

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL

**ELUCIDATING THE ROLE OF CROSS-KINGDOM RNA
INTERFERENCE BETWEEN THE FUNGAL PATHOGEN
FUSARIUM VERTICILLIOIDES AND ITS HOST PLANT MAIZE
(ZEA MAYS)**

SENA KIZILBOĞA

**A THESIS OF MASTER OF SCIENCE
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS**

ADVISOR: ASSOC. PROF. DR. FATMA AYDINOĞLU

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GEBZE TEKNİK ÜNİVERSİTESİ
LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

MANTAR PATOJENİ FUSARIUM VERTICILLIOIDES
İLE KONAK BİTKİ MISIR (ZEA MAYS)
ETKİLEŞİMİNDE ÂLEMLER ARASI RNA
İNTERFERANS ROLÜNÜN AYDINLATILMASI

SENA KIZILBOĞA

YÜKSEK LİSANS TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK
ANABİLİM DALI

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HAZİRAN 2025



MASTER of SCIENCE JURY APPROVAL FORM

A thesis submitted by Sena , KIZILBOGA, defended on 17/06/2025 before the jury formed with the 02/06/2025 date and 2025/29 numbered decision of the GTU Graduate Administration Board, has been accepted as a MASTER of SCIENCE thesis in the Department of Molecular Biology of Genetics.

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To my family

ABSTRACT

Maize is one of the most widely cultivated cereal crops worldwide, providing livelihoods for millions of people in many parts of the world. One of the most common plant pathogenic fungi is *Fusarium verticillioides*. As a result of infection, root rot, crown rot, seedling blight, ear rot, stalk rot, and seed rot diseases occur. Understanding the interactions between hosts and pathogens is critical to eliminate these threats. Recent studies to uncover these interactions have found that RNA molecules travel reciprocally between plants and pathogens and play an important role in gene silencing. The gene silencing mechanism induced by dsRNA was named RNAi. Therefore, this study aims to identify genes likely to play a role in the *F. verticillioides*-maize interaction by cross kingdom RNAi. In this study, *F. verticillioides* was applied to maize seedlings by leaf inoculation method before the first true leaves emerged. The effects of infection were evaluated in terms of morphological, physiological, biochemical and molecular parameters. To evaluate the effect of infection on maize, final leaf lengths were recorded and statistically significant differences were found in the third and fourth leaves between the resistant infected and resistant control groups.. When leaf width, leaf area, chlorophyll content and cell lengths were evaluated, it was observed that the infection affected the plant through many different parameters. Biochemical analyses showed significant changes in phenolic content and antioxidant enzyme activities, indicating oxidative stress and activation of defense mechanisms. At the molecular level, transcript profiling of host and fungal miRNAs and their target genes revealed cross-kingdom regulatory interactions. For example, *F. verticillioides*-derived fv-miR1827 was up-regulated by 815-fold, leading to a dramatic 5651-fold decrease in its maize target gene 3-ketoacyl-CoA synthase. Conversely, maize-derived Zm-miR159 was up-regulated by 5-fold in response to infection. While some of its target genes were induced, the vacuolar fusion protein MON1 was significantly down-regulated by 88%. The experimental results support the hypothesis that sRNAs mediate communication between the host and the pathogen. These findings provide insights into the molecular dialogue that takes place during maize-*F. verticillioides* interactions and hold promise for improving disease resistance through RNAi-based strategies.

Keywords: Cross-Kingdom RNAi, Plant-Pathogen Interaction, Maize, *Fusarium verticillioides*, Biotic Stress

ÖZET

Mısır, dünya çapında yetiştirilen en yaygın tahıl ürünlerinden biridir ve dünyanın birçok yerinde milyonlarca insanın geçimini sağlar. Bitki patojenik mantarları arasında en yaygın olanlardan biri *Fusarium verticillioides*'tir. Enfeksiyonun bir sonucu olarak kök çürümesi, taç çürümesi, fide yanıklığı, koçan çürümesi, sap çürümesi ve tohum çürümesi hastalıkları ortaya çıkar. Bu tehditleri ortadan kaldırmak için konakçılar ve patojenler arasındaki etkileşimleri anlamak kritik öneme sahiptir. Bu etkileşimleri ortaya çıkarmak için yapılan son çalışmalar, RNA moleküllerinin bitkiler ve patojenler arasında karşılıklı olarak taşındığını ve gen susturulmasında önemli bir rol oynadığını bulmuştur. RNA interference, dsRNA tarafından indüklenen gen susturma mekanizmasıdır. Bu çalışma, *F. verticillioides*-mısır etkileşiminde rol oynaması muhtemel genleri, âlemler arası RNAi yöntemi ile belirlemeyi amaçlamaktadır.

Bu çalışmada, *F. verticillioides*, ilk gerçek yaprakların ortaya çıkmasından sonra yaprak aşılama yöntemi ile mısır fidelerine uygulandı. Enfeksiyonun etkileri morfolojik, fizyolojik, biyokimyasal ve moleküler parametreler bakımından değerlendirildi. Enfeksiyonun mısır üzerindeki etkisini değerlendirmek için son yaprak uzunlukları kaydedildi ve dirençli enfekte ve kontrol grupları arasında üçüncü ve dördüncü yapraklarda istatistiksel olarak önemli farklılıklar bulundu. Yaprak genişliği, yaprak alanı, klorofil içeriği ve hücre uzunlukları değerlendirildiğinde enfeksiyonun bitkiye birçok farklı parametreden etki ettiği görülmektedir. Biyokimyasal analizler, fenolik içerik ve antioksidan enzim aktivitelerinde oksidatif stresi ve savunma mekanizmalarının aktivasyonunu gösteren önemli değişiklikler gösterdi. Moleküler düzeyde, konak ve mantar miRNA'larının ve hedef genlerinin transkript profillemesi, âlemler arası düzenleyici etkileşimleri ortaya koydu. Örneğin, *F. verticillioides*'ten türetilen fv-miR1827, 815 kat yukarı düzenlendi ve mısır hedef geni 3-ketoasil-CoA sentazında dramatik bir 5651 kat azalmaya yol açtı. Tersine, mısırdan türetilen Zm-miR159, enfeksiyona yanıt olarak 5 kat yukarı düzenlendi. Hedef genlerinden bazıları indüklenirken, vakuolar füzyon proteini MON1, %88 oranında önemli ölçüde aşağı düzenlendi. Deney sonuçları, sRNA'ların konak ve patojen arasındaki iletişimi aracılık ettiği hipotezini desteklemektedir. Bu bulgular, mısır-*F. verticillioides* etkileşimleri sırasında gerçekleşen moleküler diyaloga ilişkin bilgiler sağlıyor ve RNAi tabanlı stratejiler ile hastalık direncini iyileştirmek için umut vaatmektedir.

Anahtar Kelimeler: Âlemler-Arası RNAi, Bitki-Patojen Etkileşimi, Mısır, *Fusarium verticillioides*, Biyotik Stres.

ACKNOWLEDGEMENTS

I would like to express my sincere thanks and appreciation to my advisor, Assoc. Prof. Fatma AYDINOĞLU, for her consistently positive and sincere approach, invaluable support, and everything she has contributed to me throughout my graduate studies and thesis work. I would also like to thank the members of the Plant Systems Biology Laboratory, Erdem Emre Deligöz and Yaprak Narmanlı, for their support. I am especially grateful to my dear friends Betül Çakmak and Özge Vazgeçen, who were always by my side during this journey, motivated me, and helped me stay strong. Finally, I would like to thank my family for their unwavering love and support not only during my graduate studies but throughout my entire life.

I would like to thank Technological Research Council of Türkiye (TÜBİTAK) for supporting me by a scholarship throughout my master science study and for financially supporting this project by Grand number 221O321.

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LIST OF SYMBOLS AND ABBREVIATIONS

RT-qPCR	: Real time quantitative polymerase chain reaction
PTI	: Pathogen triggered immunity
R	: Resistance
ETI	: Effector triggered immunity
MAMP	: Microbe associated molecular pattern
PAMP	: Pathogen associated molecular pattern
PRR	: Pattern recognition receptor
FFSC	: <i>Fusarium fujikuroi</i> species complex
Srna	: Small RNAs
avr	: Avirulence
RLP	: Receptor-Like Proteins
RLK	: Receptor-Like Kinases
NLR	: Nucleotide-Binding and Leucine-Rich Repeat Receptors
TIR	: Toll/interleukin-1 Receptor
CC	: Coiled-coil domain
RNAi	: RNA Interference
dsRNA	: double strand RNA
CPMP	: Coat protein mediated protection
PTGS	: Post-Transcriptional Gene Silencing
siRNA	: Small interfering RNA
miRNA	: MicroRNA
RDR	: RNA-dependent RNA polymerase
DCL	: Dicer like enzyme
HEN1	: HUA ENHANCER 1
AGO	: Argonaute proteins
RISC	: RNA-induced silencing complex
°C	: Celcius
ddH ₂ O	: Double distilled water
h	: Hour
g	: Gram
%	: Percent
PDA	: Patato Dextrose Agar
μL	: Microliter
Cm	: Centimeter
FC reagent	: Folin Ciocalteu Reagent
CAT	: Catalase
POX	: Peroxide Oxidase
APX	: Ascorbate Peroxidase
GR	: Glutathione Reductase
SOD	: Superoxide Dismutase
EDTA	: Ethylene diamine tetra acetic acid
PVP	: Polyvinylpyrrolidone
ROS	: Reactive Oxygen Species
H ₂ O ₂	: Hydrogen peroksit
T _m	: Melting temperature
cDNA	: Complementary DNA
Fv	: <i>Fusarium verticillioides</i>

CT	: Cycle of threshold
LL	: Leaf length
LER	: Leaf elongation rate
FLL	: Final leaf length
Zm	: <i>Zea mays</i>
Zm_TIFY6	: TIFY domain-containing protein 6
zm_KCoA	: 3-ketoacyl-CoA synthase
zm_CR4	: Putative receptor protein kinase CRINKLY4
zm_RDRP	: RNA-dependent RNA polymerase

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1.INTRODUCTION

The genus *Fusarium*, one of the most studied fungi worldwide, is an important research topic because it is a toxin-producing plant pathogen that can even indirectly affect humans [Leslie and Summerell 2006]. They belong to the Nectriaceae family in the Hypocreales order of the Ascomycota fungi phylum. They can be found in water, air, insects, plants, organic substrates and soils. In the agricultural industry, they cause billions of dollars in losses due to drastic reductions in maize yields. They are known to cause many diseases, jeopardizing food and feed safety. *Fusarium* can infect plants from soil-borne inoculum in roots or aerial parts of plants through air or water. One of the most common pathogenic fungi is *Fusarium verticillioides*. This species mostly causes ear rot in maize and sorghum. As a result of infection, ear rot, seedling blight, stalk rot, and seed rot diseases occur. After being consumed as feed by animals, it causes many different animal diseases, including cancer. They can also synthesize the fumonisin family of mycotoxins that are associated with human esophageal cancer.

Maize, which grows as a weed in Mexico, is the domesticated form of Teosinte. Maize is classified as a tropical and temperate variety that can grow in different climatic conditions. Millions of people in many parts of the world depend on maize for their livelihood. Abiotic stresses such as drought, nutrient shortages, and aluminum toxicity, and biotic stresses such as diseases and pathogens, reduce maize productivity. Understanding the interactions between hosts and pathogens is critical to eliminating these threats [Assem et al., 2015].

Cross-kingdom RNA transfer is used by pathogens to block the host's defense system, as well as by the host to develop a strong and unique defense strategy against the pathogen. Cotton plants infected by *V. dahliae* send fungus-specific miRNAs to prevent fungal invasion [Zhang et al., 2016]. It has been found that erythrocyte miRNAs are sent to *Plasmodium falciparum* and inhibit plasmodium growth [LaMonte et al., 2012]. sRNA transfer is bidirectional. The stomata, hyphae, and wounded parts of plants make the plant vulnerable to pathogens. The plant immune system consists of two basic steps. In the first step, microbial or pathogen-associated molecular patterns (MAMPs/PAMPs) are recognized by transmembrane pattern recognition receptors (PRRs) located in the membranes of plant cells, and pathogen

triggered-immunity (PTI) is activated. In order to overcome PTI, pathogens secrete effector molecules. In return, the plant recognizes resistance genes and effector genes, and effector-triggered immunity is activated (ETI). Then, effector molecules aim to overcome ETI by producing new effector molecules from pathogens recognized by the resistance (R) genes.

RNA Interference (RNAi) was first discovered in worms as a gene silencing mechanism triggered by double-strand RNAs (dsRNAs). In plants, this mechanism is defined as post-transcriptional gene silencing (PTGS). It was shown by the discovery of a short RNA molecule showing homology to mRNA and the studies conducted that this RNA is related to PTGS (Baulcombe et al., 1999). This short RNA molecule was named small interfering RNA (siRNA). RNAi is triggered by dsRNA. siRNA and miRNAs induce PTGS by repressing translation by endonucleolytic cleavage or by creating a physical barrier. They can also trigger transcriptional gene silencing (TGS) by chromatin modifications and DNA methylation.

1.1. Purpose of the Study

F. verticillioides, a plant fungal pathogen, causes various diseases such as root rot, crown rot, ear rot, and seedling blight in maize and reduces crop yield. In addition, fumonisins, a member of the mycotoxin family produced by this pathogen, threaten human and animal health. As a result, it causes millions of dollars in economic losses every year in the world. Much research is being conducted to overcome diseases caused by *F. verticillioides*, but the goal has not been achieved. Therefore, this study aims to determine the genes that are likely to play a role in *F. verticillioides* - maize interaction by a cross-kingdom RNAi manner. In this context, possible miRNAs of both organisms and their target were identified reciprocally between the two kingdoms by bioinformatic analysis, and their expression was analyzed by the RT-qPCR method. In addition, it is aimed to look at the interaction from a multi-faceted perspective by evaluating with morphological, physiological, and enzymatic analyses to elucidate the phytopathosystem. The determined genes will shed light on future studies to strengthen the plant's defense system against *F. verticillioides*.

2. LITERATURE REVIEW

2.1. General Characteristics of *Fusarium* spp.

The genus *Fusarium*, one of the most studied fungi worldwide, is an important research topic because it is a toxin-producing plant pathogen and also a human pathogen [Leslie and Summerell 2006]. *Fusarium*, which is in the group of hyaline (light and unpigmented) filamentous fungi, is not specific to a particular region and has a cosmopolitan distribution [Walsh et al., 2004]. They belong to the Nectriaceae family in the Hypocreales order of the Ascomycota fungi phylum. They can be found in water, air, insects, plants, organic substrates, and soils. The *Fusarium* genus, a plant pathogen, was first described by Link in 1809 due to its canoe-shaped conidia. In 1925, the *Fusarium* fujikuroi species complex (FFSC) was established by Wollenweber et al. This complex, which also includes *Fusarium* species such as *F. proliferatum*, *F. subglutinans*, *F. verticillioides*, *F. temperatum*, and *F. fujikuroi*, contains more than 60 species. Fungal species within the scope of FFSC can infect many economically important plants and cause diseases [Yang et al., 2022]. Nowadays, with the development of molecular sequencing, more than 200 species have been identified in 22 species complexes. Classification is made according to molecular characterization, host association, and morphology [Al-Hatmi et al., 2016]. *Fusarium* species can produce spores sexually or asexually. Asexual spores are of three types: macroconidia, chlamydospores, and microconidia (Figure 2.1). Not all *Fusarium* species can produce all types of spores, and less than 20% of them reproduce sexually. As long as a nutrient source is available in the environment, asexual spores are produced [Ajmal et al., 2022].

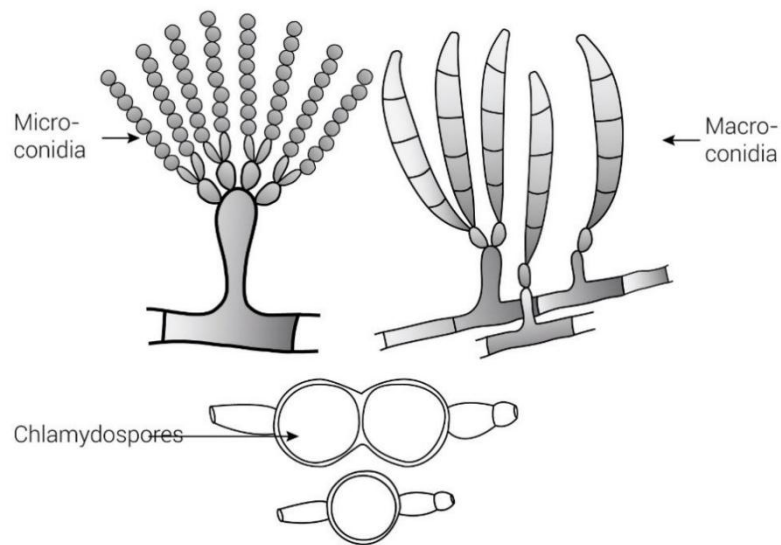


Figure 2.1: Asexual spores of *Fusarium*.

Fusarium species are among the most destructive and mycotoxin-producing fungi known and cause billions of dollars in losses in the agricultural industry due to drastic reductions in crop yields. They are known to cause many diseases, jeopardizing food and feed safety as well as economic losses [Sharma et al., 2018]. The fact that they infect foods that are consumed by humans and animals makes the situation even more important, and they are frequently reported in infectious diseases. *Fusarium* can be transmitted to plants from soil-borne inoculum in roots or the upper parts of the plant through air or water, and can cause diseases. *Fusarium* species use various infection methods. In the initial stage of infection, the pathogen relies on the living host (biotrophic), but later it starts to kill and consume the host cells (necrotrophic), so they are classified as hemibiotrophic.

Among pathogenic fungi, one of the most common is *Fusarium verticillioides* (teleomorph *Gibberella moniliformis*). The genome size of *F. verticillioides* is 42 Mb, and it has 11 chromosomes [Ma et al., 2013]. This species mostly causes ear rot in maize and sorghum, but it can also infect many other plants. Infection by *F. verticillioides* occurs during all growth and developmental stages of maize. As a result of the infection, root rot, crown rot, seedling blight, ear rot, stalk rot, and seed rot diseases occur. This fungus, which is reported to be the cause of various animal diseases, including cancer, is ingested by animals. They can also synthesize the fumonisin family of mycotoxins, which are associated with human esophageal cancer

[Howard et al., 2001]. Fumonisin disrupts sphingolipid metabolism by inhibiting the activity of the ceramide synthase enzyme due to their structure resembling the sphingolipid sphinganine [Soriano et al., 2005]. Sphingolipids, which are an important part of the cell membrane, also play an important role in cell signaling pathways. The disruption of cell signaling pathways by fumonisins may be the cause of the emergence of fumonisin-related diseases [Brown et al., 2007]. In a study in Somalia, more than eight mycotoxins were detected in samples taken from Maize [Wielogorska et al., 2019]. These included fumonisin B1 and B2, the levels of which were well above the European Union limits. The results show that fumonisin exposure is a significant public health problem. Fumonisin has also been found to cause leukoencephalomalacia in horses [Marasas et al., 1988].

2.2. The Agricultural and Economic Importance of Maize

Maize, which grows as a weed in Mexico, is the domesticated form of Teosinte. Maize is classified as a tropical and temperate variety that can grow in different climatic conditions. Maize is the most common cereal crop grown in the United States. In the United States alone, 332 million metric tons of Maize are grown annually. Maize provides the livelihood of millions of people in many parts of the world. In these lands where Maize is produced, abiotic stresses such as drought, nutrient deficiencies, and aluminum toxicity, and biotic stresses such as diseases and pathogens reduce maize productivity [Assem et al., 2015].

Plant pathogens, which cause serious damage to plant health and productivity, pose a threat to the agricultural industry and natural ecosystems. Understanding the interactions between hosts and pathogens is critical to eliminating these threats. Recent studies to uncover these interactions have found that RNA molecules travel reciprocally between plants and pathogens and play an important role in gene silencing. These RNAs are especially small regulatory RNAs (sRNA). It is known that sRNAs play a role in cell-to-cell communication through plasmodesmata, which are known as intercellular communication channels and provide symplastic continuity. It is also known that they are transported to different organs of the plant through the vascular system. Recent studies suggest that sRNAs pass between kingdoms and that these sRNAs are probably transported by exosomes or exosome-like vesicles [Knip et al., 2014].

2.3. Small RNA Exchange Across Kingdoms: Insights from Plant–Pathogen Interfaces

The transfer of sRNAs between kingdoms has been reported to occur between a wide variety of species: from fungal pathogens to plants [Weiberg et al., 2013], from bacteria to nematodes [Liu et al., 2012], from humans to the malaria-causing chromist *P. Falciparum* [LaMonte et al., 2012], from plants to insects [Mao et al., 2007], and from plants to nematodes [Ibrahim et al., 2010]. Plant pathogenic fungi have developed various strategies for nutrient acquisition from the host they infect. Three main groups implement different strategies. These are biotrophic fungi, necrotrophic fungi, and hemibiotrophic fungi. *Uromyces fabae* and *Blumeria graminis*, which belong to the biotrophic fungi group, differentiate haustoria, a specialized infection structure, to feed on living tissue. *Botrytis cinerea* and *Sclerotium sclerotiorum* are in the necrotrophic fungi group and secrete lytic enzymes and toxins to kill cells and use the released nutrients. Examples of hemibiotrophic fungi are *Colletotrichum* species, which form haustoria-like structures and switch to a destructive necrotrophic phase in the later stages of infection.

