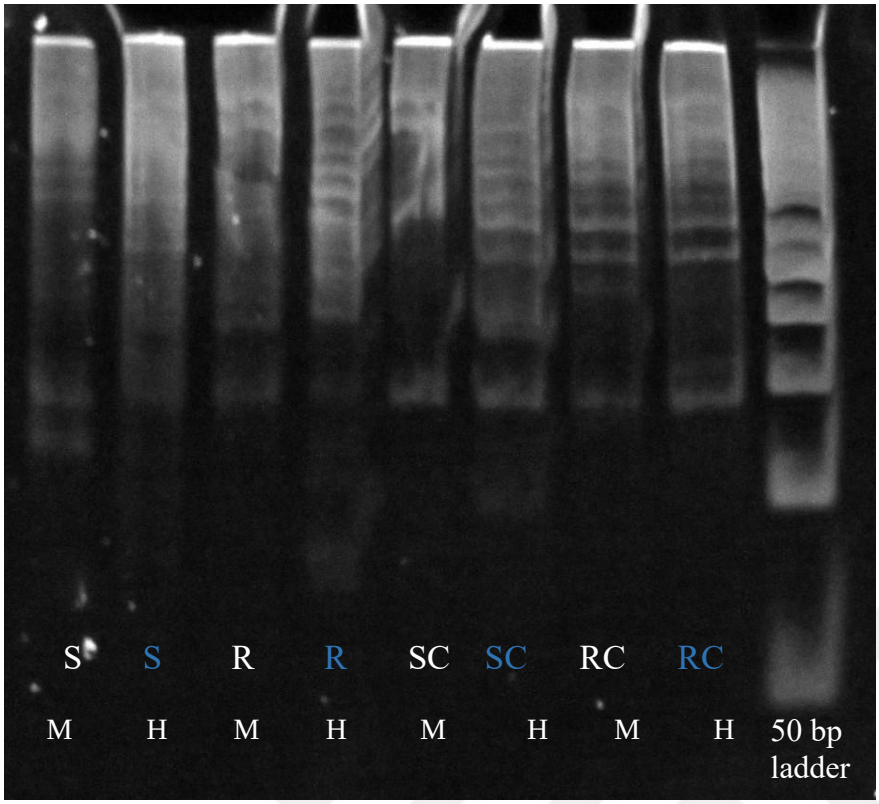

Figure 21. PAGE image of the selective PCR with the 8th combination. (E+2c, H+3c) (S: Sensitive, R: Resistant, SC: Sensitive Control, RC: Resistant Control, M: *Msp*I H: *Hpa*II) (The letters written in blue mean that the sample was cleaved with *Hpa*II and the letters in white were cleaved with *Msp*I.)