Cross-kingdom RNA transmission is used by pathogens to block the host's defense system, as well as to develop a powerful and unique defense strategy against the pathogen. For example, cotton plants infected with *V. dahliae* send out miRNAs specific to the fungus to prevent fungal invasion [Zhang et al., 2016]. Another example is that in humans, erythrocyte miRNAs were found to be sent to *Plasmodium falciparum*, the causative agent of malaria, and inhibit *plasmodium* growth [LaMonte et al., 2012]. This shows that sRNA transmission is bidirectional. While the plant uses this system for defense by targeting virulence genes against the pathogen that infects it, the pathogen uses its sRNAs as offensive weapons to suppress and disrupt the host plant's immune system.

2.4. The Plant Immune System: Layers of Defense

Pathogens have developed many different strategies to infect plants. The stomata, hyphae, and wounded parts of plants make the plant vulnerable to pathogens. Pathogenic microorganisms can enter and multiply through these openings. Fungi can enter from the epidermis cells of the plant or reach the intercellular space and inside

the cells with the help of their hyphae. Plants, which have to survive in the same ecological environment as pathogens, have their immune systems. The plant immune system consists of two basic steps. In the first step, the transmembrane pattern recognition receptors located in the membranes of plant cells recognize microbial or pathogen-associated molecular patterns, (MAMPs or PAMPs) and pathogen-triggered immunity (PTI) is activated. In order to pass PTI, pathogens secrete effector molecules. In return, the plant recognizes the effector genes with resistance (R) genes, and effector-triggered immunity (ETI) is activated [Jones et al., 2006]. PTI and ETI are of vital importance for the plant in this war between the plant and the pathogen. Investigation of these systems is very important for crop yield.

2.4.1. Pathogen Triggered Immunity vs Effector Triggered Immunity

Although plant immunity consists of 2 main steps, scientists have explained plant immunity with a zigzag model consisting of 4 phases (Figure 2.2). In the 1st phase, PAMPs are recognized by PRRs located in the cell membrane, and PAMP-triggered immunity is activated. As a result, if the plant is successful, the pathogen cannot establish a colony and fails. However, if the pathogen can escape this phase, it targets plant proteins with effector molecules (avirulence). Pathogen effector molecules suppress or reduce the effect of PTI and disable plant immunity. This phase is defined as Effector-triggered susceptibility (ETS) (phase 2). In the third phase, plant resistance (R) proteins recognize effector molecules and activate effector-triggered immunity (ETI). R proteins can perceive effector molecules directly by binding to them or indirectly. The change in the proteins targeted by the effector molecules can again be detected by the R genes and ETI is activated. If ETI is successful, it indicates that the plant has developed resistance to this pathogen. ETI also causes a hypersensitive cell death response at the site of infection. In the fourth stage, the pathogen must change its effector molecules or produce new effector molecules in order to escape the resistance genes. Pathogens that produce new effector molecules that can escape ETI continue to exist as a result of natural selection. Similarly, plants develop protective strategies by diversifying their R genes and continue to keep ETI active.

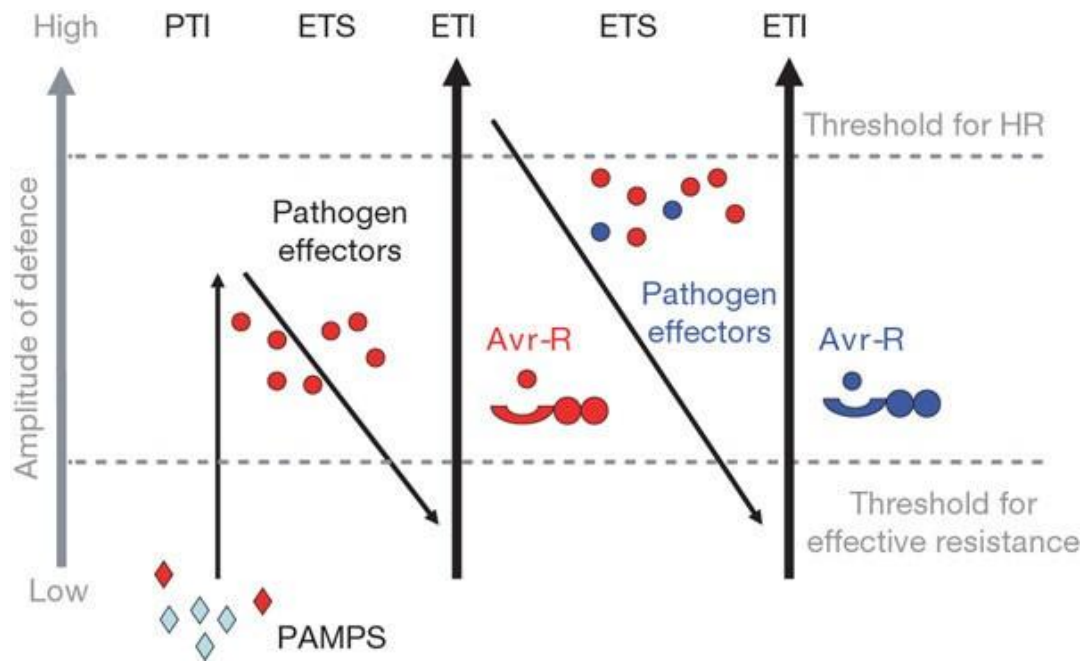


Figure 2.2: The zigzag model of plant immunity. In the 1st stage, effector molecules (PAMPs/MAMPs) are shown with red diamonds. These effector molecules are detected by PRRs and PTI is activated. In the second stage, pathogens that pass the 1st stage secrete effector molecules (red circles). These molecules suppress PTI or show properties that increase the spread and nutrition of the pathogen in the plant. In this case, effector triggered susceptibility (ETS) occurs. In the third stage, when the secreted effector molecules are recognized by the plant R genes, an effector triggered immune response occurs. ETI is stronger in terms of the response it creates compared to PTI, and if the strength of this response exceeds a certain threshold (threshold for HR), a hypersensitive response occurs and cell death occurs at the infection site. In stage 4, effector molecules aim to overcome ETI by producing new effector molecules (blue circles) from pathogens recognized by the R genes. In response, the plant promotes the formation of new alleles of the R genes and ETI is activated again [Jones et al., 2006].

PRRs that recognize PAMPs on the plant cell surface are glycosylated transmembrane proteins. In order to localize to the cell surface, they are first folded and processed in the endoplasmic reticulum for post-translational modifications. They are then transported to the Golgi apparatus and the trans Golgi network where they undergo additional modifications. Correct folding and modifications of PRRs play an important role in the perception of PAMPs [Godfrey et al., 2012]. In order for PRRs to recognize a molecule as an elicitor, this molecule must not be present in the host plant and must contain specific molecular patterns that identify pathogens. Examples include chitin for fungi and peptidoglycan and flagellin for bacteria [Boller et al., 2009]. Examples of PAMPs and PRRs that specifically sense these include Flagellin Sensing2 (FLS2),

a leucine-rich repeat receptor kinase (LRR-RK) that binds to flg22 and elicits the PTI response. Another example is the LRR-RK EFR found in *Arabidopsis*, which binds to bacterial EF-Tu, and the LRR receptor-like proteins EIXI/2 in tomato, which recognize fungal xylanase. Also, the LysM receptor-like protein CEBiP found in rice, which recognizes fungal chitin as a PAMP [Zipfel et al., 2010].

In plant immunity, it has been determined that R proteins of plant can directly or indirectly recognize avirulence (avr) proteins of the pathogen. Pathogens subjected to selection pressure by R genes can diversify their avr proteins and become virulent. In response, new alleles of R genes appear. R genes can be found in the cell membrane or inside the cell. One type of R proteins are transmembrane proteins with an LRR domain on the cell surface, and these proteins may also contain an intracellular kinase domain (Receptor-Like Proteins (RLP)/Receptor-Like Kinases (RLK)). The majority of identified R proteins are intracellular nucleotide-binding and leucine-rich repeat receptors (NLR). NLR proteins contain more than one domain. NLR proteins contain the LRR domain, a nucleotide-binding (NB) domain and an N-terminal domain composed of a Toll/interleukin-1 receptor (TIR) or a coiled-coil (CC) domain (Figure 2.3) [Petit-Houdenot et al., 2017].

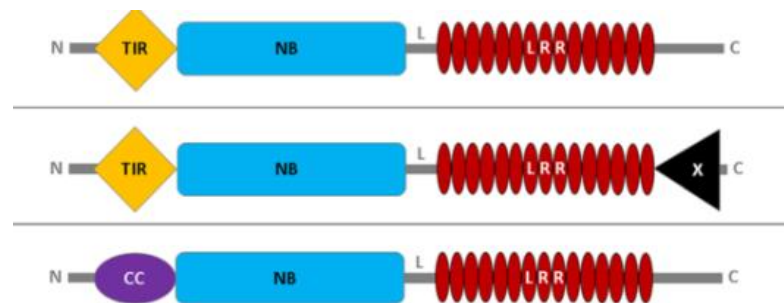


Figure 2.3: An overview of NLR protein domain composition. NLR proteins contain an LRR domain, a nucleotide-binding (NB) domain and an N-terminal domain composed of a Toll/interleukin-1 receptor (TIR) or a coiled-coil (CC) domain. The black triangle X represents the variable domain [Hofberger et al., 2014].

The presence of multiple domains of R proteins allows them to recognize avr proteins simultaneously and trigger plant immunity rapidly. The recognition of avr proteins by R proteins has been described in different models. The elicitor-receptor model is that the R protein directly recognizes the avr protein and generates a defense response [Keen et al., 1990; Jia et al., 2000; Dodds et al., 2006; Catanzariti et al., 2010;

Steinbrenner et al., 2015]. In the guard model, as mentioned before, the R protein detects modifications in the protein targeted by the avr protein and generates a defense response [Dangl et al., 2001]. The decoy model is based on the recognition by R proteins of modifications in ‘decoy’ plant proteins that mimic the protein targeted by the avr protein [van der Hoorn et al., 2008]. According to the recently developed integrated decoy model, atypical domains that mimic effector targets are incorporated into the structure of NLR proteins and assume decoy functions [Cesari et al., 2014a; Le Roux et al., 2015, Sarris et al., 2015].

2.5. Mechanism of RNAi

RNAi is triggered by dsRNA. dsRNA can arise endogenously from the transcription of microRNA (miRNA) genes, from RNA transcripts produced from inverted repeat loci, or from overlapping, bidirectional or convergent transcription. In addition, aberrant RNAs produced by transposons can be converted to dsRNA with the help of RNA-dependent RNA polymerase (RDR). These resulting dsRNAs are cleaved into 21-24 nucleotide duplexes by the ribonuclease III-type Dicer like (DCL) enzyme. The resulting RNA duplexes are divided into two separate classes: miRNAs and siRNAs. While siRNAs are formed by the cleavage of long dsRNAs that are perfect matches or close to it; miRNAs are formed by the cleavage of imperfectly folded precursor RNAs [Pumplin & Voinnet, 2013]. In order for siRNAs and miRNAs to remain intact, they are protected in the nucleus by 2' O methylation by the methyltransferase HUA ENHANCER 1 (HEN1). These RNAs are exported to the cytoplasm with the help of the HASTY exportin protein embedded in the nuclear envelope [Kaur et al., 2008]. HEN1 stabilized siRNA and miRNA duplexes are loaded onto Argonaute (AGO) proteins and form RNA-induced silencing complex (RISC). siRNA and miRNA loaded onto RISC play a role in silencing target mRNA and induce PTGS by repressing translation by endonucleolytic cleavage or physical barrier. They can also trigger transcriptional gene silencing (TGS) by chromatin modifications and DNA methylation [Siomi et al., 2009; Jinek et al., 2008].

2.6. The Role of RNA Interference in Plant Immunity and Defense Response

RNA Interference (RNAi) was first discovered in worms as a gene silencing mechanism triggered by dsRNAs [Guo & Kemphues, 1995]. It was found that by injecting sense or anti-sense RNA sequences, the genes targeted by these RNAs were suppressed. From this observation, it was thought that sense or antisense RNAs provided gene suppression, but later in the studies of Fire et al., [1998], it was shown that sense or antisense RNAs trigger gene suppression not alone but by forming dsRNAs. The gene silencing mechanism induced by dsRNA was called RNAi [Kusaba et al., 2004]. Before RNAi was fully discovered, RNAi-related phenomena were demonstrated in plants. Co-suppression is one of these phenomena. Co-suppression is a gene silencing mechanism mediated by a sense transgene. While it was expected that the expression of the related gene would increase along with the homologous endogenous genes when the transgene was integrated into the genome, the opposite expression was observed. Several different reasons for this situation are presented in the literature. One of these is related to the high levels of transcription of the transgene. Another is that the transgene produces low levels of transcription from the antisense strand, resulting in the formation of dsRNA. Inversely repeated transgenes also cause dsRNA production naturally [Montgomery & Fire, 1998]. Another phenomenon associated with RNAi is Coat protein mediated protection (CPMP). A sense coat protein transgene provides virus resistance. Initially, this resistance was thought to be due to the proteins produced by the transgene, but it was found that virus resistance still existed when the translation of the proteins was blocked. In this case, it was shown that virus resistance occurs post-transcriptionally at the RNA level [Kusaba et al., 2004].

In plants, post-transcriptional gene silencing (PTGS) has been described as an RNA-degradation mechanism [Vaucheret et al., 2001]. It was shown by the discovery of a short RNA molecule showing homology to mRNA and the studies conducted that this RNA is related to PTGS [Baulcombe et al., 1999]. Later, a small RNA molecule was found in an in vitro RNAi system in *Drosophila*. This short RNA molecule was named small interfering RNA (siRNA). As a result of these findings, it was suggested that PTGS and RNAi mechanisms use the same gene silencing mechanism [Hannon et al., 2002].

3. MATERIALS AND METHODS

3.1. Maize Lines and Growing Conditions

In this study, *Fusarium verticillioides* tolerant ADK310 (R) and susceptible ADK1188 (S) breeding line of maize were used. Seeds were supplied by Maize Research Institute (Sakarya/TURKEY). Maize seeds were treated with 70% ethyl alcohol for 3 min each time. Then, they were sterilized with 5% bleach for 3 min. Rinsing was done with ddH₂O for 2 min each time. Sterile seeds were sown in vials filled with autoclaved peat/vermiculite (1/1, g/g) soil mixture. The vials were left to germinate in the plant growth cabinet (Panasonic). The plant growth cabinet was operated at 25°C with 16 h of light period and 16°C with 8 h of dark period, light intensity of 20,000 lux and humidity of 70%. Plants germinated in 5 days and were taken to the plant growth room. The plant growth room operates at 25±2°C, light intensity 20,000 lux and humidity 55-70% with a 16-hour light period followed by an 8-hour dark period. Plants were watered with 0.5x Hoagland solution every 2 days. Control and infected groups used in this study were grown physically separately from each other in plastic greenhouses under the same conditions (Figure 3.1).



Figure 3.1: Maize seeds germinated in the plant growth chamber (A) and Maize seedlings grown in plastic greenhouses in the plant growth room (B).

3.2. Fungus Strain, Growth Conditions and Leaf Inoculation

The *F. verticillioides* (Fv) strain to be used in this study was obtained from MAEM. The fungal isolate was grown in petri dishes containing Potato Dextrose Agar (PDA) medium at $25\pm 2^{\circ}\text{C}$ for 10 days.

Fv culture grown in PDA medium for 10 days was scraped with a sterile scalpel and spores were obtained by filtering through filter paper. Before the first leaves of the germinated maize seedlings fully bloomed, 10 μl inoculum containing 105 spores of the fungus isolate were given into the rolled first leaves for each plant (Figure 3.2). This process was repeated twice for two repeating days.

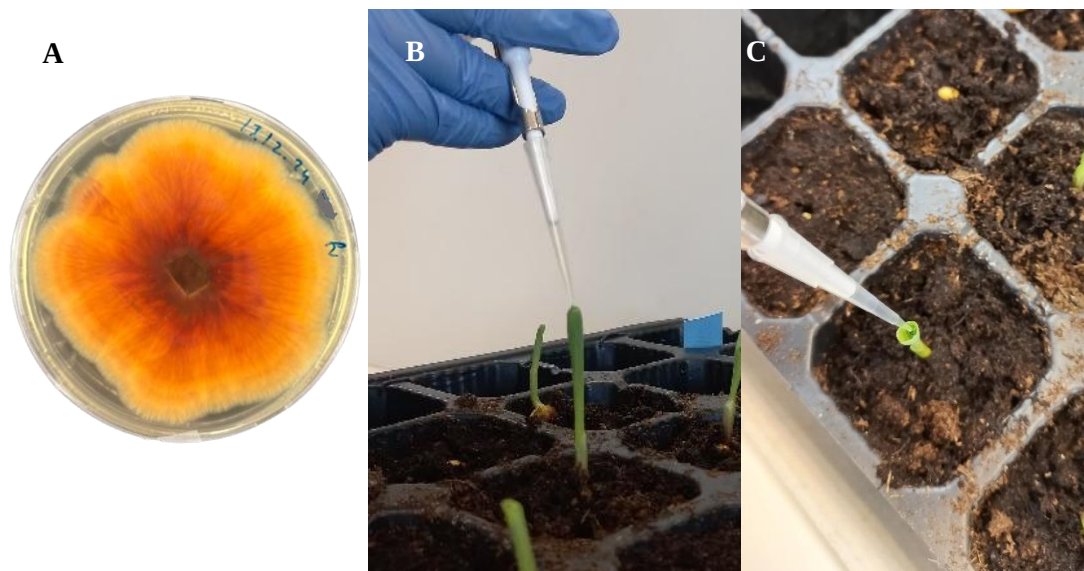


Figure 3.2: *Fusarium verticillioides* in PDA medium at 10 days old (A) Infection of maize plants with F.v using leaf inoculation method (B-C).

3.3. Leaf Length, Leaf Area and Leaf Width Measurement

In order to observe the effect of Fv infection on the leaf growth and development of maize seedlings, the length of the third and fourth leaves of the control and Fv infected groups were measured with a ruler from the moment the leaves started to appear until fully got matured. The measurements were made from the beginning of the stem in the soil to the tip of the leaf. The difference between the groups was determined using Student's T-test ($p < 0.05$).

When the third and fourth leaves of the seedlings in control and Fv-infected groups were fully matured, their leaf area was determined by using the Image J (www.imagej.nih.gov) application [Jingwen, et al., 2012]. Photographs of the third and fourth leaves cut from the ligule parts were taken. The taken photographs were loaded into the Image J application and the leaf area was measured using the polygon tool. Student's T-test was used to show statistically significance of difference between the groups ($p < 0.05$).

Leaf widths of the control and infected groups of R and S lines were measured with a ruler. Student's T-test was used to show statistically significance of difference between the groups ($p < 0.05$).

3.4. Determination of Chlorophyll Amount

To determine the degree of biotic stress caused subsequent to Fv infection, the photosynthesis mechanism, chlorophyll content, chlorophyll a, chlorophyll b and carotenoid amounts were examined.

3.4.1. Chlorophyll content

When the third and fourth leaves of the seedlings in control and Fv-infected groups were fully matured, the chlorophyll content of the plants was determined using the SPAD meter (SPAD-520 Plus) [Yuan et al., 2016]. For this, measurements were taken from the tip, middle and base parts of the third and fourth leaves of the plants in both groups. The chlorophyll content of each leaf was calculated by taking the average of these three measurements. Then, the average of the chlorophyll content of 12 plants in the control group and the Fv-inoculated group was taken and its standard deviation was calculated. Student's T-test was used to determine significance of the difference between the groups. P-value less than 0.05 were considered statistically significant difference between the groups.

3.4.2. Chlorophyll a, Chlorophyll b and Carotenoids

On the 5th day after leaf Fv inoculation, biochemical analyses were performed to determine the amounts of chlorophyll a, chlorophyll b and carotenoids on the second leaf. For these analyses, 5 plants were used and 2 cm sections were taken from the middle part of the leaves and placed in glass tubes with 1 ml of 100% ethanol. The glass tubes were incubated in the dark for 24 hours at +4°C. Then, the samples were measured with a spectrophotometer (Shimadzu, UV1280) in a quartz cuvette at 661.6 nm, 644.8 nm and 470 nm wavelengths. The data obtained in the measurement results were calculated according to formula represented Lichtenthaler [2001] to determine the amounts of chlorophyll a, chlorophyll b and carotenoids. Student's T-test was used to determine whether the difference between two groups was statistically significant, and $p < 0.05$ were considered significant.

3.5. Cell Length Measurement of Control and Infected Maize Leaf Samples

On the fifth day after inoculation, 2 cm samples were taken from the second leaves and fully mature third and fourth leaves and kept in 100% ethanol (EtOH) for 2 days. EtOH was renewed at 1-day intervals to ensure transparency of the leaves. Leaf samples were placed in lactic acid to prevent long-term deterioration. Samples were placed on slides and lactic acid was dropped on them. Imaging was performed using a Differential Interference Contrast (DIC) microscope (Leica DM2500) at 10X and 20X magnifications. Cell lengths were measured in micrometers (μm) using ImageJ software (<https://imagej.nih.gov>).

3.6. Total Phenolic Content Measurement

The total phenolic content was determined by the Folin Ciocalteu (FC) reagent method according to Ainsworth (2007). For the experiment, firstly, 1 cm² (20 mg) sample was taken from the third leaf of each of the 3 plants inoculated with Fv and the 3 plants in the control group, the growth of which had completely stopped, and they were placed in 2 ml Eppendorf tubes containing steel balls, quickly thrown into liquid nitrogen and stored at -80°C to be used in the analyses. 2 ml of ice-cold 95% (v/v) methanol (stored at -20 °C) was added to the Eppendorf tubes to homogenize the leaf tissue. The tubes

were mixed for 5 min at 30 Hz using a mixer miller (Retsch, LB.MM400). Then, they were incubated for 2 hours in the dark at room temperature. After incubation, centrifugation was performed at 13,000 g for 5 min at room temperature. The supernatant was placed in new 2 ml tubes and 100 μ l of the supernatant was taken for phenolic measurement. The remaining supernatant was stored at -20°C. In addition, 100 μ l of each concentration prepared for gallic acid standard (500, 250, 125, 62.5, 31.25 and 15.625) and 100 μ l of 95% methanol for blank were taken into tubes. 200 μ l of FC reagent was added to the samples, standard and blank and vortexed thoroughly. It was waited for 5 to 10 min to prevent phenols from reacting with air and 800 μ l of 700 mM alcali- Na_2CO_3 was added to each tube. It was incubated at room temperature for 2 hour. 200 μ l was added to 96-well microplate (TPP, Switzerland) for each sample, standard and blank in 3 replicates. Absorbance was measured at 765 nm using Spectrophotometer device (Thermo Scientific Varioskan Flash). After measurements were taken, a standard curve was created and the concentrations of the samples were calculated according to the created curve. The difference between the Fv and mock groups was statistically analyzed using Student's t-test ($p < 0.05$).

The FC reagent consists of phosphomolybdic acid ($\text{H}_3\text{PMo}_{12}\text{O}_{40}$) and phosphotungstic and is yellow in color. With this method, electrons in phenolic compounds are transferred to phosphomolybdic/phosphotungstic acid complexes. This reaction takes place in an alkaline environment and Na_2CO_3 is used for the appropriate pH. As a result of electron transfer, the reduced compounds form a blue color and absorbance is measured with a spectrophotometer at a maximum of 765 nm. This color change is directly proportional to the reducing activity of phenolics. The reducing activity of a phenolic can be standardized using gallic acid [Dominguez-López et al., 2023]. There are some points to consider regarding this method. One of these is that other oxidation substrates present in the plant extract may compete with the FC reagent and be reduced by phenols or air oxidation may also occur after the sample is alkalized. Therefore, the FC reagent is added before alkalizing the sample. With this method, total phenolic and other oxidation substrates in a sample are measured. It is widely used because it is simple and reproducible.

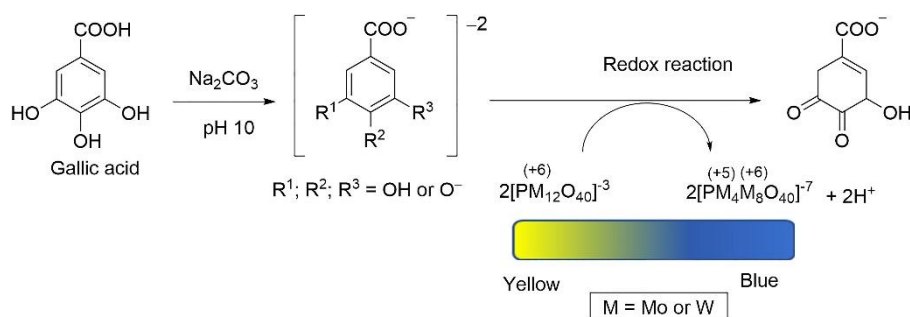


Figure 3.3: The principle behind FC method [Dominguez-López et al., 2023].

3.7. Identification of *Fusarium verticillioides* Spread Throughout the Leaf

In order to evaluate the spread of *F. verticillioides* inoculation to the upper leaves and along the leaf, control and infected third and fourth leaves of the R and S groups were used. The leaves were first washed with 70% ethanol to perform surface sterilization and then rinsed with ddH₂O. Discs were taken from the tip, middle and base regions of the sterilized leaves and planted in PDA medium to test for the presence of the pathogen.

3.8. Enzyme Activities Involved in Antioxidant System

To determine the effect of Fv infection on antioxidant system, the activity of Catalase, Peroxide Oxidase, Ascorbate Peroxidase, Glutathione Reductase and Superoxide Dismutase antioxidant enzymes were analyzed in maize second leaves for both control and Fv-infected groups. performed to measure the changes in the activities of Catalase, Peroxide Oxidase, Ascorbate Peroxidase, Glutathione Reductase and Superoxide Dismutase enzymes, which are enzymes in the antioxidant system, as a result of Fv infection of maize. In order to perform these tests, protein isolation was first performed.

3.8.1. Protein Isolation From Leaf Tissue

For protein isolation, 2 cm long samples were taken from the second harvested leaves of R and S maize lines. The analysis were conducted on three biological replicates for both groups and lines. Tissue samples were taken into 2 ml eppendorf tubes containing

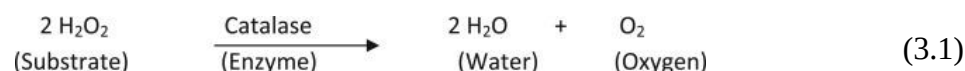
sterile steel beads and immediately fixed in liquid nitrogen. They were stored at -80°C until analyses. Total protein isolation was performed according to Kayihan et al. (2016). For protein isolation, samples were taken from -80 °C and placed in liquid nitrogen and homogenized using the mixer miller device (Retsch, LB.MM400) for 40 sec, at 15 Hz. It is important that the tissue is completely pulverized. 1 ml of extraction buffer (50 mM KH_2PO_4 , 1 mM EDTA, 2% PVP, pH:7.8) was added onto the homogenized samples. Then, it was vortexed thoroughly and centrifuged at 10,000 rpm for 30 min at +4°C. After centrifugation, the supernatant was carefully removed and transferred to new tubes. The samples were stored at -20°C until further analysis.

The total protein amount in samples was determined according to Bradford (1976). For this purpose, Bovin Serum Albimune (BSA) (Sigma) standards were first prepared. Concentrations were determined as 0.125 mg/ml, 0.25 mg/ml, 0.5 mg/ml, 0.75 mg/ml, 1 mg/ml and 2 mg/ml. 5 µl of each prepared BSA solution was taken and 250 µl of Bradford reagent (SIGMA) was added to it. 255 µl of Bradford reagent was used for blank. 5 µl of protein samples isolated from leaf tissue were taken and 250 µl of Bradford reagent was added onto it. The 96 well plate (TPP, Switzerland) was used for measurement. Samples in each well were pipetted thoroughly and incubated in the dark for 10 min. Absorbance values were measured at 595 nm with a Spectrophotometer (Thermo Scientific Varioskan Flash). A standard curve graph was created in Microsoft Excel using dilute solutions prepared using BSA, and the amounts of protein samples were determined by referencing this graph.

3.8.2. Catalase Enzyme Activity

Catalase enzyme activity was determined by taking Aebe (1984) as reference. To measure catalase enzyme activity, 900 µl of 50 mM KH_2PO_4 , 50 µl of 200 mM H_2O_2 and 50 µl of protein samples were used. 900 µl of KH_2PO_4 and 100 µl of H_2O_2 were used for blank. Absorbance was measured at 240 nm with a spectrophotometer (Shimadzu, UV1280). 1.5 ml quartz cuvettes were used. The decrease in absorbance values at 0, 30, 60 and 90 seconds was noted [Chance and Maehly, 1955]. 3 technical replicates were made for each biological replicate. Student T-test was used to see the statistical differences between the groups ($p < 0.05$).

Catalase enzyme protects the cell against oxidative stress and acts as an antioxidant. It plays an important role in the breakdown of reactive oxygen species (ROS). This enzyme is found in many aerobic and anaerobic organisms. Catalase is an enzyme that can hydrolyze more than one million molecules of hydrogen peroxide (H₂O₂), one of its substrates, and has one of the highest turnover numbers (Figure 3.4) [Kaushal et al., 2018].



3.8.3. Peroxidase Enzyme Activity

Peroxidase enzyme activity was determined by referring to Quesada et al. (1990). 850 µl of 50 mM KH₂PO₄, 50 µl of 10 mM H₂O₂, 50 µl of 0.02% dianisidine, and 50 µl of protein samples were used to measure peroxidase enzyme activity. 900 µl of KH₂PO₄, 50 µl of H₂O₂, and 50 µl of dianisidine were used for the blank. Absorbance was measured at 460 nm with a spectrophotometer (Shimadzu, UV1280). 1.5 ml quartz cuvettes were used. The increase in absorbance values at 0, 30, 60, and 90 seconds was noted [Chance et al.,1995]. Three technical replicates were made for each biological replicate. Student T-test was used to see the statistical difference between the groups (p<0.05).

Peroxidases use H₂O₂ to oxidize compounds. The breakdown of H₂O₂ by these enzymes in plants provides a defense against stress, removal of toxic compounds, and a defense response against pathogens [Singh et al., 2013].

3.8.4. Ascorbate Peroxidase Enzyme Activity

Ascorbate enzyme activity was performed using the reference Nakano and Asada (1981). To measure ascorbate peroxidase enzyme activity, 850 µl of 50 mM KH₂PO₄ (pH: 7.0), 50 µl of 20 mM H₂O₂, 50 µl of 5mM ascorbic acid and 50 µl of protein samples were used. For the blank, 900 µl of KH₂PO₄, 50 µl of H₂O₂ and 50 µl of ascorbic acid were used. Absorbance was measured at 290 nm with a spectrophotometer (Shimadzu, UV1280). 1.5 ml quartz cuvettes were used. The

increases in absorbance values at 0, 30, 60 and 90 seconds were noted. 3 technical replicates were made for each biological replicate. Student T-test was used to see the statistical differences between the groups ($p < 0.05$).

Ascorbate peroxidase (APX) is an important component of the antioxidant defense system in plants. It plays a critical role in the degradation of ROS concentration in the cell. APX uses ascorbate (ASH) as an electron donor and degrades hydrogen peroxide. Finally, water and dehydroascorbate (DHA) molecules are released [Kashif Tanwir et al., 2020; Afzal et al., 2014].

3.8.5. Glutathione Reductase Enzyme Activity

Glutathione reductase enzyme activity was determined using Çakmak et al. (2006) as a reference. To measure glutathione reductase enzyme activity, 850 μ l 50 μ M KH_2PO_4 : 0.1 μ M EDTA buffer, 50 μ l 0.5 μ M GSSG, 50 μ l 0.12 μ M NADPH and 50 μ l protein samples were used. 900 μ l KH_2PO_4 : 0.1 μ M EDTA buffer, 50 μ l GSSG and 50 μ l NADPH were used for the blank. Absorbance was measured at 340 nm with a spectrophotometer (Shimadzu, UV1280). 1.5 ml quartz cuvettes were used. The decrease in absorbance values at 0, 30, 60 and 90 seconds was noted. Three technical replicates were made for each biological replicate. Student T-test was used to see the statistical difference between the groups.

Glutathione reductase is one of the most important enzymes of the antioxidant defense system. This enzyme reduces glutathione disulfide (GSSG) to GSH. It also provides NADPH oxidation. NADPH plays a role in maintaining the GSH/GSSG redox balance (Dorion et al., 2021).

3.8.6. Superoxide Dismutase Enzyme Activity

SOD enzyme activity was determined according to Banowetz (2004). To determine SOD enzyme activity, 250 Mm KH_2PO_4 (Ph: 7.8), 0.75 mM nitroblue tetrazolium (NBT), 100 mM methionine, 5 mM EDTA, 2 μ M riboflavin solutions were prepared. 44 μ l KH_2PO_4 (pH: 7.8), 20 μ l NBT, 26 μ l methionine, 4 μ l EDTA, 90 μ l riboflavin and 20 μ l protein (5-10 mg/ml) were added to each well using a 96-well TPP cell culture dish. For the blank, 60 μ l KH_2PO_4 (pH: 7.8), 20 μ l NBT, 26 μ l methionine, 4 μ l EDTA, 90 μ l riboflavin were used. Before the measurement, it was shaken for 1

min and absorbance was measured at 560 nm using a spectrophotometer (Thermo Scientific Varioskan Flash). Then, the plate was incubated under fluorescent light for 10 min. After the incubation, it was shaken again for 1 min and absorbance was measured at 560 nm. After the absorbance values were obtained, SOD enzyme activity was calculated according to Kono (1978). Statistical analysis of the difference between the experimental groups was performed using Student's T-test ($p < 0.05$).

Superoxide dismutase enzymes convert superoxide radicals into hydrogen peroxide and molecular oxygen (O_2). Catalase and peroxidases convert hydrogen peroxide into water and oxygen. As a result, the two dangerous compounds superoxide and hydrogen peroxide are converted into water, thus protecting the cells [Weydert et al., 2009].

3.9. In Silico Identification of Potential Gene Candidates in Cross-Kingdom RNAi

This study addressed two hypotheses: First, *Fusarium verticillioides* (Fv) miRNAs target *Zea mays* (maize) resistance genes (R genes) and decoy them via host endogenous RNA interference (RNAi) mechanism resulting in invasion the plant. Second, *Zea mays* miRNAs are transferred to Fv and proceed to the degradation of target transcript via fungal RNAi machinery to prevent itself against the fungus infection. To test these hypothesis, first of all bioinformatics tool psRNATarget was used to determined miRNAs and their target genes operating reciprocally between both organisms. For this end, maize genome database Zm-B73-Reference-NAM-5.0 was used (www.maizegdb.org). For Fv genome database, *F. verticillioides* 7600 (assembly ASM14955v1; <https://www.ncbi.nlm.nih.gov/genome>) was used. Maize miRNA sequences were downloaded from MicroRNA Sequence Database (miRBase) (www.mirbase.org). Fv miRNA were predicted from RNAseq data produced in our previous study on Fv infected cron root tissue of maize. The target prediction analysis were performed with for psRNATarget, max. expect value=4, hspsize=20; target accessibility=25, flanking length=17bp upstream and 13bp downstream and central mismatch=9-11 nt. First, possible matches between Fv miRNA sequences and maize mRNAs were analyzed. Similarly, matches between maize miRNAs and Fv genes were determined. During the analysis, genes with low E values and p values were primarily selected. In order to evaluate the functional importance of the selected genes,

the results of a previously conducted transcriptomic study were utilized and genes with high expression levels were determined.

3.9.1. Designing Primers

NCBI Primer Blast Tool was used for the selected maize and Fv mRNAs. The sequence of the relevant gene was loaded in fasta format and the PCR product size was adjusted between 70-130 and the melting temperature (T_m) was set to minimum 57°C, optimum 60°C and maximum 63°C. As a result, primers with T_m close to optimum and low self complementarity (self complementarity ≤ 4) and self 3' complementarity (self 3' complementarity ≤ 3) values were selected from the given primers. The selected primers were checked for the possibility of forming self dimer ($\Delta G > -9$ kcal/mol) and heterodimer ($\Delta G > -9$ kcal/mol) using IDT OligoAnalyzer™ Tool. In addition, the primer sequences for the selected sRNAs were loaded into IDT OligoAnalyzer™ Tool and primer sequences were obtained.

Table 3.1: List of the primers used in this study.

Gene Name	Sequence (5'→3')
26s Proteasome Regulatory Subunit Rpn-1 (Fveg_06397)	
Forward Primer	GCGAATTTGATACCTTGCGCT
Reverse Primer	ATCTGCGGTGTTGTGTTCCC
Palmitoyl-CoA (Fveg_02271)	
Forward Primer	TCAAGAGCCTGTGCGAGTTT
Reverse Primer	GGCTCGTGGAAGTAGATGCT
Glutathione Peroxidase (Fveg_04790)	
Forward Primer	GAACGGCAAAAAGTGTCGCT
Reverse Primer	AATCCTCGTGTCGCTGTCTG
Chitin Synthase 4 (Fveg_01403)	
Forward Primer	TGACACCCGGCTCAATCTTC
Reverse Primer	TAGGCGTGCTTGAGTAGGGA
Vacuolar Fusion Protein Mon1 (Fveg_08091)	
Forward Primer	CGCTGGTGCTTTGTTGACTT
Reverse Primer	GCACATGTTTAACGGGCCTT
Sphingolipid Delta-4 Desaturase (Fveg_03105)	

Table 3.1: List of the primers used in this study.

Forward Primer	ATCAAGGCTCACCCCGAGAT
Reverse Primer	ATCCATGCGAGGAAGACCTG
Cell Wall Integrity And Stress Response Component (Fveg_06829)	
Forward Primer	GTGCTTCCTTCAGTGCATCA
Reverse Primer	CACTCTCTTCTCGGCAACCC
TIFY6	
Forward Primer	TGATCAGCCTTTGCTGGCCT
Reverse Primer	TGCCAAAGCCAAACAACACAG
3-Ketoacyl-CoA Synthase	
Forward Primer	AGCAGCTCAAGTTCCTCAAGT
Reverse Primer	GAAGTCCGGGATGTACGGA
CRINKYL4	
Forward Primer	CACCGAGGGATCTGCACTTT
Reverse Primer	ATAGCACTGGATGGCACCAC
RDRP	
Forward Primer	TGCATCCTCAGATCAACTCCG
Reverse Primer	GCCATCGCTACTCTCGTCTC
Fv-Mir8764	
Forward Primer	CGG CTT GTT CTA GCA ACT AC
Fv-Mir1827	
Forward Primer	CGT TGA GGA ACT TGA GGA G
Fv-Mir5855	
Forward Primer	GTT AAC TCC ACG GAC ATG G
Fv-Mir7214	
Forward Primer	CTC GTT CCT CGG ATG CTT C
Fv-Mir6557	
Forward Primer	TTA GGC TAA AGC GTC CGA
Zm-Mir528a-3p	
Stem Loop RT Primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAA TGGA
Forward Primer	GAT ATC CTG TGC CTG CCT CT
Zm-Mir398a-3p	
Stem Loop RT Primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCG GGGG
Forward Primer	GAT TCT GTG TTC TCA GGT CG
Zm-Mir1432-5p	
Stem Loop RT Primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGT CGGT
Forward Primer	GGGGCTCAGGAGAGATGACACCGA
Zm-Mir159a/b	
Stem Loop RT Primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCA GAGC
Forward Primer	CGGCGGTTTGGATTGAAGGGA

3.10. Total RNA Isolation From Leaf Tissue

For total RNA isolation, the second leaves of R maize plants were harvested and 2 cm leaf samples, 1 cm from the upper part and 1 cm from the lower part of the leaf, were placed in 2 ml Eppendorf tubes containing sterile steel beads. The samples were immediately placed in liquid nitrogen and stored at -80°C for use in experiments. For RNA isolation, samples taken from -80°C were immediately thrown into liquid nitrogen and homogenized for 30 seconds at 25 frequency in the mixer miller (Retsch, LB.MM400). It is important that the samples are powdered. The homogenized samples were immediately thrown into liquid nitrogen. 750 µl of Trizol Reagent (Invitrogen) at room temperature was added to each sample. Vortexing was done for 30-60 seconds and incubated for 10 minutes. After the incubation was completed, 150 µl of chloroform was added and up-down was done approximately 20 times. It is important to mix thoroughly at this stage. It was waited for 2 minutes before centrifuging. Then, centrifuging was done at 4°C at 11,000 rpm for 15 minutes. Chloroform causes phase separation. Therefore, the samples should be gently removed from the centrifuge. 400 µl of isopropanol was added to new 1.5 ml Eppendorf tubes during the centrifugation stage. The isopropanol must be cold. 400 µl of supernatant was added to these new tubes. It was gently up-downed (20 times) and incubated at 4°C for 10 min. Afterwards, centrifugation was performed at 11,000 rpm for 10 min (4°C). The supernatant was decanted and 1 ml of ethanol was added and washed. During washing, the pellet was taken in and out several times, taking care not to let it escape into the pipette. Centrifugation was performed at 11,000 rpm for 5 min (4°C). After centrifugation, the supernatant was decanted, taking care not to let the pellet fall, and the tubes were inverted and left to dry on sterile filter paper for 15 min. The dried pellet became transparent and 25 µl -50 µl of nuclease-free water was added. It was stored at -20°C for use in subsequent experiments. NanoDrop (Thermo Scientific, NanoDrop Lite Spectrophotometer) was used to measure the amount and purity of the obtained RNA.