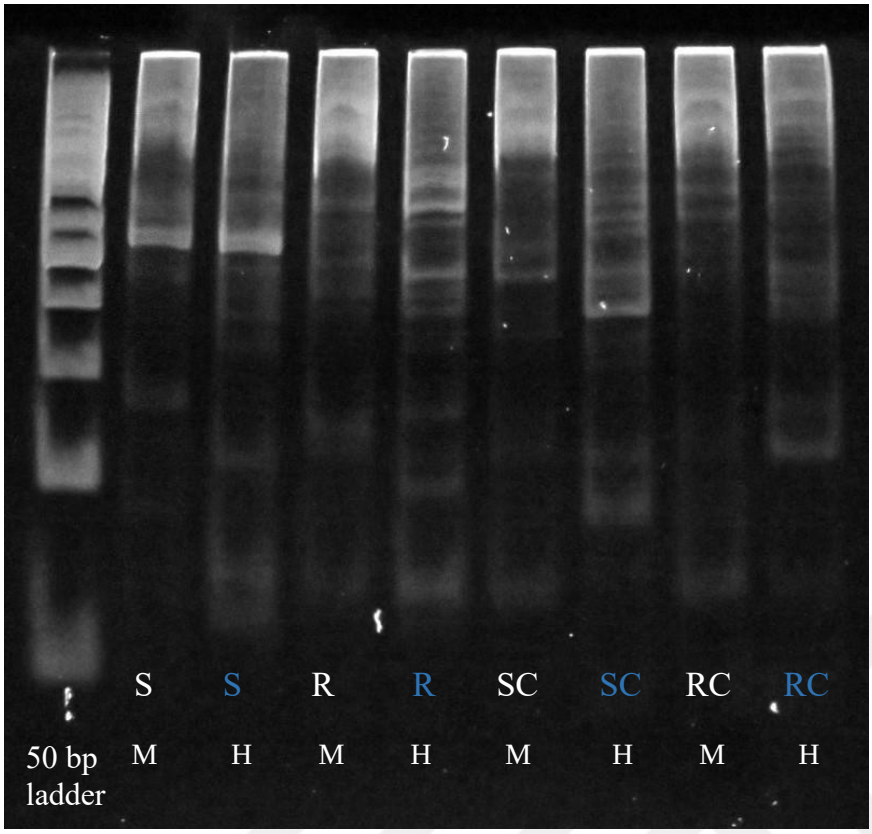

Figure 22. PAGE image of the selective PCR with the 9th combination. (E_f, H+3a) (S: Sensitive, R: Resistant, SC: Sensitive Control, RC: Resistant Control, M: *Msp*I H: *Hpa*II) (The letters written in blue mean that the sample was cleaved with *Hpa*II and the letters in white were cleaved with *Msp*I.)