3.11. cDNA Synthesis of Total RNA samples

High-Capacity Reverse Transcription kit (ThermoFisher) was used for Maize and Fv mRNAs. All-in-one cDNA kit (Applied Biological Materials) was used for Fv miRNAs and stemloop-RT PCR method was used for Maize miRNAs.

3.11.1. cDNA synthesis of Maize and Fv mRNAs

cDNA synthesis of Maize and Fv mRNAs was performed according to the protocol in the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher) manual. The components in the kit were thawed on ice. The amounts specified in the protocol were put into PCR tubes to make a total volume of 10 μ l (Table 3.2). The amount of sample required for 1.2 μ g RNA was added to make up to 10 μ l with NFW. Thermal cycler (Biometra, Analytik Jena) conditions are shown in Table 3.3. A total volume of 20 μ l cDNA was synthesized.

Table 3.2: Amounts of content required for cDNA synthesis.

Component	Volume
10X RT Buffer	2 μ L
25X dNTP Mix (100 mM)	0.8 μ L
10X RT Random Primers	2.0 μ L
MultiScribe™ Reverse Transcriptase	1.0 μ L
Nuclease-free H ₂ O	4.2 μ L
Total per reaction	10.0 μ L

Table 3.3: Thermal cycler conditions used for cDNA synthesis from maize and Fv mRNAs.

Settings	Step 1	Step 2	Step 3	Step 4
Temperature	25°C	37°C	85°C	4°C
Time	10 min	120 min	5 min	Hold

3.11.2. cDNA synthesis of Maize miRNAs

Maize miRNAs cDNA synthesis was performed according to Varkonyi-Gasic et al. (2007). High Capacity cDNA Reverse Transcription kit (ThermoFisher) was used for cDNA synthesis. 2 μ l of 10x RT buffer, 0.5 μ l of 10Mm Dntp mix, 0.25 μ l of

MultiScribe™ Reverse Transcriptase, 4 µl of stem-loop RT primer and 3.25 µl of nuclease-free water were added to the PCR tube. Samples were added in the amounts required for 3 µg of total RNA and NFW was added to complete to 10 µl. 20 µl of reaction was prepared. Samples were incubated in a heat block at 65°C for 5 min and immediately placed on ice for 2 min incubation. Then, samples were placed in a thermal cycler. Conditions required for pulsed RT are shown in Table 3.4.

Table 3.4: Thermal cycler conditions used for cDNA synthesis from maize miRNAs.

Settings	Step 1	Step 2 (60 cycle)	Step 3	Step 4	Step 5	Step 6
Temperature	16°C	30°C	42°C	50°C	85°C	4°C
Time	30 min	30 sec	30 sec	1 sec	5 min	Hold

3.11.3. cDNA synthesis of Fv miRNAs

cDNA synthesis of Fv miRNAs was performed according to miRNA All-In-One Cdna synthesis kit (Applied Biological Materials) manuel. 10 µl of 2X miRNA cDNA Synthesis SuperMix and 2 µl of Enzyme Mix included in the kit were added to the PCR tube for a 20 µl reaction. The amount required for 2 µg of RNA samples was added and the reaction was completed to 20 µl. Thermal cycler conditions are shown in Table 3.5.

Table 3.5: Thermal cycler conditions used for cDNA synthesis from Fv miRNAs.

Settings	Step 1	Step 2	Step 3	Step 4
Temperature	37°C	50°C	85°C	4°C
Time	30 min	15 min	5 min	Hold

3.12. Quantitative RT-PCR (qRT-PCR) For Transcription Level Determination

RNA samples in which cDNA synthesis was performed were used to determine transcript levels for selected genes. qRT-PCR was performed using BlasTaq™ 2X

qPCR MasterMix kit (Applied Biological Materials). Maize and Fv mRNA samples were prepared according to Table 3.6. Universal reverse was used instead of reverse primer for Maize and Fv miRNAs. The prepared samples were started to react according to the conditions in Table 3.7 using a thermal cycler. After the results were obtained, normalization was performed using Tubulin genes. Transcript levels were determined with the $2^{-\Delta\Delta CT}$ method (Pfaffl 2001). Whether the change in transcription levels was statistically significant was calculated using the Student's T-test method ($p < 0.05$).

Table 3.6: Amounts of content required for RT-qPCR

Component	Volume
BlasTaq™ 2X qPCR MM	10 μ l
Forward Primer (10 μ M)	0.5 μ l
Reverse Primer (10 μ M)	0.5 μ l
Template DNA	3 μ l
Nuclease-free H ₂ O	Up to 20 μ l

Table 3.7: Thermal cycler conditions used for RT-qPCR.

Settings	Step 1	Step 2- Step 3 (40 cycle)	
Temperature	95°C	95°C	60°C
Time	3 min	15 sec	1 min

4.RESULTS

With the leaf inoculation method, Fv was applied to the maize seedlings before the first leaves fully bloomed. The seedlings displayed visible disease symptoms at 5 days post inoculation (dpi). The degree of *F. verticillioides* infection on maize was determined on second leaves of seedlings by measuring the length of mature cells, the amount of chlorophyll a,b, carotenoid and total phenolics, the activity of antioxidant system enzymes, and the transcript level of miRNAs and their target genes operating between two organism for mutual defence.

4.1. Cultivation of Maize Plants

Yellowing, burning and necrotic spots were observed on the first and second leaves of R infected plants (Figure 4.1b). Plants in the R control group (Figure 4.1a) did not show any signs of infection or disease. Plants in the S infected group showed decreased chlorophyll a and total chlorophyll content compared to the S control group ($p<0.05$). Plants in the R control group were taller in terms of plant height than the R infected group ($p<0.01$). The height of plants in the S infected group decreased compared to the S control group but there was no statistically significant difference.



Figure 4.1: No visible disease symptoms resulting from Fv infection were observed in the R control group, indicating healthy and unaffected plants (Figure 4.1a). In contrast, plants in the R infected group exhibited clear symptoms of Fv infection, including wilting and lesions, demonstrating the impact of the pathogen (Figure 4.1b). A side-by-side comparison of the overall health and appearance of both the R control and infected plants is presented in Figure 4.1c, highlighting the differences caused by the infection. (R: resistant; S: susceptible)

4.2. Effect of *F.verticillioides* on Plant Growth and Leaf Development

After the first leaf emerged, the leaf lengths of these leaves were measured to determine whether the Fv inoculum given to the second leaf passed to the third and fourth leaves and its effect on leaf lengths. The formation process of the difference between leaf lengths is seen in Figure 4.2 and Figure 4.3. It is seen in Figure 4.2 that the difference between the lengths of the third leaf in the RC and RI groups started to occur after the fourth day and that the leaf lengths were close to each other in the first 4 days. While the leaf elongation of the RI group ended on the 10th day, the elongation of the RC group stopped on the 11th day. It is seen that the difference between the third leaves in the SC and SI groups started to occur after the 6th day. While the

elongation of the third leaves of the SI group stopped on the 8th day, the SC continued to elongate until the 10th day.

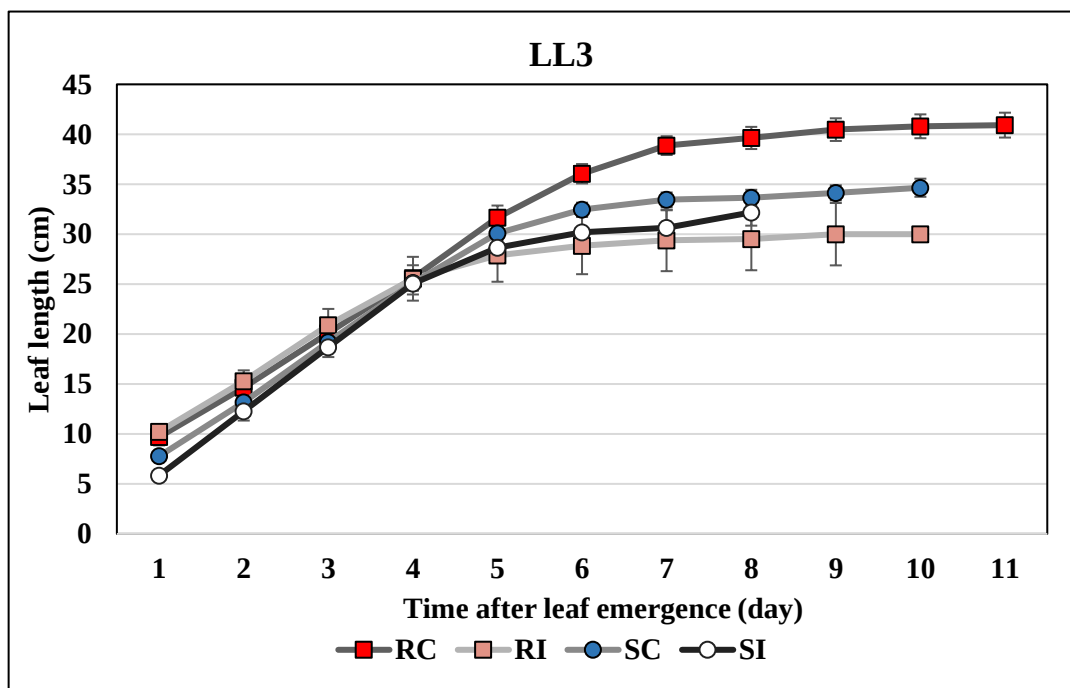


Figure 4.2: The length of the third leaf (LL3) of the ADK1188 (S) and ADK310 ® seedlings under control (C) and *F. verticillioides* infection (I). Measurements were taken from the moment the third leaves of the maize plants began to appear. 6 plants were used in the ADK310(R) control group, 10 plants in the R infected group, 10 plants in the ADK1188(S) control group and 6 plants in the S infected group. Measurements were continued until the leaves matured and stopped growing. RC=ADK310 line control; SC=ADK1188 line control; RI; ADK310 line *F. verticillioides* inoculated; SI=ADK1188 line *F. verticillioides* inoculated. Error bars indicate standard deviation. n=3, ave±sd.

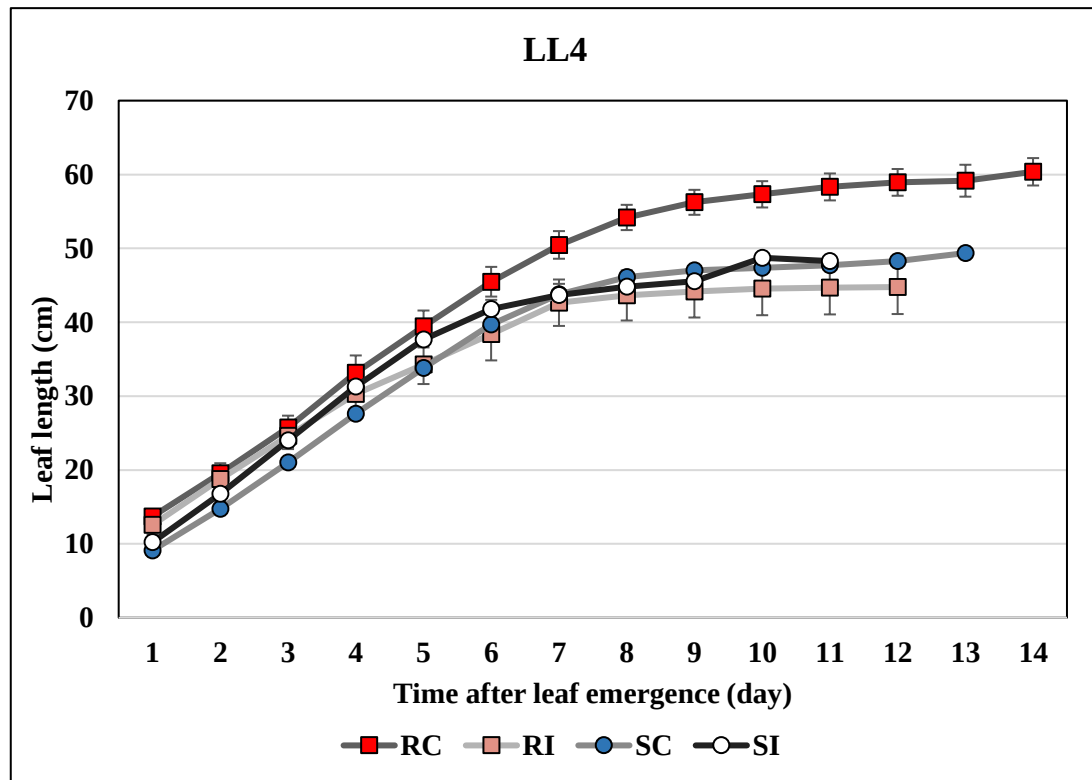


Figure 4.3: The length of the fourth leaf (LL4) of the ADK1188 (S) and ADK310 ® seedlings under control (C) and *F. verticillioides* infection (I). Measurements were taken from the moment the fourth leaves of the maize plants began to appear. 6 plants were used in the ADK310(R) control group, 10 plants in the R infected group, 10 plants in the ADK1188(S) control group and 6 plants in the S infected group. Measurements were continued until the leaves matured and stopped growing. RC=ADK310 line control; SC=ADK1188 line control; RI; ADK310 line *F. verticillioides* inoculated; SI=ADK1188 line *F. verticillioides* inoculated. Error bars indicate standard deviation. n=3, ave±sd.

When the final leaf length lengths (Figure 4.4) were compared, the third leaves of the plants in the RI group decreased by approximately 17% compared to the RC group, which was statistically significant ($p < 0.01$). The third leaves of the plants in the SI group did not change compared to the SC group ($p > 0.05$). When the fourth leaf was examined, it was seen that the plants in the RI group decreased statistically significantly by approximately 23% compared to the RC group ($p < 0.01$). In the SI group, there was not a change in the fourth leaf compared to the SC group ($p > 0.05$).

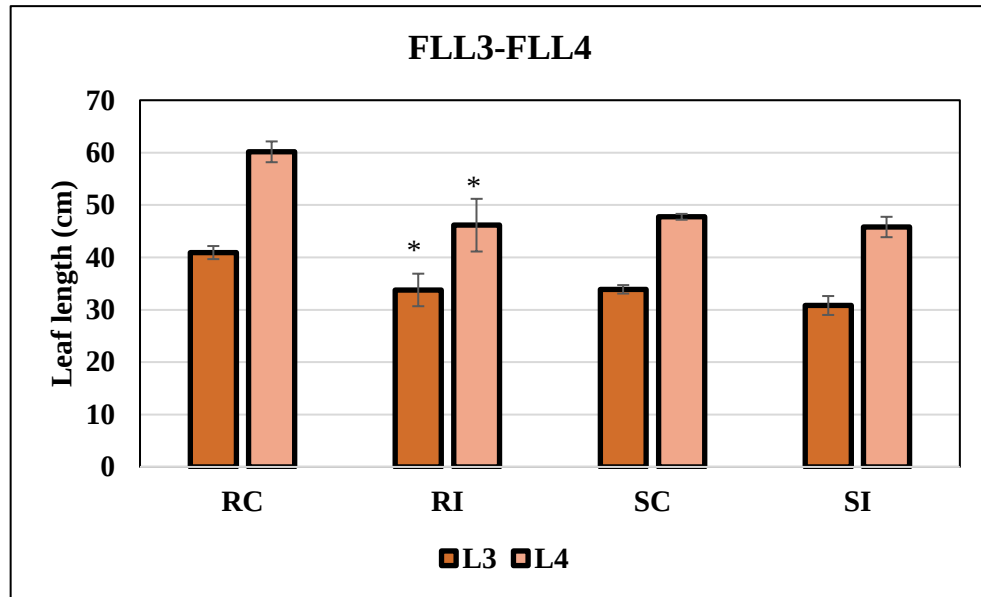


Figure 4.4: The final length of the third (FL3) and fourth (FL4) leaf of the ADK1188 (S) and ADK310 (R) seedlings under control (C) and *F. verticillioides* infection (I). Final leaf length of maize seedlings for leaf 3 and leaf 4. Statistical significance of the difference between groups was determined using Student's T-test ($p < 0.05$). RC=ADK310 line control; SC=ADK1188 line control; RI; ADK310 line *F. verticillioides* inoculated; SI=ADK1188 line *F. verticillioides* inoculated.

Daily leaf length measurements were used to determine whether there was a difference between the control and infected plants' leaf elongation rates (LER). The leaf elongation rate of the third leaf of the RC group (LER3) was 2.21 ± 0.13 . The LER3 of the RI group was 2.12 ± 0.74 . The LER3 of the RI group did not change compared to the RC group ($p > 0.05$). The leaf elongation rate of the fourth leaf of the RC group (LER4) was 2.7 ± 0.44 . The LER4 of the RI group was 2.46 ± 0.7 . The LER4 of the RI group did not change compared to the RC group ($p > 0.05$). The leaf elongation rate of the third leaf of the SC group (LER3) was 2.41 ± 0.13 . LER3 of SI group was 2.67 ± 0.19 . SI group LER3 had a statistically significant increase of 11% compared to SC group ($p < 0.05$). Leaf elongation rate (LER4) of the fourth leaf of SC group was 2.41 ± 0.13 . SI group LER4 had a statistically significant increase of 14% compared to SC group ($p < 0.05$).

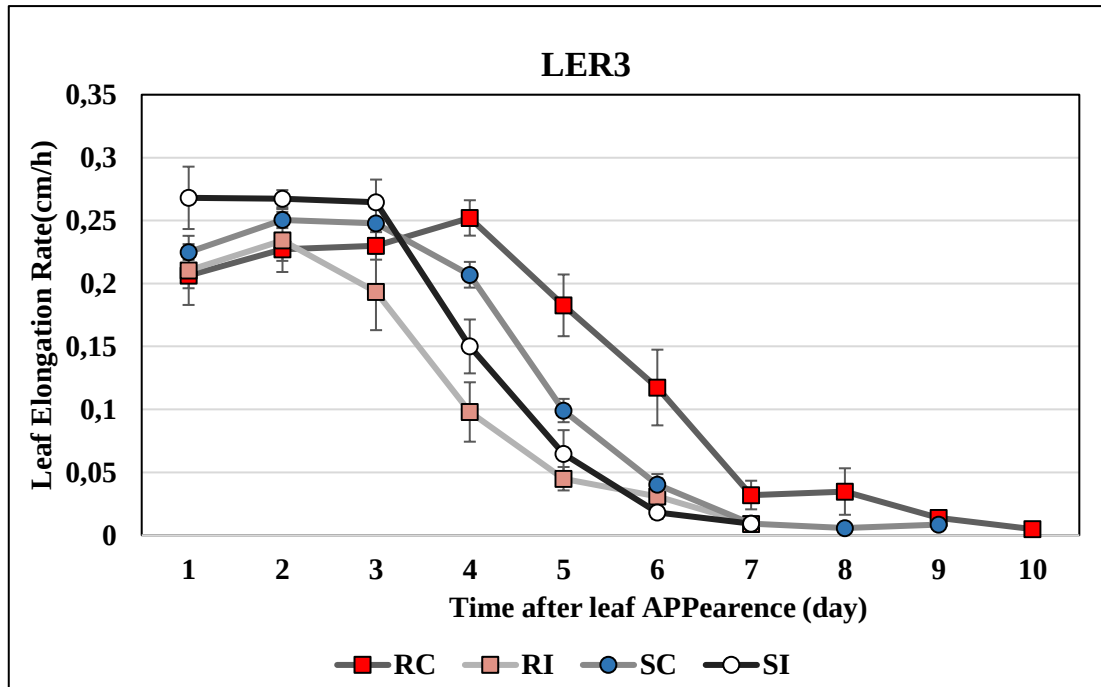


Figure 4.5: The elongation rate of third leaf (LER3) of ADK1188 (S) and ADK310 (R) seedlings under control (C) and *F. verticillioides* infection (I). Statistical significance of the difference between groups was determined using Student's T-test ($p < 0.05$). RC=ADK310 line control; SC=ADK1188 line control; RI; ADK310 line *F. verticillioides* inoculated; SI=ADK1188 line *F. verticillioides* inoculated.