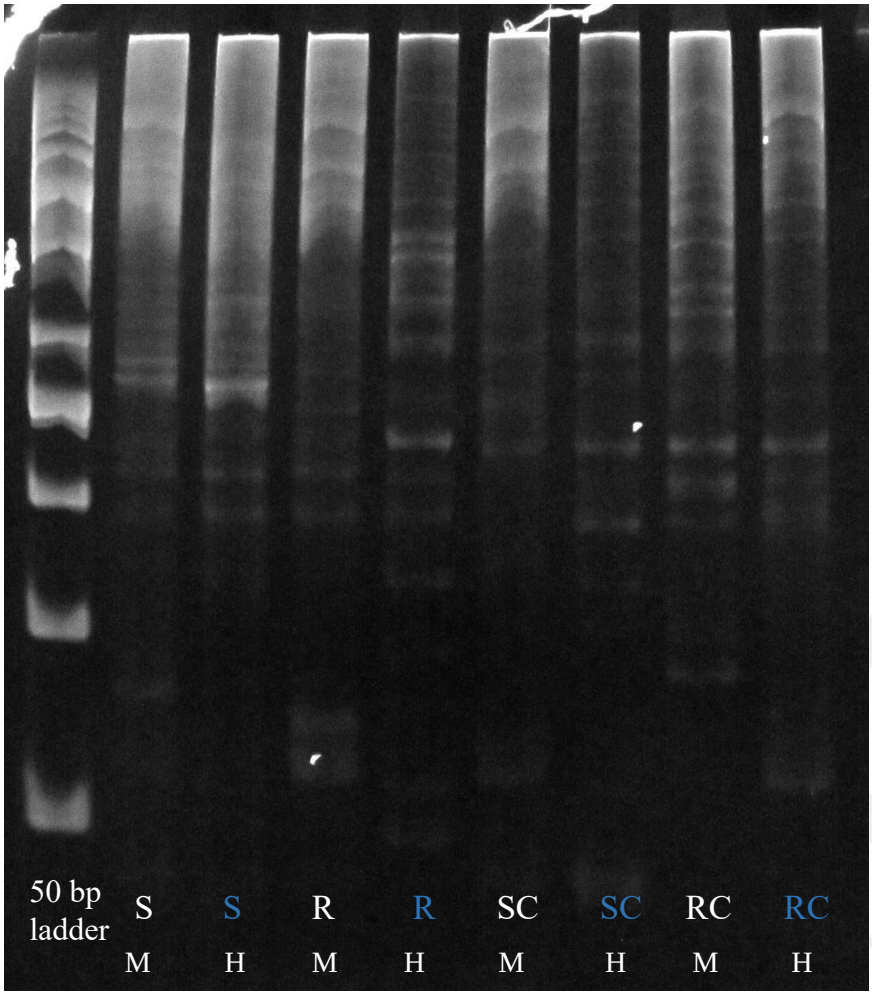

Figure 23. PAGE image of the selective PCR with the 10th combination. (E_f, H+3c) (S: Sensitive, R: Resistant, SC: Sensitive Control, RC: Resistant Control, M: *Msp*I H: *Hpa*II) (The letters written in blue mean that the sample was cleaved with *Hpa*II and the letters in white were cleaved with *Msp*I.)

CHAPTER 5

RESULTS AND RECOMMENDATIONS

5.1. Scoring Results

The evaluation of the bands in the gel images was made using the scoring method, and the analysis file was prepared by giving values of 0 and 1. The results obtained are given in the tables below (Tables 7, 8, 9, 10, 11, 12, 13, 14, 15, 16).

Table 7
Band scores of 1st primer combination. (E_e, H,d)

E-e/ H-d	Sensitive treated (*Msp*I)	Sensitive treated (*Hpa*II)	Resistant treated (*Msp*I)	Resistant treated (*Hpa*II)	Sensitive untreated (*Msp*I)	Sensitive untreated (*Hpa*II)	Resistant untreated (*Msp*I)	Resistant untreated (*Hpa*II)
	1	1	1	1	1	1	1	1
	1	1	1	1	0	0	1	1
	0	0	1	1	1	1	1	1
	0	0	1	1	0	1	1	1

Table 8
Band scores of 2nd primer combination. (E_e, H_e)

E-e/ H-e	Sensitive treated (*Msp*I)	Sensitive treated (*Hpa*II)	Resistant treated (*Msp*I)	Resistant treated (*Hpa*II)	Sensitive untreated (*Msp*I)	Sensitive untreated (*Hpa*II)	Resistant untreated (*Msp*I)	Resistant untreated (*Hpa*II)
	1	1	1	1	1	1	1	1
	0	0	1	1	0	1	1	0
	1	0	1	1	1	1	1	0
	0	0	1	1	0	1	1	1

Table 9
Band scores of 3rd primer combination. (E+2a, H+3a)

E+2a/ H+3a	Sensitive treated (*Msp*I)	Sensitive treated (*Hpa*II)	Resistant treated (*Msp*I)	Resistant treated (*Hpa*II)	Sensitive untreated (*Msp*I)	Sensitive untreated (*Hpa*II)	Resistant untreated (*Msp*I)	Resistant untreated (*Hpa*II)
	1	1	1	1	1	1	1	1
	0	1	1	1	0	1	1	1

Table 10
Band scores of 4th primer combination. (E+2a, H+3c)

E+2a/ H+3c	Sensitive treated (*Msp*I)	Sensitive treated (*Hpa*II)	Resistant treated (*Msp*I)	Resistant treated (*Hpa*II)	Sensitive untreated (*Msp*I)	Sensitive untreated (*Hpa*II)	Resistant untreated (*Msp*I)	Resistant untreated (*Hpa*II)
	1	1	1	1	1	1	1	1
	0	0	1	1	1	1	1	0
	0	0	1	1	0	1	1	1

Table 11

Band scores of 5th primer combination. (E+2b, H+3a)