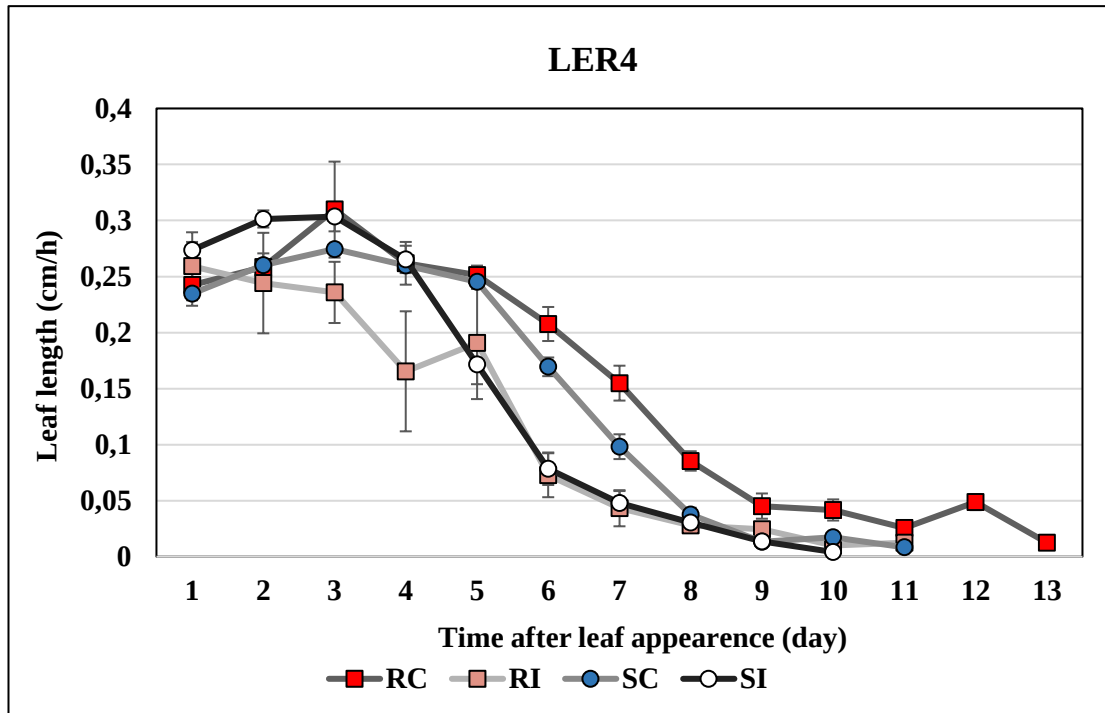


Figure 4.6: The elongation rate of fourth leaf (LER4) of ADK1188 (S) and ADK310 (R) seedlings under control (C) and *F. verticillioides* infection (I). Statistical significance of the difference between groups was determined using Student's T-test ($p < 0.05$). RC=ADK310 line control; SC=ADK1188 line control; RI; ADK310 line *F. verticillioides* inoculated; SI=ADK1188 line *F. verticillioides* inoculated.

To understand whether Fv affects maize leaf width, leaf width was measured with the help of a ruler. While the third leaf width of the RI group did not change compared to the RC group ($p > 0.05$), the third leaf width of the SI group decreased by approximately 10% compared to the SC group, which was statistically significant ($p < 0.01$). While the fourth leaf width of the RI group did not change compared to the RC group ($p > 0.05$), the fourth leaf width of the SI group decreased by approximately 14% compared to the SC group, which was statistically significant ($p < 0.01$).

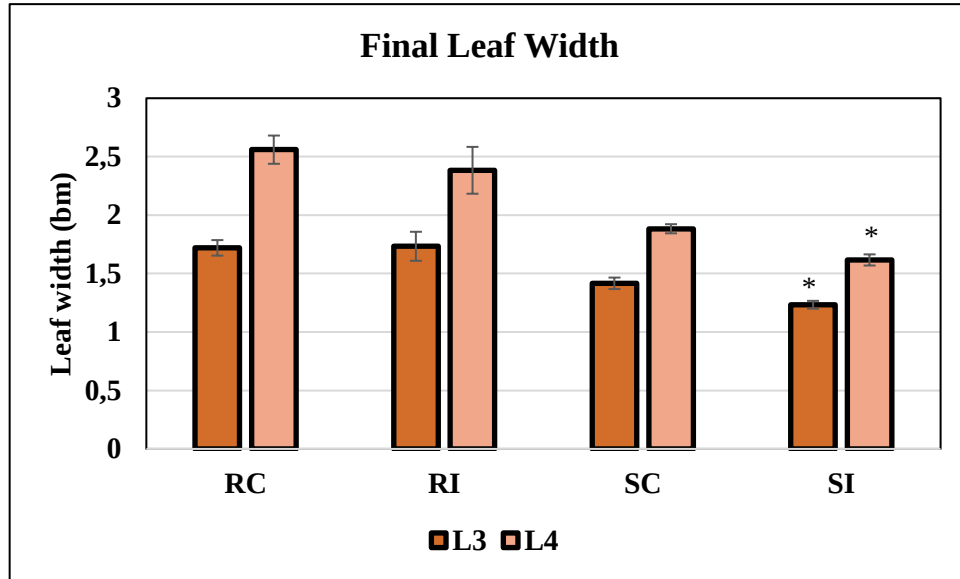


Figure 4.7: The width of leaf 3 (L3) and leaf 4 (L4) of ADK1188 (S) and ADK310 (R) seedlings under control (C) and *F. verticillioides* infection (I). Statistical significance of the difference between groups was determined using Student's T-test ($p < 0.05$). RC=ADK310 line control; SC=ADK1188 line control; RI; ADK310 line *F. verticillioides* inoculated; SI=ADK1188 line *F. verticillioides* inoculated.

As a result of the measurements made for leaf area, it was observed that the third and fourth leaf of the RI group did not change compared to the RC group ($p > 0.05$). The third leaf area of the SI group decreased by 30% and the fourth leaf area decreased by 26% compared to the SC group, which was statistically significant ($p < 0.05$).

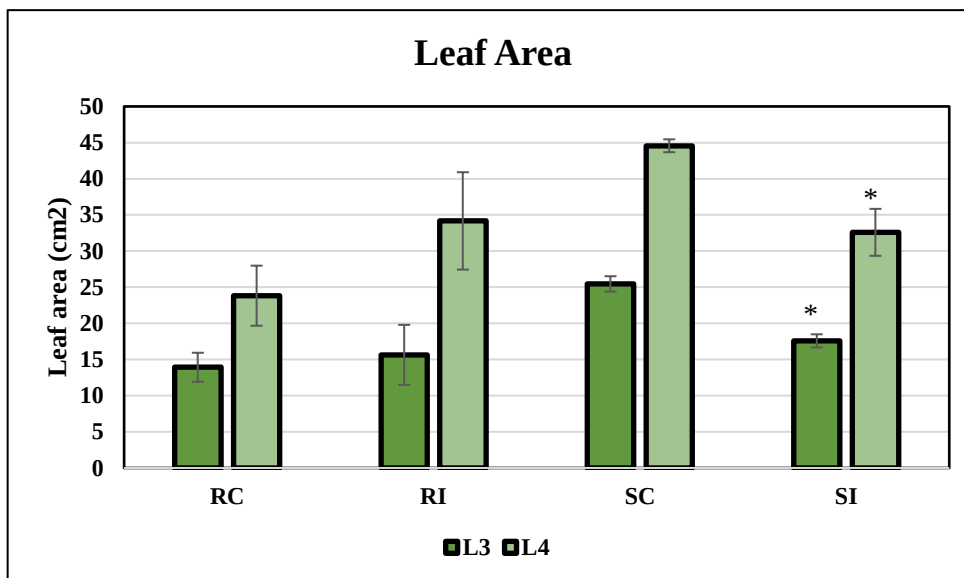


Figure 4.8: The area of leaf 3 (L3) and leaf 4 (L4) of ADK1188 (S) and ADK310 (R) seedlings under control (C) and *F. verticillioides* infection (I). Statistical significance of the difference between groups was determined using Student's T-test ($p < 0.05$). RC=ADK310 line control; SC=ADK1188 line control; RI; ADK310 line *F. verticillioides* inoculated; SI=ADK1188 line *F. verticillioides* inoculated.

4.3. Effect of *F. verticillioides* on Plant Photosynthetic Efficiency

SPADmeter was used to compare the chlorophyll density of the third and fourth leaves between the the control and infected group of R and S line. The third leaf chlorophyll density of the RI group did not change compared to the RC group ($p > 0.05$). The third leaf chlorophyll density of the SI group decreased by approximately 30% compared to the SC group, which was statistically significant ($p < 0.01$). The fourth leaf chlorophyll density of the RI group did not change compared to the RC group ($p > 0.05$). The fourth leaf chlorophyll density of the SI group did not change compared to the SC group ($p > 0.05$)(Figure 4.9).

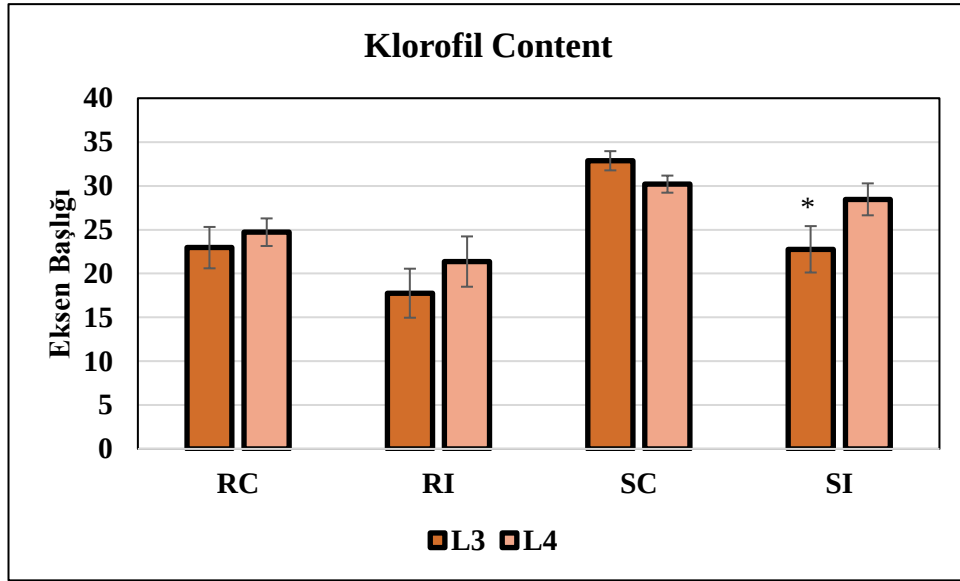


Figure 4.9: The chlorophyll content of leaf 3 (L3) and leaf 4 (L4) of ADK1188 (S) and ADK310 (R) seedlings under control (C) and *F. verticillioides* infection (I). Statistical significance of the difference between groups was determined using Student's T-test ($p < 0.05$). RC=ADK310 line control; SC=ADK1188 line control; RI; ADK310 line *F. verticillioides* inoculated; SI=ADK1188 line *F. verticillioides* inoculated.

4.3.1. Chlorophyll a, b and Carotenoid Content of *F. verticillioides* Infected Mazie Seedlings

Based on the analyses performed on the second leaf, the chlorophyll a, chlorophyll b and carotenoid content of the RI group did not change compared to the RC group ($p > 0.05$). The chlorophyll a content of the SI group decreased significantly by 19% compared to the SC group ($p < 0.05$). The chlorophyll b and carotenoid content of the SI group did not change compared to the SC group ($p > 0.05$).

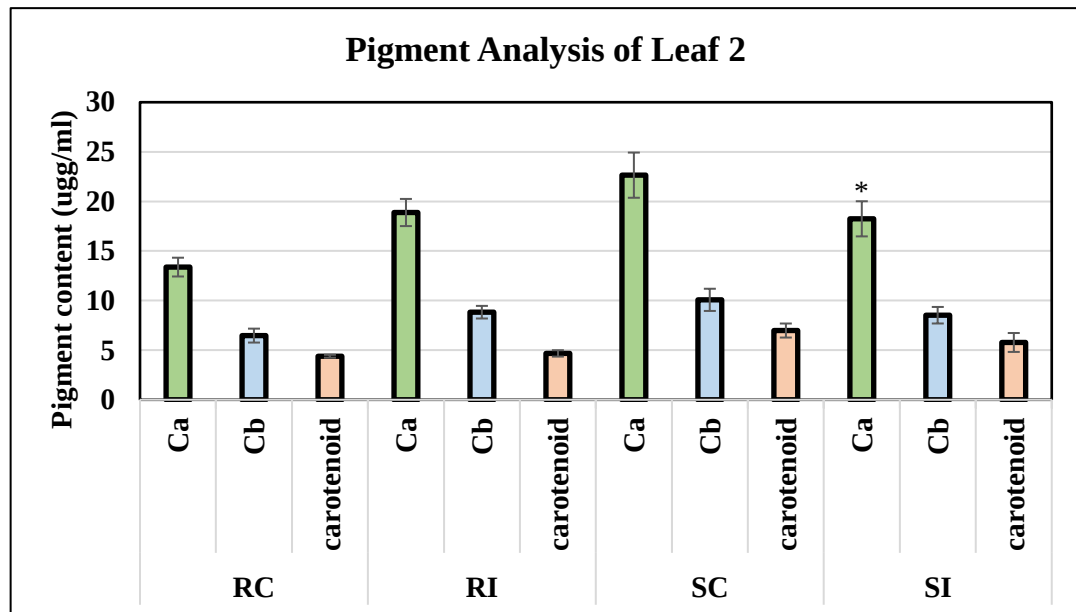


Figure 4.10: The chlorophyll a, chlorophyll b and carotenoid content of second leaf of the tolerant (R) and sensitive (S) maize line infected by *F. verticillioides*. Statistical significance of the difference between groups was determined using Student's T-test ($p < 0.05$). RC=ADK310 line control; SC=ADK1188 line control; RI; ADK310 line *F. verticillioides* inoculated; SI=ADK1188 line *F. verticillioides* inoculated.

4.4. Effect of *Fusarium verticillioides* on Cell Length

To examine the effect of Fv on cell lengths, samples taken from the second, third and fourth leaves were photographed under a light microscope with 10X magnification. Cell lengths were measured with the ImageJ application. Lengths were measured with the ImageJ application. Second leaf cell length did not change in the RI group compared to the RC group ($p > 0.05$). In the SI group, cell length decreased by approximately 40% compared to the SC group, which was statistically significant ($p < 0.01$). The third leaf cell length of the RI group decreased statistically significantly by approximately 23% compared to the cell length of the RC group ($p < 0.01$). The third leaf cell length of the SI group did not change compared to the SC group ($p > 0.05$). The fourth leaf cell length of the RI group decreased statistically significantly by approximately 17% compared to the cell length of the RC group ($p < 0.05$). The fourth leaf cell length of the SI group did not change compared to the cell length of the SC group ($p > 0.05$).

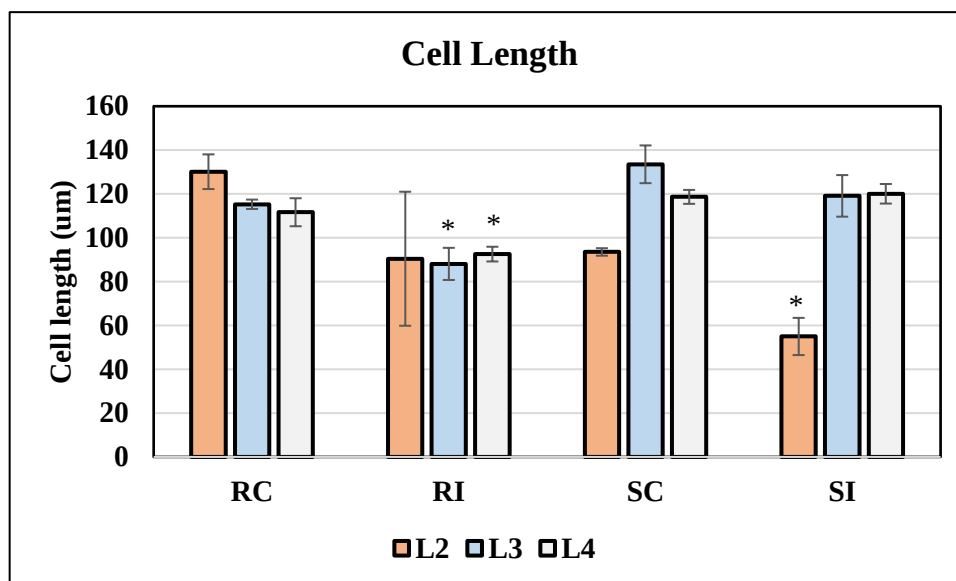


Figure 4.11: The length cells of leaf 2 (L2), leaf 3 (L3) and leaf 4 (L4) of the tolerant (R) and sensitive (S) maize line infected by *F. verticillioides* (I). Statistical significance of the difference between groups was determined using Student's T-test ($p < 0.05$). RC=ADK310 line control; SC=ADK1188 line control; RI; ADK310 line *F. verticillioides* inoculated; SI=ADK1188 line *F. verticillioides* inoculated.

4.5. Accumulation of Phenolic Compounds in Maize Under *F. verticillioides* Infection

Phenolics are secondary metabolites that play a role in the defense system of plants. They strengthen the defense as antifungal, antimicrobial and antioxidant agents during the invasion of microorganisms (Tariq & Ahmed, 2024). In this study, the change in the amount of phenolics in two different maize lines was investigated with the infection of Fv pathogen in maize. The phenolic concentration of the RI group increased by 166% statistically significantly compared to the RC group ($p < 0.01$). The phenolic content of the SI group did not change compared to the SC group ($p > 0.05$).

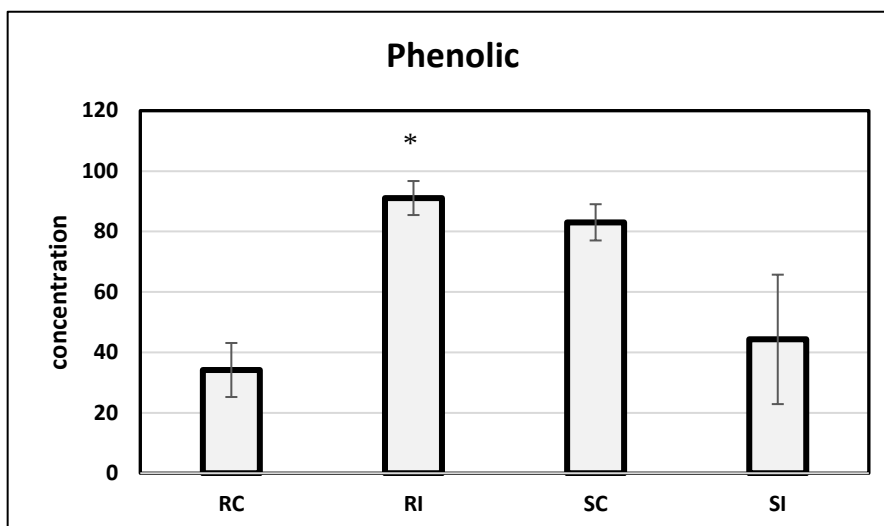


Figure 4.12: The total phenolic content of second leaf of the tolerant (R) and susceptible (S) maize line infected by *F. verticillioides* (I). Statistical significance of the difference between groups was determined using Student's T-test ($p < 0.05$). RC=ADK310 line control; SC=ADK1188 line control; RI; ADK310 line *F. verticillioides* inoculated; SI=ADK1188 line *F. verticillioides* inoculated.

4.6. Spread of *F. verticillioides* throughout the leaf

In order to show that Fv given to the maize by the leaf inoculation method is taken by the plant and to determine in which parts of the leaf it is concentrated, third and fourth leaves were surface sterilized with 70% ethanol. Leaf disc were taken from the tip, middle and base parts of the leaves and added to the PDA medium (Figure 4.13). Fv growth could not be observed on the plate containing the third and fourth leaves of SI plants. The Fv diameter width of RI plants in the third leaf increased statistically significantly by approximately 43% compared to the fourth leaf ($p < 0.05$). There was no significant difference between the tip, middle and base parts ($p > 0.05$) (Figure 4.14).

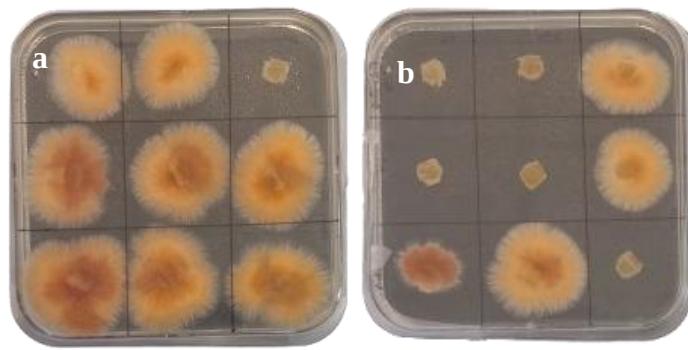


Figure 4.13: *F.verticillioides* growth on PDA after surface sterilization of maize leaves 3 and 4. RC group leaf 3 (a); RI group leaf 4 (b). Statistical significance of the difference between groups was determined using Student's T-test ($p<0.05$). RC=ADK310 line control; RI; ADK310 line *F. verticillioides* inoculated.

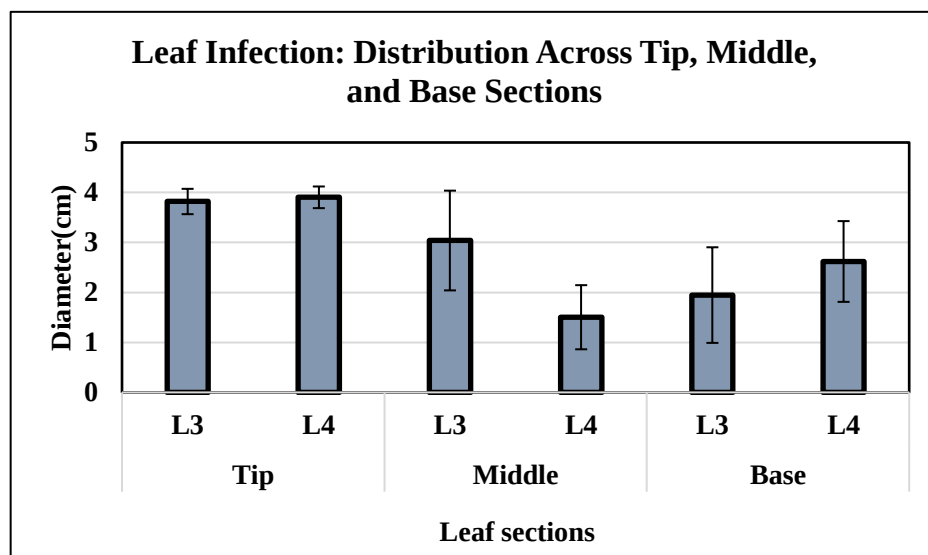


Figure 4.14: The fungal growth area from infected third and fourth leaf tissue. Statistical significance of the difference between groups was determined using Student's T-test ($p<0.05$). RI; ADK310 line *F. verticillioides* inoculated.

4.7. Response of Enzymes In The Maize Antioxidant System to *F.verticillioides* Infection

4.7.1. Catalase Enzyme Activity

The response of catalase enzyme, one of the important enzymes of plant antioxidant system, to Fv infection was determined by enzyme activity test. For the measurement

of catalase enzyme activity, 2 cm samples were taken from the second leaves of R and S maize seedlings. After protein isolation from the samples, enzyme activity test was applied using hydrogen peroxide as a substrate. Catalase enzyme activity of RI group did not change compared to RC group ($p>0.05$). Enzyme activity of SI group decreased by approximately 29% statistically significantly compared to SC group ($p<0.05$).

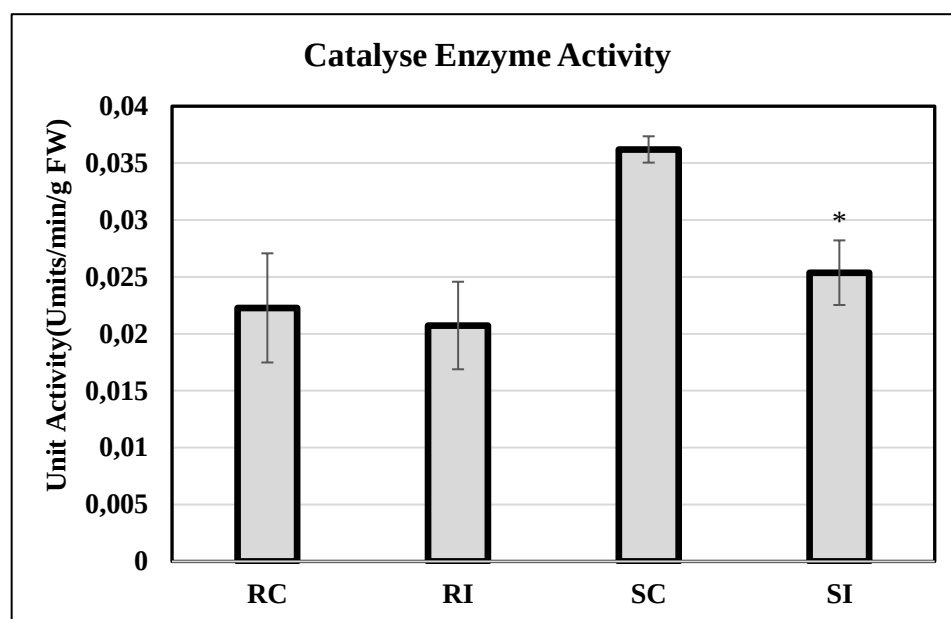


Figure 4.15: Catalase enzyme activity in second leaf (L2) of the tolerant (R) and sensitive (S) maize line infected by *F. verticillioides* (I). Statistical significance of the difference between groups was determined using Student's T-test ($p<0.05$). RC=ADK310 line control; SC=ADK1188 line control; RI; ADK310 line *F. verticillioides* inoculated; SI=ADK1188 line *F. verticillioides* inoculated.

4.7.2. Peroxidase Enzyme Activity

Peroxidase (POX) degrades hydrogen peroxide in the cell and creates a strong antioxidant response against biotic and abiotic stress. Enzyme activity was determined in the stress response to Fv infection. For the measurement of POX enzyme activity, 2 cm samples were taken from the second leaves of R and S plants. Protein isolation was performed from the samples and enzyme activity tests were performed. The POX enzyme activity of the RI group increased by 74% compared to the RC group ($p>0.05$). The enzyme activity of the SI group did not change compared to the SC group ($p>0.05$).

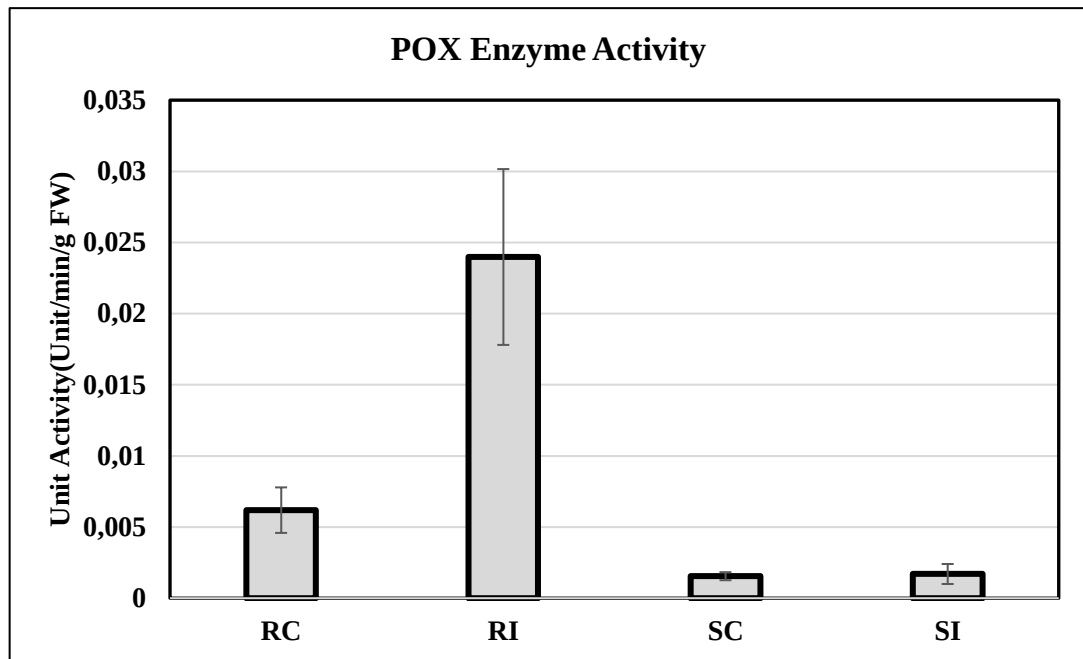


Figure 4.16: Peroxidase enzyme activity in second leaf (L2) of the tolerant (R) and sensitive (S) maize line infected by *F. verticillioides* (I). Statistical significance of the difference between groups was determined using Student's T-test ($p < 0.05$). RC=ADK310 line control; SC=ADK1188 line control; RI; ADK310 line *F. verticillioides* inoculated; SI=ADK1188 line *F. verticillioides* inoculated.

4.7.3. Ascorbate Peroxidase Enzyme Activity

Ascorbate Peroxidase (APX), which is involved in the antioxidant system, plays a role in reducing the ROS concentration in the cell. It is expected to degrade ROS that occur in response to Fv-induced biotic stress. For the measurement of APX enzyme activity, 2 cm samples were taken from the second leaves of R and S plants. Protein isolation was performed from the samples taken and enzyme activity tests were performed. APX enzyme activity of the RI group did not change compared to the RC group ($p > 0.05$). APX enzyme activity of the SI group increased statistically significantly by approximately 68% compared to the SC group ($p < 0.05$).

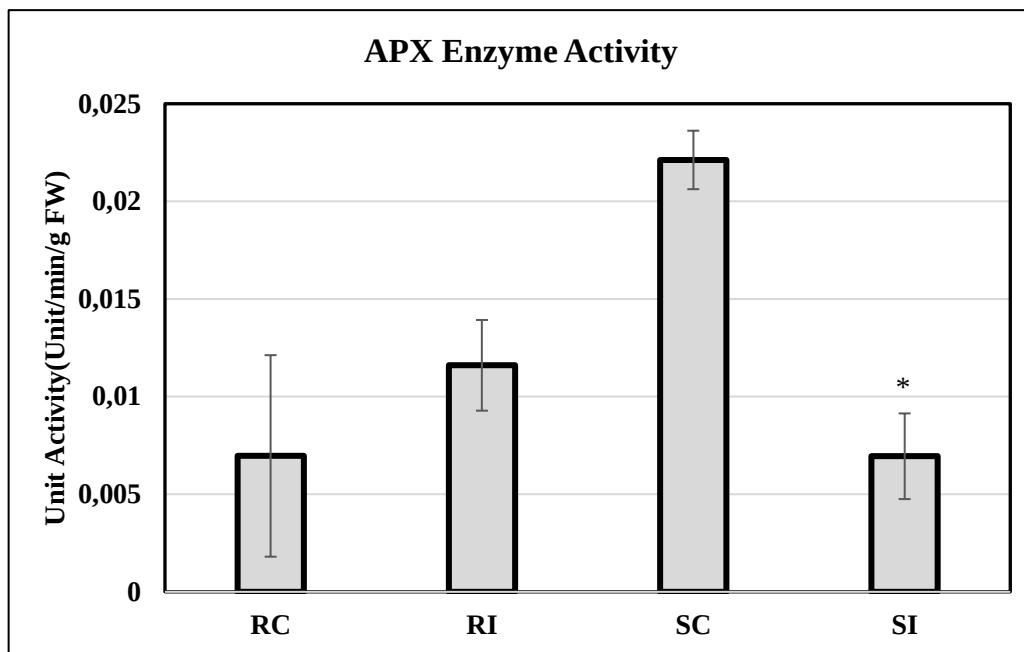


Figure 4.17: Ascorbate peroxidase enzyme activity in second leaf (L2) of the tolerant (R) and sensitive (S) maize line infected by *F. verticillioides* (I). Statistical significance of the difference between groups was determined using Student's T-test ($p < 0.05$). RC=ADK310 line control; SC=ADK1188 line control; RI; ADK310 line *F. verticillioides* inoculated; SI=ADK1188 line *F. verticillioides* inoculated.

4.7.4. Superoxide Dismutase Enzyme Activity

In the case of Superoxide dismutase (SOD), two dangerous compounds, superoxide and hydrogen peroxide, are converted into water, thus protecting the cells. For the measurement of SOD enzyme activity, 2 cm samples were taken from the second leaves of R and S plants. After protein isolation from the samples, enzyme activity tests were performed. The SOD enzyme activity of the RI group did not change compared to the RC group ($p > 0.05$). The SOD enzyme activity of the SI group increased by approximately 128% compared to the SC group, which was statistically significant ($p < 0.05$).

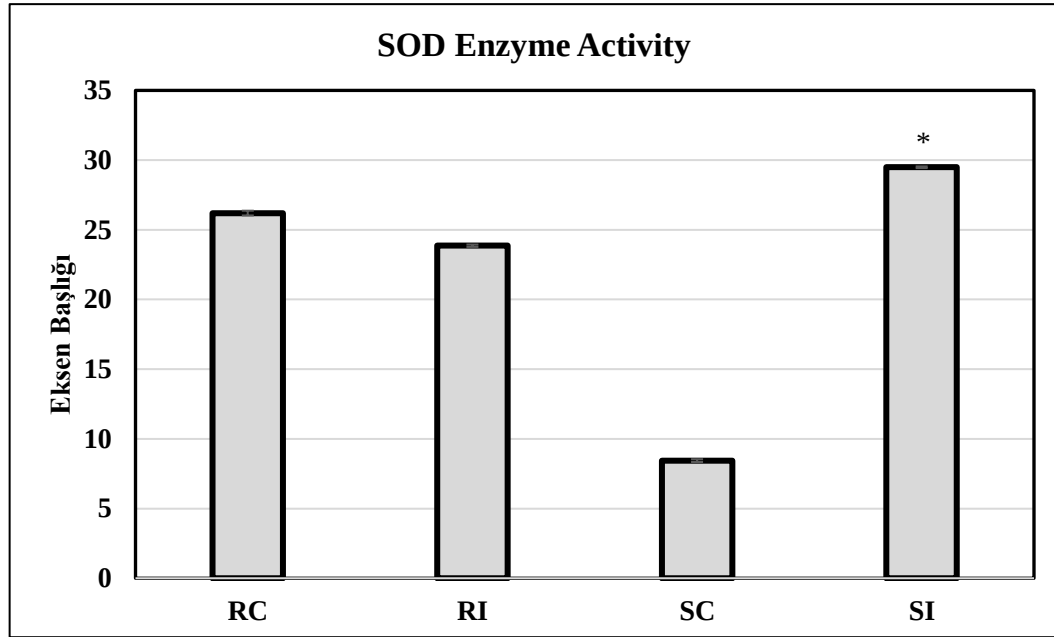


Figure 4.18: Superoxide dismutase enzyme activity in second leaf (L2) of the tolerant (R) and sensitive (S) maize line infected by *F. verticillioides* (I). Statistical significance of the difference between groups was determined using Student's T-test ($p < 0.05$). RC=ADK310 line control; SC=ADK1188 line control; RI; ADK310 line *F. verticillioides* inoculated; SI=ADK1188 line *F. verticillioides* inoculated.

4.7.5. Glutatyon Reductase

Glutathione reductase (GR) plays a role in the functioning of the antioxidant system by converting potentially toxic GSSG to GSH. Enzyme activity test was performed to determine the response of this enzyme to Fv stress. For the measurement of GR enzyme activity, 2 cm samples were taken from the second leaves of R and S plants and protein isolation was performed. GR enzyme activity was determined from the samples taken. GR enzyme activity of the RI group did not change compared to the RC group ($p > 0.05$). GR enzyme activity of the SI group did not change compared to the SC group ($p > 0.05$).

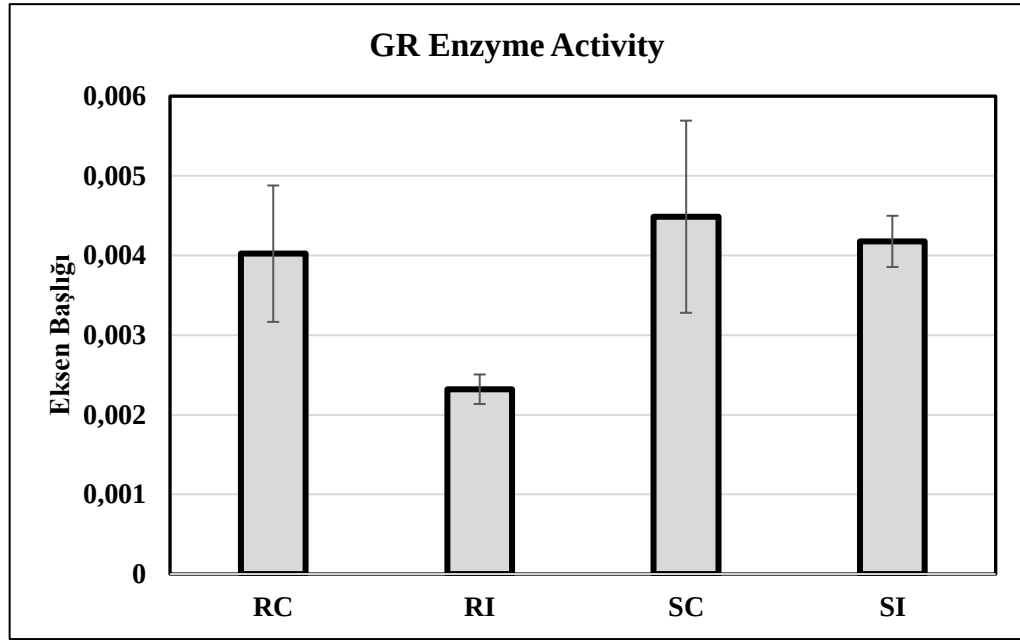


Figure 4.19: Glutathion reductase enzyme activity in second leaf (L2) of the tolerant (R) and sensitive (S) maize line infected by *F. verticillioides* (I). Statistical significance of the difference between groups was determined using Student's T-test ($p < 0.05$). RC=ADK310 line control; SC=ADK1188 line control; RI; ADK310 line *F. verticillioides* inoculated; SI=ADK1188 line *F. verticillioides* inoculated.

4.8. In Silico Prediction of Candidate Gene Selection Transferred Between Two organism For Mutual Defence

Two hypotheses are put forward in this study: First, it is suggested that miRNAs transferred to the maize plant as a result of *Fusarium verticillioides* infection silence genes involved in the plant's defense response through the 'cross-kingdom RNAi' mechanism. Second, *Zea mays* miRNAs are thought to target mRNAs vital to *Fusarium verticillioides* via the 'cross-kingdom RNAi' mechanism. The miRNAs and their target genes identified in line with these two hypotheses were determined using psRNATarget and RNAhybid and are listed in Table 4.1.

Table 4.1: The miRNAs and their target genes identified were determined using psRNA Target and RNAhybrid bioinformatic tools.

MiRNA_Acc	Traget_Acc	E	UP E\$	miRNA_aligned_fragment	Target_aligned_fragment	Traget_Desc.
zma-miR398a-3p	XM_01889476 9.1	0.5	-1.0	UGUGUUCUCAGGUCGCCCCCG	AUGGGGCGACCUGGGAACACA	<i>Fusarium verticillioides</i> 7600 26S proteasome regulatory subunit rpn-1 (FVEG_06397), mRNA
zma-miR398a-3p	XM_01889525 4.1	4.5	-1.0	UGUGUUCUCAGGUCGCCCCCG	CAGAGGAGACCUGAGAACAGU	<i>Fusarium verticillioides</i> 7600 cell wall integrity and stress response component (FVEG_06829), mRNA
zma-miR528a-3p	XM_01888948 4.1	1	-1	CCUGUGCCUGCCUCUCCAUAU	ACUGGAAGGGGCGGGCACAGG	<i>Fusarium verticillioides</i> 7600 palmitoyl-CoA hydrolase (FVEG_02271), mRNA
zma-miR159a-3p	XM_01889267 6.1	3.0	-1.0	UUUGGAUUGAAGGGAGCUCUG	CUGAGC-CCUUCAAUCUAAA	<i>Fusarium verticillioides</i> 7600 glutathione peroxidase (FVEG_04790), mRNA
zma-miR159a-3p	XM_01888835 0.1	4,5	-1	UUUGGAUUGAAGGGAGCUCUG	GCCAGUUUCCUUCGAUCUGAU	<i>Fusarium verticillioides</i> 7600 chitin synthase 4 (FVEG_01403), mRNA
zma-miR159a-3p	XM_01889690 8.1	4.0	-1.0	UUUGGAUUGAAGGGAGCUCUG	AAGGGUUCCCCUUUGUUCAAA	<i>Fusarium verticillioides</i> 7600 vacuolar fusion protein MON1 (FVEG_08091), mRNA
zma-miR1432-5p	XM_01889069 0.1	3.0	-1.0	CUCAGGAGAGAUGACACCGAC	CUCGGUGUUGUCUCUCUUCAG	<i>Fusarium verticillioides</i> 7600 sphingolipid delta-4 desaturase (FVEG_03105), mRNA
NC031675.1:5198 746-5198764	Zm00001d0294 48_T001	1	-1	UGUUCUAGCAACUACGACA	UGUAGUAGUUGCUAGAACA	gene_biotype:protein_coding transcript_biotype:protein_coding description:TIFY6
NC_031679.1:165 1804-1651827	Zm00001d0303 43_T001	2	-1	GAGGAACUUGAGGAGCUUGCCGAC	CAGCUCAAGUCCUCAAGUCCCUC	gene_biotype:protein_coding transcript_biotype:protein_coding description:3-ketoacyl-CoA synthase
NC_031685.1:116 5834-1165855	Zm00001d0234 25_T001	1	-1	ACUCCACGGACAUGGUAACCCC	GCGGUUACCAUGUCUGUGGGGU	gene_biotype:protein_coding transcript_biotype:protein_coding gene_symbol:CR4 description:Putative receptor protein kinase CRINKLY4
NC_031680.1:306 7191-3067214	Zm00001d0468 75_T001	2	-1	UUCCUCGGAUGCUUCUCUUCUGAG	UGCAGAAGAGAGGUAUUCGAGGAG	gene_biotype:protein_coding transcript_biotype:protein_coding description:RNA-dependent RNA polymerase

4.9. The expression Levels of miRNA and their Target Genes Reciprocally during *Zea mays* and *F. verticillioides* interaction

The miRNAs and mRNAs that are likely to play a role in Cross-Kingdom RNAi were identified and specific primers were designed for these genes. The accuracy of the primers was tested by PCR. The expression profiles pointed out how sRNAs affect the transcript levels of the mRNAs they target by crossing kingdoms.