E+2b/ H+3a	Sensitive treated (*Msp*I)	Sensitive treated (*Hpa*II)	Resistant treated (*Msp*I)	Resistant treated (*Hpa*II)	Sensitive untreated (*Msp*I)	Sensitive untreated (*Hpa*II)	Resistant untreated (*Msp*I)	Resistant untreated (*Hpa*II)
	1	1	1	1	1	1	1	1
	0	1	0	1	1	1	1	1
	0	0	0	0	1	0	0	0
	0	0	0	1	0	1	1	1
	0	1	0	1	1	1	1	1

Table 12

Band scores of 6th primer combination. (E+2b, H+3c)

E+2b/ H+3c	Sensitive treated (*Msp*I)	Sensitive treated (*Hpa*II)	Resistant treated (*Msp*I)	Resistant treated (*Hpa*II)	Sensitive untreated (*Msp*I)	Sensitive untreated (*Hpa*II)	Resistant untreated (*Msp*I)	Resistant untreated (*Hpa*II)
	1	1	1	1	1	1	1	1
	1	1	1	1	0	1	1	1
	0	0	0	1	0	1	1	1
	1	1	1	1	0	1	1	1
	1	1	1	1	0	1	1	1

Table 13

Band scores of 7th primer combination. (E+2c, H+3a)

E+2c/ H+3a	Sensitive treated (*Msp*I)	Sensitive treated (*Hpa*II)	Resistant treated (*Msp*I)	Resistant treated (*Hpa*II)	Sensitive untreated (*Msp*I)	Sensitive untreated (*Hpa*II)	Resistant untreated (*Msp*I)	Resistant untreated (*Hpa*II)
	1	1	1	1	1	1	1	1
	0	1	1	0	1	0	1	1
	0	1	0	0	0	1	1	1
	0	1	1	1	0	1	1	0
	1	1	0	0	1	1	1	1
	1	1	0	1	0	0	0	0
	0	0	1	1	0	1	0	0
	1	1	1	1	1	1	1	1
	1	0	0	0	0	0	0	0
	0	0	0	0	0	1	0	0
	0	0	0	1	0	0	0	0

Table 14

Band scores of 8th primer combination. (E+2c, H+3c)

E+2c/ H+3c	Sensitive treated (*Msp*I)	Sensitive treated (*Hpa*II)	Resistant treated (*Msp*I)	Resistant treated (*Hpa*II)	Sensitive untreated (*Msp*I)	Sensitive untreated (*Hpa*II)	Resistant untreated (*Msp*I)	Resistant untreated (*Hpa*II)
	1	1	1	1	1	1	1	1
	0	1	1	1	1	1	1	0
	0	1	1	1	1	1	1	1
	1	1	1	1	1	1	1	1
	1	1	1	1	1	1	1	1

Continued from Table 14

	0	1	1	1	0	1	1	1
	0	0	0	1	0	1	1	1
	0	1	1	1	0	1	0	0
	1	1	1	1	1	1	1	1
	1	0	0	1	0	0	0	0
	0	0	0	1	0	1	0	0
	0	0	0	1	0	0	0	0

Table 15
Band scores of 9th primer combination. (E_f, H+3a)

E-f/ H+3a	Sensitive treated (*Msp*I)	Sensitive treated (*Hpa*II)	Resistant treated (*Msp*I)	Resistant treated (*Hpa*II)	Sensitive untreated (*Msp*I)	Sensitive untreated (*Hpa*II)	Resistant untreated (*Msp*I)	Resistant untreated (*Hpa*II)
	1	1	1	1	1	1	1	1
	1	0	1	0	1	0	1	1
	1	0	1	0	1	0	1	0
	0	1	0	1	1	1	1	1
	1	1	1	1	0	1	1	0
	1	1	0	0	0	1	0	0
	1	1	0	0	1	0	0	0
	1	1	1	1	1	1	1	1
	0	0	1	1	0	1	0	1
	0	1	1	1	0	1	0	1
	0	1	0	1	0	1	0	0
	1	0	0	1	0	0	0	1
	0	1	1	0	1	1	0	1
	0	0	0	1	0	1	0	0
	0	1	0	1	1	0	1	0

Table 16
Band scores of 10th primer combination. (E_f, H+3c)

E-f/ H+3c	Sensitive treated (*Msp*I)	Sensitive treated (*Hpa*II)	Resistant treated (*Msp*I)	Resistant treated (*Hpa*II)	Sensitive untreated (*Msp*I)	Sensitive untreated (*Hpa*II)	Resistant untreated (*Msp*I)	Resistant untreated (*Hpa*II)
	1	1	1	1	1	1	1	1
	1	0	1	0	1	0	1	1
	0	0	1	0	1	1	1	1
	0	0	1	1	1	1	1	1
	0	0	0	1	0	1	1	1
	0	1	0	1	0	0	1	0
	1	1	0	1	1	1	1	1
	1	0	1	0	0	1	0	1
	1	1	0	1	1	0	0	0
	0	0	1	1	1	0	0	0
	1	0	1	1	1	1	1	1
	1	1	1	1	0	1	1	1
	1	1	1	1	0	1	1	1
	0	0	0	1	0	1	0	0

Continued from table 16

	0	0	0	1	0	1	0	0
	0	0	0	0	0	0	1	0
	1	0	0	0	0	0	1	0
	0	0	1	0	1	0	0	1
	0	0	0	1	0	0	0	0
	0	0	0	0	1	1	0	0

5.2. Phylogenetic Analyses and Results

The DNA fragments obtained in the analyses were profiled and the bands that differed between the samples were analyzed. As a result of 10 different primer combinations, a total of 81 bands were analyzed and 53 of them were methylation sensitive loci (MSL), and 28 non-methylated loci (NML) were obtained. The distinctions observed in MSL are epigenetic, while the distinctions observed in NML are genetic. The quantity of polymorphic MSL bands was calculated as 52 (98%), and NML as 9 (32%). The frequencies of the methylation profiles of the samples are given in Table 17. The highest number of fragments is found unmethylated, followed by hyper methylated fragments. The least number of fragments with hemi-methylation of outer cytosine and internal cytosine methylation were detected.

Table 17

Methylation profile frequencies of the samples.

Methylation Status	Sensitive infected	Sensitive uninfected	Resistant infected	Resistant uninfected
Type I *Hpa*II+/*Msp*I+ (Unmethylated)	0.3457	0.7407	0.5061	0.5432

Continued from Table 17

Type II *Hpa*II+/*Msp*I- (Hemimethylation of outer cytosine)	0.1975	0	0.2716	0.0740
Type III *Hpa*II-/*Msp*I+ (Inner cytosine methylation)	0.1234	0.2593	0.0987	0.1358
Type IV *Hpa*II-/*Msp*I- (Hyper methylation)	0.3333	0	0.1234	0.2469

The phylogenetic tree obtained according to the methylation profile and the Neighbor-Joining Tree method revealed that the samples showed different methylation profiles in terms of the response of the plant to the disease (Figure 15).