4.9.1. The expression level of *Zea mays* miRNA and Their Predicted *F. verticillioides* genes

While the transcription level of Zm-miR398a in RI tissue did not change compared to RC ($p>0.05$), the targets of zm-mir398a, Fv_06397 (26S proteasome regulatory subunit Rpn-1) and Fv_06829 (cell wall integrity and stress response component), showed a statistically significant increase by 3-fold and 152-fold, respectively, in RI tissue compared to control ($p<0.01$). As a result of these findings, it is thought that Fv_06397 and Fv_06829 genes may play a role in plant defense during pathogen attack.

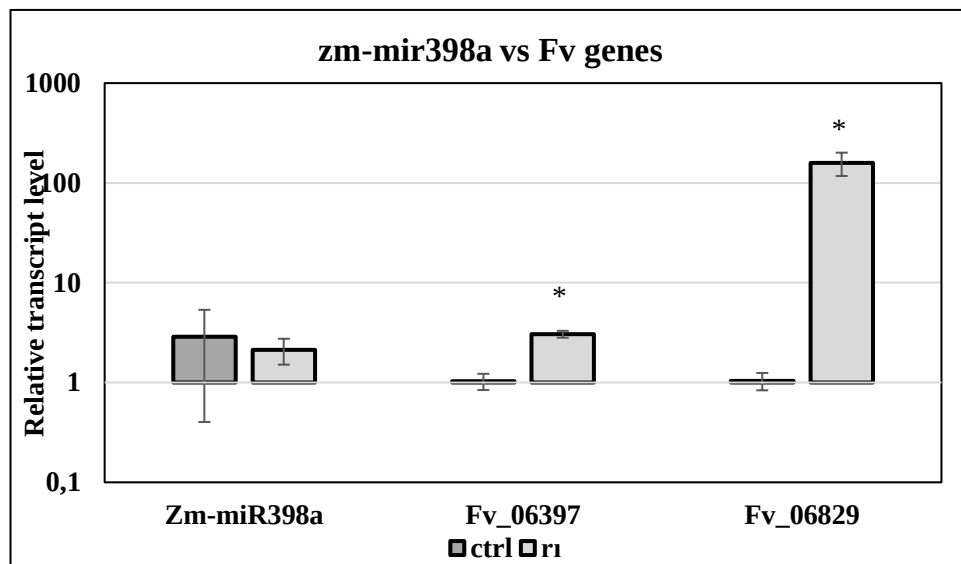


Figure 4.20: The relative transcript levels of Zm-miR398a and its targets, Fv_06397 and Fv_06829, are shown. Statistical significance of the difference between groups was determined using Student's T-test ($p<0.05$). RI; ADK310 line *F. verticillioides* inoculated; ctrl: control maize leaf for sRNA; Fv culture grown on PDA for mRNA.

The relationship between the palmitoyl-CoA hydrolase (Fv_02271) gene, which is the target of zm-mir528a, was examined by RT-qPCR. While the transcription level of zm-miR528a decreased by 89% in RI tissue compared to RC ($p>0.05$), Fv_02271, which is the target of zm-mir528a, showed a statistically significant increase of 129% ($p<0.05$).

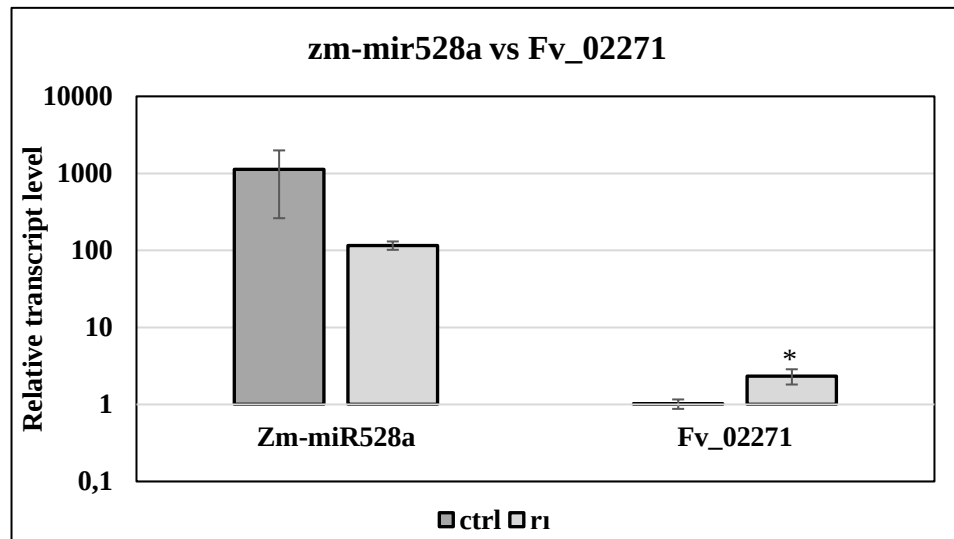


Figure 4.21: The relative transcript levels of Zm-miR528a and its target, Fv_02271, are shown. Statistical significance of the difference between groups was determined using Student's T-test ($p<0.05$). RI; ADK310 line *F. verticillioides* inoculated; ctrl: control maize leaf for sRNA; Fv culture grown on PDA for mRNA.

The transcription level of Zm-miR1432 in RI tissue did not change compared to the control ($p>0.05$), while its predicted fungal targets, sphingolipid delta-4 desaturase (Fv_03105), showed a statistically significant 22-fold increase ($p<0.05$).

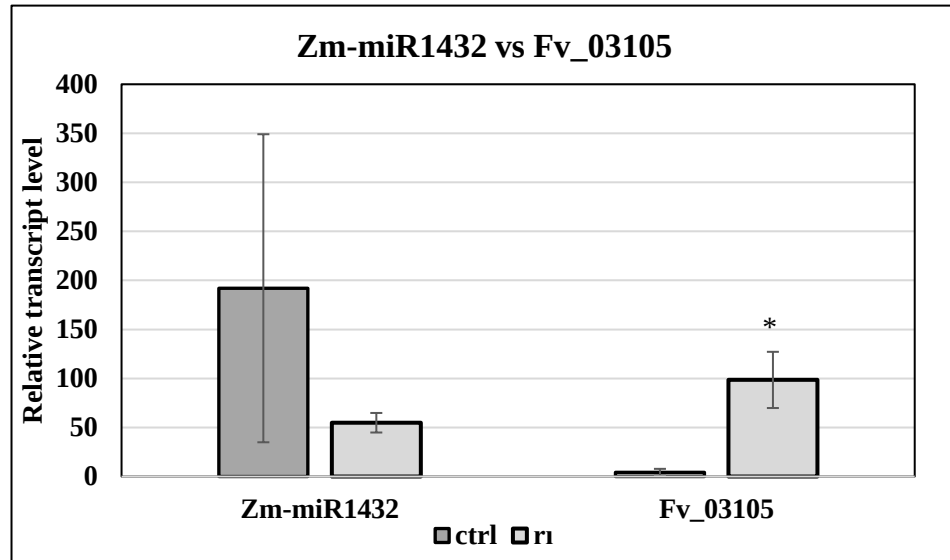


Figure 4.22: The relative transcript levels of Zm-1432 and it target, Fv_03105 are shown. Statistical significance of the difference between groups was determined using Student's T-test ($p<0.05$). RI; ADK310 line *F. verticillioides* inoculated; ctrl: control maize leaf for sRNA; Fv culture grown on PDA for mRNA.

The transcription level of Zm-miR159 in RI tissue was significantly increased by 5-fold compared to the control ($p<0.01$), while the targets of zm-mir159, glutathione peroxidase (Fv_04790) and chitin synthase 4 (Fv_01403), showed a statistically significant increase of 12- and 402-fold, respectively ($p<0.05$). Vacuolar fusion protein MON1 (Fv_08091) was statistically significantly decreased by 88% ($p<0.05$). This change in transcription levels suggests an antagonistic effect between Zm-miR159 and its target Fv_08091. This suggests that Fv_04790 and Fv_01403 genes are not directly targeted by Zm-miR159a.

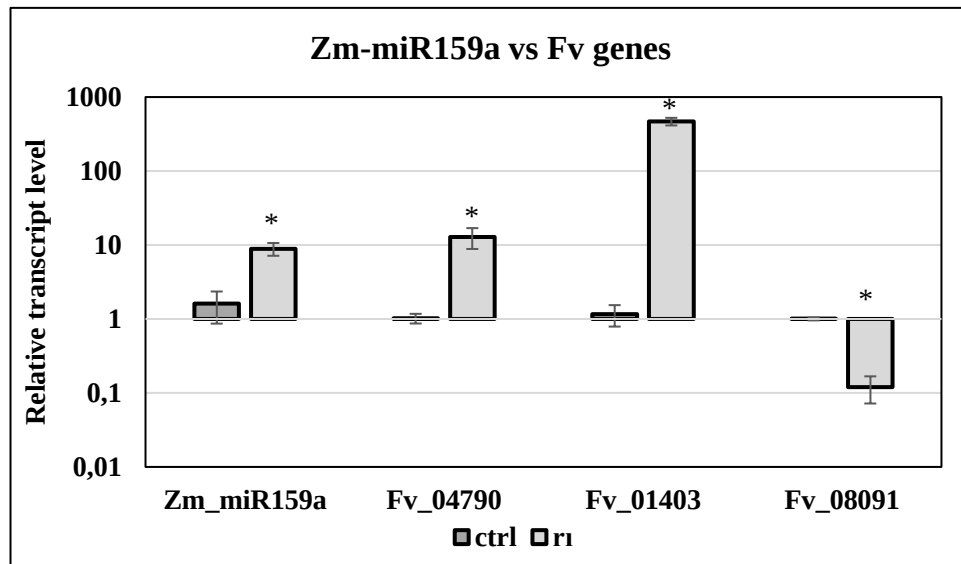


Figure 4.23: The relative transcript levels of Zm-159a and its targets, Fv_04790, are shown. Fv_01403 and Fv_08091. Statistical significance of the difference between groups was determined using Student's T-test ($p < 0.05$). RI; ADK310 line *F. verticillioides* inoculated; ctrl: control maize leaf for sRNA; Fv culture grown on PDA for mRNA.

4.9.2. The expression level of *F. verticillioides* miRNAs and Their Predicted *Zea mays* genes

The transcription levels of Fv-miR8764 miRNA and its target gene TIFY domain-containing protein 6 (zm_TIFY6) were examined. Fv-miR8764 increased statistically significantly by 2214 times ($p < 0.01$), while zm_TIFY6 decreased by 83% in infected second leaf of R seedlings compared to control group ($p > 0.05$).

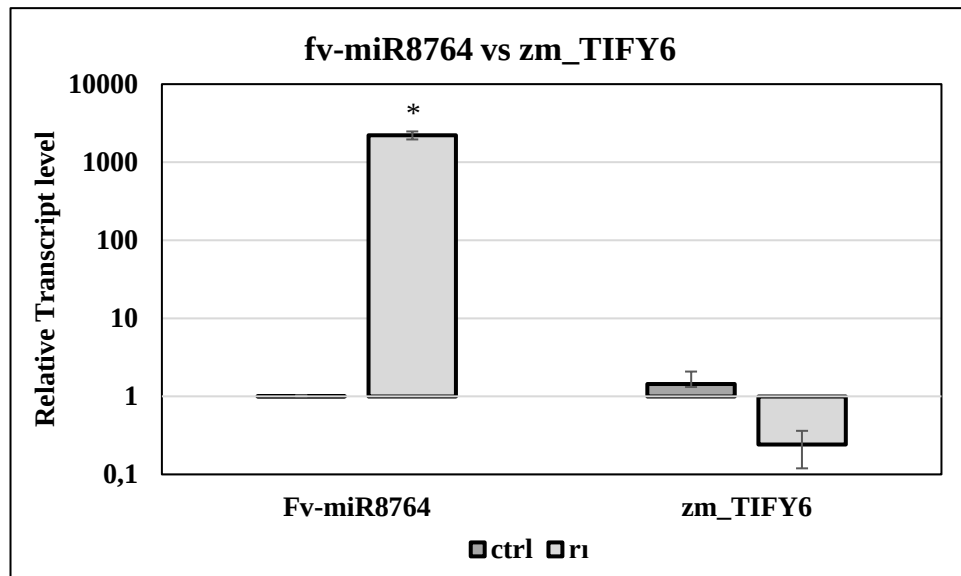


Figure 4.24: The relative transcript levels of Fv_miR8764 and its targets, Zm-TIFY6. Statistical significance of the difference between groups was determined using Student's T-test ($p < 0.05$). RI; ADK310 line *F. verticillioides* inoculated; ctrl: control maize leaf for sRNA; Fv culture grown on PDA for mRNA.

The transcription levels of Fv-miR1827 miRNA and its predicted *Zea mays* target 3-ketoacyl-CoA synthase (zm_KCoA) genes were examined. While fv-miR1827 increased statistically significantly by 815 times ($p < 0.05$), zm_KCoA statistically significantly decreased by 5651 times ($p < 0.05$). This change in transcription levels suggests an antagonistic effect between Fv-miR1827 and its target zm_KCoA.

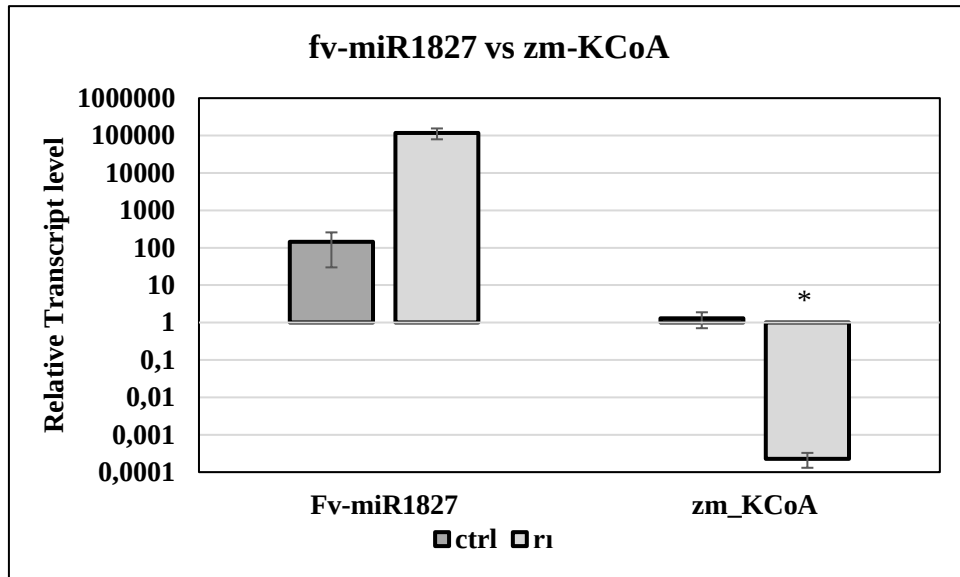


Figure 4.25: The relative transcript levels of Fv_miR1827 and its targets, Zm-KCoA. Statistical significance of the difference between groups was determined using Student's T-test ($p < 0.05$). RI; ADK310 line *F. verticillioides* inoculated; ctrl: control maize leaf for sRNA; Fv culture grown on PDA for mRNA.

The transcription levels of Fv-miR5855 miRNA and its target Putative receptor protein kinase CRINKLY4 (zm_CRINKYL4) genes were examined. Fv-miR5855 increased statistically significantly by 22000 fold ($p < 0.01$), while zm_CRINKYL4 did not change ($p > 0.05$).

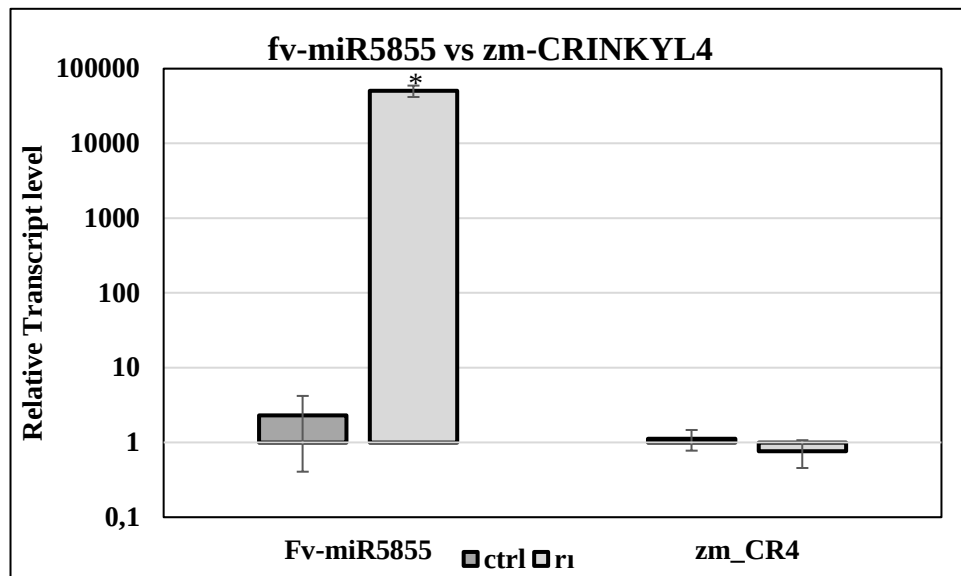


Figure 4.26: The relative transcript levels of Fv_miR5855 and its targets, Zm-CRINKYL4. Statistical significance of the difference between groups was determined using Student's T-test ($p < 0.05$). RI; ADK310 line *F. verticillioides* inoculated; ctrl: control maize leaf for sRNA; Fv culture grown on PDA for mRNA.

The transcription levels of Fv-miR7214 miRNA and the RNA-dependent RNA polymerase (zm_RDRP) genes it targets were examined. While fv-miR7214 increased statistically significantly by 3400 fold ($p < 0.05$), zm_RDRP decreased by 71% ($p > 0.05$).

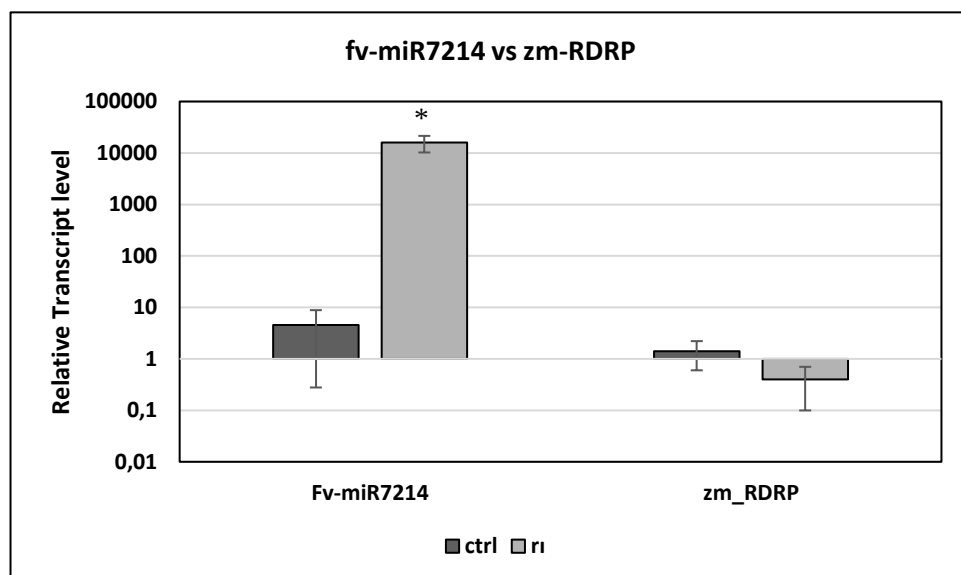


Figure 4.27: The relative transcript levels of Fv_miR7214 and its targets, Zm-RDRP. Statistical significance of the difference between groups was determined using Student's T-test ($p < 0.05$). RI; ADK310 line *F. verticillioides* inoculated; ctrl: control maize leaf for sRNA; Fv culture grown on PDA for mRNA.

5.DISCUSSION AND CONCLUSION

In this study, *Fusarium verticillioides* infection on tolerant (R) and sensitive (S) maize lines at seedling stage were investigated at morphological, physiological, biochemical and transcriptional levels. The observation showed that R line displayed more severe symptoms than S line oppositely to expectation. In the study conducted by Wang et al. (2016), it was determined that as a result of the application of *F. verticillioides* to two different maize lines, resistant (BT-1) and susceptible (N6), by seed inoculation method, the susceptible line showed more severe symptoms. This situation shows that Fv infects resistant and susceptible lines in different organs to different degrees. In addition, Fv is an endophytic pathogen and some studies have shown that it protects maize against different pathogens and supports its growth. Lee et al. (2009) showed that when *F. verticillioides* co-inoculated with another maize fungal pathogen *Ustilago maydis*, it protected maize from the disease and helped reduce its symptoms compared to the group inoculated *Ustilago maydis* alone.