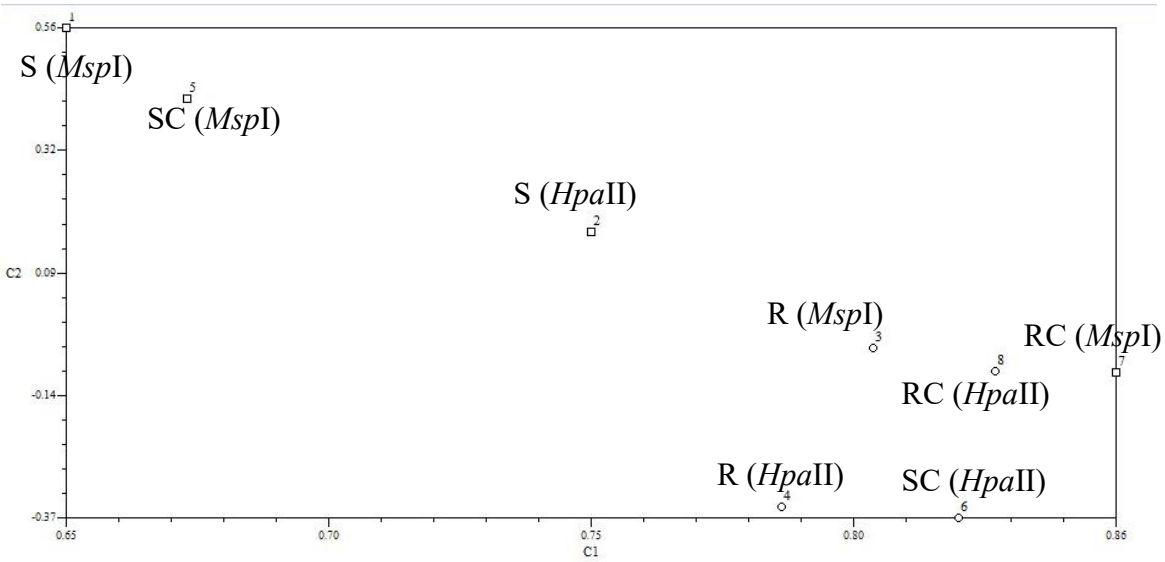

Figure 24. Principal Coordinate Analysis (PCoA) graphic showing the methylation pattern similarity distances of the varieties.

Table 18
Eigenvalues and percentages of variance of the factors

Factor	Eigenvalue	Total variance (%)	Cumulative variance (%)
1	4.83689648	60.4612	60.4612
2	0.79222183	9.9028	70.3640
3	0.59402264	7.4253	77.7893
4	0.49409336	6.1762	83.9654
5	0.40522823	5.0654	89.0308
6	0.35676603	4.4596	93.4904
7	0.28830177	3.6038	97.0941
8	0.23246965	2.9059	100.0000

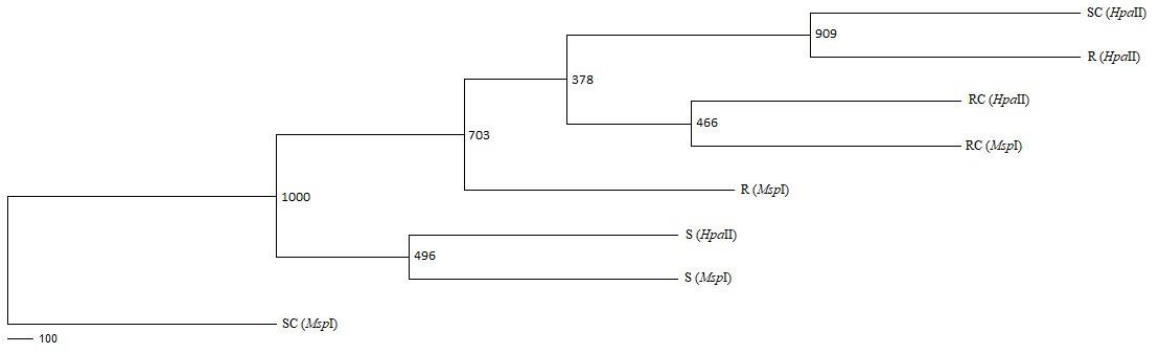

Figure 25. Neighbor-joining tree (Saitou and Nei, 1987) according to MSAP data.

Bootstrap numbers are implied nearby the arms.

5.3. Discussion

Sunflower is a highly preferred oil source because it has no variety problems, requires little labor, and can be grown easily in every region. It is possible to increase the yield obtained from sunflower with biotechnological developments (Kaya. 2016).

Epigenetic was first defined in 1940 and is defined as hereditary alterations that do not result from the sequence in the DNA but occur in gene expression. Today, with advancing technology, studies in this field have increased significantly. With the emergence of the effects of epigenetics on the organism, it has been stated that cells with the same genetic sequence reveal different profiles and different phenotypes occur. The different expression patterns of genes were effective in the formation of a cell type that differed from the parent cell (Güler and Peynircioğlu, 2016).

DNA methylation is one of the important epigenetic regulations that affect the expression of genes. In research conducted to date, it has been determined that cytosine methylation is effective in embryonic and prenatal development, cancer, bacterial host defense, transgene silencing, hormone regulation, biotic and abiotic stress, genome folding, and speciation, heterosis, and imprinting events in most organisms (Karaca and İnce, 2019).

In this study, IMI-044-A which does not carry the Pl_{17} gene and SUN 2259-CL which carries the Pl_{17} gene, with and without *Plasmopara halstedii* infection, registered by Trakya Agricultural Research Institute, were used. DNA methylation levels were compared using the methylation-sensitive amplification polymorphism (MSAP) method with using 10 different primer combinations between downy mildew resistant and sensitive genotypes.

With this technique, CCGG sequences were cleaved equally with *Hpa*II and *Msp*I enzymes. Outer cytosine methylated CCGG sequences were not cleaved with *Hpa*II, but were cleaved with *Msp*I, and unmethylated sequences were cleaved with both enzymes. Internal cytosine methylation is cleaved with *Hpa*II but not cleaved with *Msp*I. During the PCR stage, If there is methylation in the gene, the enzymes will not be able to cleave the DNA sequence and the amplification will be successful (Tekin 2003).

In the frame of this study, a total of 81 bands were obtained. While 52 of these bands (98%) were polymorphic Methylation Sensitive Loci (MSL), 9 of them (32%) were calculated as Non-Methylated Loci (NML).

Methylated cytosine is called the "fifth base of DNA" because of its important role in regulating gene expression and it is different from normal cytosine (Lister and Ecker, 2009). For this reason, it shows the necessity of evaluating the methylation pattern in addition to the nucleotide sequences (Toparslan et al. 2015).

In this study, 4 different methylation patterns were observed. Type I (*Hpa*II+/*Msp*I+) represents the unmethylated region, Type II (*Hpa*II+/*Msp*I-) represents the hemi-methylated outer cytosine region, Type III (*Hpa*II-/*Msp*I+) represents internal cytosine methylation, and Type IV (*Hpa*II-/*Msp*I-) represents the hypermethylated region also refers to a completely methylated region.

The most observed pattern in all sunflower varieties was the unmethylated pattern, followed by fully methylated pattern and the least observed one with hemimethylated and methylated internal cytosine.

The PCoA graphic and the dendrogram shows us that the methylation profiles of sunflower varieties different between the downy mildew disease resistant and sensitive genotypes. It is also seen that the varieties have different methylation profiles in terms of sensitive control and resistant control which are not infected with the downy mildew disease.

By using the NTSYS-pc (Rohlf, 2000), which is a software package commonly used in numerical taxonomy and multivariate analysis, we obtained a principal coordinate analysis graph. The primary purpose of a PCoA graph is to show the relationships between samples based on a distance or dissimilarity matrix. Samples that are plotted close to each other are more similar, while those that are further apart are more dissimilar.

In this study, all resistant varieties are located close to each other. This cannot be concluded that the same for sensitive individuals, because one sensitive variety that has not been treated with the pathogen and has been cut with the *Hpa*II enzyme is located closer to resistant individuals. A difference could be mentioned at the genomic level, which was the result expected. Although the effect of the disease treatment on the methylation level is not very critical, it can be said that there is a difference because the individuals treated with and without the disease are close to each other but they are on different coordinations.

NTSYS-pc programme (Rohlf, 2000) also gave the eigenvalues and percentages of the factors. Programme detected 8 factors, but one of them gave a higher value than the others. A factor with a higher eigenvalue captures more of the total variance present in the data compared to factors with lower eigenvalues. This suggests that the corresponding factor is more significant in representing the underlying structure of the data.

The pedigree obtained by using Phylip 3.6 programme (Felsenstein, 1989) showing the methylation similarities of the varieties. Neighbour-joining tree (Saitou and Nei, 1987) and the bootstrap numbers, analyzed with 1000 replicates (Felsenstein, 1985) are indicated near the branches. It can be interpreted that the difference in methylation levels is associated with the disease since the sensitive individual who has not been treated with the disease is on a different branch from the sensitive individuals who have been treated with the disease.

Bulut et al. (2020) examined the methylation differences in the capsule and other organs of the opium poppy and suggested that it has an effect on BIA biosynthesis. Different DNA methylation status indicates that it may have important biological implications when it comes to alkaloid content in the opium poppy (Bulut et. al., 2020).

In another study conducted in 2021, Kanoosh et al., performed molecular analysis of DNA methylation for hybrid potency of cytoplasmic male sterility in sunflower using methylation-sensitive amplification polymorphism (MSAP), revealing that the four primers used were successful in characterizing a total of 36 specific loci; 35 of these loci were found to be methylation sensitive (MSL) (Kanoosh et el. 2021).

In 2017, Chwialkowska et. al, developed a new method called MSAP-Seq, improving the traditional MSAP technique. This method allows the analysis of methylation regions throughout the genome with the Next Generation Sequencing (NGS) technique. The validation of the technique has been established with *Hordeum vulgare*. This technique provides direct analysis of MSAP amplicons without the hassle of PAGE. MSAP-Seq, a simple and fast method, allows direct analysis of DNA methylation with high efficiency with NGS technology. But this is not a cost effective as traditional MSAP methods (Chiwalkowska et. al, 2017). The msap-seq method has also been tested on clonal white poplar populations, and it is mentioned in the publication that this method is much more preferable, especially for plant species whose genomes have not yet been sequenced (Guarino et. al., 2020).

In a study examining methylation levels in angiosperms, the Bisulfite Sequencing technique was used. Although this technique is very effective in determining DNA methylation patterns and prevalence (Niederhuth et. Al, 2016), it is a more costly process than the MSAP technique, but it is possible to obtain more detailed results than the results obtained in MSAP. Although four types of methylation are determined according to the presence of PAGE bands in MSAP scoring, the status of half or full methylation cannot be expressed very sharply in these types. In this case, sequencing analyses can provide more reliable information.

The MSAP technique has been used before to determine methylation levels in the banana genome and it has been stated that this technique is a derivative of amplified fragment length polymorphism (AFLP). The MSAP procedure was first derived from AFLP, in 1997 (Reyna-Lopez et. al., 1997). It has also been explained that the ligation step in such techniques depends on many variables. In the analysis, it has been revealed that the MSAP technique has a 0.2% error margin (Baurens et. al., 2003).

Previously, it was published that *msap* was used as an R package. It was a tool used to calculate methylation-sensitive amplified polymorphism data in a statistical sense (Perez-Figueroa, 2013). However, this package cannot be used in new versions of R programs. For this reason, *msap* tool could not be used in this study, the percentages of polymorphisms manually calculated.

Different epigenetic profiles from downy mildew-sensitive and resistant sunflowers suggest that methylation affects downy mildew disease response.

In conclusion, different DNA methylation status indicates that it may have essential biological implications when it comes to downy mildew in sunflowers. However, the differences seen between resistant and sensitive genotypes suggest that much remains to be discovered related to the fundamental reasons of alteration in this molecular aspect. Given its role in gene expression and its ability to vary independently of genetic variation, understanding these causes will be crucial for a comprehensive perception of the significance of DNA methylation in biotic stress effects.

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APPENDICES

APPENDIX 1

GENERULER 50 BP DNA LADDER