After inoculation, the lengths of the third and fourth leaves were measured every day from the day they started to appear until fully matured. It was detected that the R and S seedlings in control groups took longer to reach maturity than the infected groups. This growth inhibition also caused reduced final leaf length of third and fourth leaf of R and S seedlings infected by compared to control groups. The FLL3 of RI was statistically significantly reduced by 17% compared to RC group and the final leaf length of the RI fourth leaf was statistically significantly 23% shorter than the RC group. In order to understand the difference in leaf length between the control and infected groups, the length of the mature cells measured. This helped to make a connect between organ size and cell length [Rymen et al., 2010]. When the cell lengths of the third leaves were examined, the RI group decreased significantly by 23% compared to the RC group. Based on these data, it was seen that reduced leaf elongation rate were accompanied reduced cell length resulted in shortened leaf in R line due to Fv infection.

Third and fourth leaf leaf elongation rate measurements were made in R and S breeding lines under control and Fv infection. SI group LER3 had a statistically significant increase of 11% compared to SC group. SI group LER4 had a statistically significant increase of 14% compared to SC group. Granier et al. (1999) showed in their study

that the stress to which sunflower leaves are exposed in the early stages reduces the leaf area by half. Bernstein et al. (1995) showed in their study that salt stress inhibits leaf growth in sorghum to certain extents.

When we examined the leaf width, the third leaf width of the SI group decreased by 10% and the fourth leaf width decreased by 14% compared to the SC group. There was a statistically significant decrease in the S line but not in the R line in infected leaf compared to control due to genotypic differences. Leaf width was found to increase under some stress conditions, but this varies according to the stress severity and plant species. For example, Wang et al. (2024) observed that *Atriplex canescens* leaf width decreased under drought stress. Yao et al. (2023) showed that leaf width increased under salt stress in their study with *Lycium barbarum L.*

It was observed that the third leaf area and the fourth leaf area in RI group did not change compared to the RC group. The third leaf area decreased by 30% and the fourth leaf area decreased by 26% in the SI group compared to the SC group which was statistically significant. Studies on leaf area have shown that leaf areas decrease under infection [Aslam et al., 2015]. The fact that there was no change in leaf area between the RC and RI groups in the R line but a significant decrease between the SC and SI groups in the S line is thought to be due to genetic differences between the two lines.

As a biotic stress, the effects of Fv on plant leaf length, leaf elongation rate, leaf width and leaf area parameters were observed. After these physiological analyzes, it was investigated how Fv affected the relative chlorophyll content (SPAD) and chlorophyll a, b and carotenoid contents of maize. When the chlorophyll content of the SI group was examined, a 30% decrease was seen compared to the SC group and this decrease was statistically significant. In the study conducted by Wang et al. (2024), it was observed that SPAD contents decreased under drought stress in *Atriplex canescens* plants compared to the control group. Chlorophyll a, chlorophyll b and carotenoid content of the RI group did not change compared to the RC group. In the SI group, chlorophyll a decreased by 19% which is statistically significant compared to the SC group. Chlorophyll b and carotenoid content did not change. Shah et al. (2017) showed in their study that chlorophyll content decreases under stress conditions. This suggests that stress conditions reduce the chlorophyll content of plants and therefore their photosynthetic capacity. Closing the stomata of plants that want to escape from stress conditions affects photosynthesis. Closing the stomata under stress conditions reduces

water loss through transpiration and prevents pathogens from entering the plant using the stomata [Bawa et al., 2023].

Phenolics are secondary metabolites that play a role in the defense system of plants. They strengthen the defense as antifungal, antimicrobial and antioxidant agents during the invasion of microorganisms. The defense system induced by phenolics includes physical changes such as suberization and lignification, metabolic changes such as the synthesis of pathogenesis-related proteins and the synthesis and accumulation of metabolites such as phytoalexins. They play a role in protecting plants from damage caused by pathogens [Tariq & Ahmed, 2024]. In this study, the change in the amount of phenolics in two different maize lines was investigated with the infection of Fv pathogen in maize. The phenolic concentration of the RI group increased by 166% statistically significantly compared to the RC group. The phenolic content of the SI group did not change compared to the SC group. The significant increase in the R line shows that phenolics play an important role in the defense response of this line against Fv. The change in the S line is not significant, but the decreasing trend in phenolics can be thought to be due to the endophytic property of Fv. Fv, which is endophytically present, can play a positive role in the growth of maize [Lee et al., 2009]. In the study conducted by Alhaithloul et al. (2021), it is observed that the total phenol content of tomato seedlings (*Solanum lycopersicum* L.) increases significantly under abiotic stress.

In order to determine the transfer of Fv from the second leaf to the upper leaves and its spread along the leaf, disc-shaped samples were taken from the infected third and fourth maize leaves. After surface sterilization, the samples were planted in PDA medium and the diameters of the fungal growth zones formed were measured. Separate samples were taken from the tip, middle and base regions for each leaf and the evaluation was made. In the RI group, the isolates obtained from the third leaf showed statistically significant (43%) higher growth than the fourth leaf ($p < 0.05$). However, no significant difference was detected between the tip, middle and base regions on the same leaf. On the other hand, *F. verticillioides* growth was not observed in the SI group on the PDA medium. Since Fv growth was not observed in the SI group, RT-qPCR analysis was performed only for the R group. Oren et al. (2003) showed in their study that maize seedlings were infected with Fv soil inoculum and Fv was found in the upper leaves in the later stages.

Catalase enzyme catalyzes hydrogen peroxide H_2O_2 and O_2 . The presence of catalase enzyme shows that the plant has a defensive response to stress. In this context, catalase enzyme activities of infected and control R and S lines were examined. While catalase enzyme activity did not change in the RI group compared to the RC group, it decreased by 29% in the SI group compared to the SC group, which was statistically significant. Mandal et al. (2008) examined CAT enzyme activity in tomato (*Solanum lycopersicum* L.) plants infected with *Fusarium oxysporum*. CAT activity tended to decrease 24 hours after infection and an increase in H_2O_2 accumulation was observed in parallel with this situation. This decrease in catalase and the increase in hydrogen peroxide levels indicate that the antioxidant defense response is inadequate and the pathogen has invaded and settled in the plant tissues. Hanaka et al. (2017) found that the activity of *Zea mays* leaves catalase increased after exposure to *Trigonotylus caelestialium*, a plant bug. In another study, Banerjee et al. (2022) observed that catalase enzyme activity increased due to increased ROS activity in the leaves and roots of wheat (*Triticum aestivum*) seedlings infected with *Fusarium oxysporum*. As a result, the increase or decrease in catalase enzyme in pathogen-plant interactions varies according to how strong the plant's antioxidant system is, and this determines whether the pathogen infects the plant.

Peroxidase enzyme is an important part of the antioxidant defense system that is involved in the antioxidant system and degrades H_2O_2 . Peroxidase enzyme activity was examined in R and S maize lines. The RI group showed a 74% increase in POX enzyme activity compared to the RC group which was not statistically significant. The SI group did not show a change in POX enzyme activity compared to the SC group. Maschietto et al. (2016) investigated the change in peroxidase enzyme activity as a result of *Fusarium verticillioides* infection using resistant (CO441 and CO433) and susceptible (CO354 and CO389) maize lines. High levels of peroxidase activity were observed in the resistant lines even before inoculation, but low levels of enzyme activity and H_2O_2 accumulation were found in the susceptible lines after infection.

Ascorbate Peroxidase, another member of the antioxidant system, provides defense against biotic and abiotic stresses by reducing ROS concentration in the cell. APX enzyme activity of the RI group did not change compared to the RC group. APX enzyme activity of the SI group decreased statistically significantly by approximately 68% compared to the SC group. Superoxide Dismutase converts superoxide, a harmful

ROS species for the cell, to H₂O₂ and then to H₂O and O₂. The SOD enzyme activity of the RI group did not change to the RC group. The SOD enzyme activity of the SI group increased by approximately 128% compared to the SC group, which was statistically significant. Glutathione reductase is an enzyme that is part of the antioxidant system that converts the toxic substance GSSG into GSH. GR enzyme activity of the SI and RI groups did not change compared to the SC and RC groups. Kumar et al. (2009) reported that an increase in CAT, SOD and GR enzyme activities was observed as a result of inoculation of maize plants with *Fusarium verticillioides* and this was an indication of the antioxidant defense response developed by maize against the pathogen. In another study conducted by Aydinoglu et al. (2023), APX and SOD activities did not change significantly in response to *Fusarium verticillioides* infection in *Nicotiana benthamiana* seedlings, whereas GR activity showed a statistically significant increase.

As a result of morphological, physiological and enzymatic analyses performed on two different maize lines infected with Fv infection, defense response against pathogen stress was determined in different parameters in both lines. Firstly, as a result of morphological observation, more severe symptoms (yellowing, necrosis) were determined in RI group compared to SI group. While there was a significant difference in final leaf length, third and fourth leaf cell length and phenolic content data in R group, there was no significant difference in S group. In addition, Fv growth was not observed in S group in third and fourth leaves planted on PDA, while it was observed in R group. Considering these results, gene identification and RT-qPCR analyses were performed in R maize line. Genes sRNAs and genes that are likely to play a role in Fv-maize phytopathosystem were selected using psRNATarget bioinformatics tool.

First, the hypothesis that Zm-miRNAs target Fv genes was tested. It was determined that Zm-mir398a miRNA targets 26S proteasome regulatory subunit Rpn-1 (Fv_06397) and cell wall integrity and stress response component (Fv_06829) genes in the Fv genome. One of the targets of miR398a, 26S proteasome regulatory subunit Rpn-1, is a part of the Ubiquitin–proteasome system (UPS). UPS provides targeted destruction of proteins involved in various pathways in the cell and the elimination of misfolded or damaged proteins. The 26S proteasome complex is at the center of the UPS system and is a multi-subunit structure. Rpn1 is one of the non-ATPase rpn proteins of this complex. The existence of this system has been demonstrated in

animals, plants and fungi [Staszczak et al., 2021]. In the annotation study conducted by Sasserone et al. (2020), it was found that the RPN-1 subunit of the 26S proteasome of the *Fusarium oxysporum* strain had 99% similarity to a sequence called FOP28-B01. The FOP28-B01 sequence is similar to a protein belonging to the nudix hydrolase family, and nudix hydrolases have been reported as virulence factors in plant pathogens such as oomycetes, bacteria, and fungi. In a study by Yao et al. (2012), it was shown that RPN1a mutant *Arabidopsis* plants became more susceptible to the fungal pathogen *G. cichoracearum* and to virulent and avirulent bacterial Pto DC3000 strains. In a study by Vladimir Brukhin et al. (2005), RPN1a was found to be a lethal gene for embryogenesis in *Arabidopsis*. As a result of these findings, it was shown that RPN1a plays a role in innate plant immunity and that this gene is also found in *Fusarium* species. Another target of mir398a, the Cell wall Integrity and Stress Response Component gene, plays a role in the regulation of cell wall integrity and stress responses in *Saccharomyces cerevisiae* via the protein kinase C1- Mitogen-Activated Protein Kinase1 (PKC1-MPK1) pathway. In the study, Wall Integrity and Stress Response Component 1 (WSC1), WSC 2 and WSC3 genes were identified. The identification of WSC genes in this pathway makes them important for cell wall integrity [Verna et al., 1997].

Various studies have been conducted on the stress response of mir398a under biotic and abiotic stress. Jagadeeswaran et al. (2009) reported that miR398 in *Arabidopsis* plant was down-regulated under abiotic (ozone fumigation and salt stress) and biotic (*Pseudomonas syringae*) stress and that the genes it targeted were up-regulated. This study shows that mir398 is important in biotic and abiotic stress responses.

As a result of RT-qPCR analysis within the scope of this thesis study, while Zm-mir398a did not change compared to the control group, Fv_06397 and Fv_06829 genes increased 3-fold and 152-fold, respectively, which are statistically significant. The increase in these genes shows the virulence of Fv and the effective functioning of its own defense system.

Palmitoyl coenzyme A (Palmitoyl CoA), one of the genes targeted by Zm-mir528a miRNA in the Fv genome, is an acyl-CoA thioester involved in β -oxidation and is transported to the mitochondrial matrix by the carnitine transport system. It is also used as a substrate by taking part in sphingosine biosynthesis [Goodridge et al., 2024]. No study was found in the literature on palmitoyl CoA hydrolase. In a study conducted

by Fabio Gherlone et al. (2024), it was determined that the palmitoyl CoA ligase gene was associated with the FUM16 gene, which plays a role in the self-protection mechanism against FB1 fumonisins in *Fusarium verticillioides*, through analyses and in vitro enzyme tests. This shows the importance of Palmitoyl CoA genes for *Fusarium*. In the literature for Zm-mir528a, a study by Manja Božić et al. (2024) found that zm-528a miRNA regulates calcium-dependent protein kinase 27 (CDPK27). CDPKs are known to play an important role in the regulation of stress response in plants. In addition, it was determined that zm-528a miRNA plays a role against cold stress in 5-day-old maize seedlings. In this study, while the transcription level of zm-miR528a decreased by 89% in RI tissue compared to RC which is not significant, Fv_02271, showed a statistically significant increase of 129%. These results indicate that Fv_02271 is not a direct target of Zm-mir528a, but its expression level is significantly increased in the interaction with maize, indicating that its self-protection mechanism is active.

The target of Zm-mir1432 was determined as sphingolipid delta-4 desaturase (Fv_03105). Sphingolipids are one of the important components of the cell membrane. No study was found in the literature specifically related to sphingolipid delta-4 desaturase. However, in a study, a bond formation in the synthesis of Fusarisude, a fungal sphingolipid, was catalyzed by the enzyme $\Delta 10(E)$ -sphingolipid desaturase ($\Delta 10(E)$ -SD). This compound is an immunosuppressive and medicinal potential compound used in the treatment of liver damage and colitis. $\Delta 8(E)$ -SD found in *Fusarium graminearum* has 55.7% amino acid similarity with $\Delta 10(E)$ -SD. This enzyme also increases the survival rate of fungi under stress conditions such as high salt concentration and low temperature [Tian et al., 2015]. Zm-mir1432 is known to play a role in the defense response in plants. In the study conducted by Wang et al. (2023), corn seeds were treated with *Trichoderma harzianum* T28, a beneficial fungus naturally found in soil, and its role in the development of resistance against the maize pathogen *Cochliobolus heterostrophus* infection was investigated. In this triple interaction, mir1432 is one of the differentially expressed miRNAs and was up-regulated. In another study conducted by Li et al. (2021), it was found that when mir1432 was expressed in ovaries against pathogen attack in rice, disease resistance decreased, and when it was down-regulated, disease resistance increased. When the expression of a rice gene targeted by mir1432 (LOC_Os03g59790) increased, disease

resistance increased, but grain yield decreased. This suggests that mir1432 fine-tunes the defense response and crop yield in rice and plays an important role in their regulation. As a result of RT-qPCR performed in this thesis study, the transcription level of Zm-miR1432 in RI tissue decreased by 71% compared to the control which is not significant and sphingolipid delta-4 desaturase (Fv_03105), showed a statistically significant 22-fold increase. The increased expression level of sphingolipid delta-4 desaturase supports Fv survival and virulence. The decreased level of Zm-miR1432 may indicate the formation of a defense response in maize according to the study of Li et al. (2021).

In this study, glutathione peroxidase (Fv_04790), chitin synthase 4 (Fv_01403), and Vacuolar fusion protein MON1 (Fv_08091) were selected as the target genes of Zm-mir159a. In the study conducted by Park et al. (2023), it was reported that glutathione metabolism has an important role in coping with oxidative stress induced by the host in *Fusarium graminearum*. In the same study, it was shown that glutathione mutant *Fusarium graminearum* fungus could not grow in minimal medium and was more sensitive to oxidative stress. Chitin, one of the basic components of the fungal cell wall, is synthesized by Chitin synthases (CHS). In the study conducted by Kong et al. (2012), CHS genes of *Magnaporthe oryzae* fungus were characterized in order to reveal the functions of CHS genes in causing disease. As a result of this study, it was found that mutant chs1 and chs7 genes reduced fungal virulence, CHS1 played an important role in spore formation, which is the basic process of the infection cycle, and the chs6 mutant reduced hyphal growth. Wang et al. (2003) showed that in cases where the Mon1 gene was mutant in yeast, vacuoles did not fuse and a tethering/docking stage was required. In a study conducted by Chen et al. (2021), Zmir159a, which targets these genes, was shown to be up-regulated in rice variants resistant to *Magnaporthe oryzae*, which causes blast disease in rice, and to play an important role in the immune response. In a study conducted by Zhang et al. (2016), it was found that cotton plants target genes essential for fungal virulence (isotrichodermin C-15 hydroxylase (HiC-15) and Ca²⁺-dependent cysteine protease (Clp-1)) by delivering mir159 miRNA to the hyphae of the fungus *Verticillium dahliae*, a plant pathogen. This study is a study that reveals the role of mir159 in cross-kingdom RNAi. As a result of the experiments conducted within the scope of this thesis, the transcription level of Zm-miR159 in RI tissue was significantly increased

by 5-fold compared to the control, while the targets of zm-mir159, glutathione peroxidase (Fv_04790) and chitin synthase 4 (Fv_01403), showed a statistically significant increase of 12- and 402-fold, respectively. Vacuolar fusion protein MON1 (Fv_08091) decreased by 88% which was statistically significant.

The hypothesis that Fv-miRNAs target maize genes was tested. First, FvmiR8764 and its target zm_TIFY6 were investigated. The TIFY gene family was discovered in *Arabidopsis* and found to be a transcription factor found in land plants. The TIFY gene family consists of 4 subgroups. These are TIFY, JAZ, PPD and ZML. The TIFY subgroup contains only the TIFY domain. This gene family consists of 27 members in maize [Vanholme et al., 2007]. In the study conducted by Wang et al. (2020), the response of TIFY genes under drought stress in *Populus trichocarpa* roots was investigated. The response of PtrTIFY genes to drought stress was examined by RT-qPCR method and PtrTIFY2 was found to be downregulated. As a result of the experiments conducted within the scope of the thesis, Fv-miR8764 increased statistically significantly by 2214 times, while zm_TIFY6 decreased by 83% which is not significant.

Fv-miR1827 miRNA and its target 3-ketoacyl-CoA synthase were identified by in silico analysis. β -ketoacyl-CoA synthase (KCS), one of the enzymes that catalyze the biosynthesis of very long chain fatty acids (VLCFA), is of critical importance for the stress response of plants. ZmKCS proteins have been shown to be found in the plasma membrane, mitochondria, and chloroplasts [Bach et al., 2010]. In the study conducted by Chen et al. (2024), it was thought that the ZmKCS17 gene decreased by less than 10-fold under salt and drought stress and may play a key role in the stress response of maize. As a result of RT-qPCR experiments within the scope of this study, fv-miR1827 increased statistically significantly by 815 times, zm_KCoA decreased by 5651 times which is statistically significant. This interaction indicates that the cross-kingdom RNAi mechanism occurs in the maize-Fv phytopathosystem.

Fv-miR5855 miRNA and its target Putative receptor protein kinase CRINKLY4 (CR4) were identified. The CR4 gene, a member of the receptor protein kinase family, was first identified in maize. It is known that the CR4 gene plays an important role in leaf development and epidermal cell differentiation. In *cr4* mutant maize plants, leaves were shorter and curled, and epidermal cell shapes were abnormal and tumor-like structures were observed [Gandhi et al., 2023]. In addition, in a study conducted by E-

Zereen & Ingram, (2012), where CR4 played a role in biotic stress response, it was determined that mutant *cr4 Arabidopsis* leaves were susceptible to *Botrytis cinerea* infection. As a result of RT-qPCR performed in this thesis study, Fv-miR5855 increased statistically significantly by 22000 fold, while zm_CRINKYL4 did not change.

Finally, Fv-miR7214 miRNA and the RNA-dependent RNA polymerase (zm_RDRP) genes were identified. RDRPs are known to play a role in post-transcriptional gene silencing and defense in plants. They catalyze the dsRNA synthesis required for gene silencing via the RNAi mechanism [He et al., 2009; Hunter et al., 2016]. In the study conducted by He et al. (2009), it was revealed that ZmRDRP1 gene plays an important role in the defense response of maize against Sugarcane Mosaic Virus (SCMV). Mutant *ZmRdRP1* gene was silenced and transgenic plants were reported to be more susceptible and showed severe disease symptoms than wild type plants. These results show that RDRP gene has an important role in the fight against pathogens of maize. As a result of the experiments conducted within the scope of this thesis study, fv-miR7214 increased statistically significantly by 3400 fold, zm_RDRP decreased by 71% which was not statistically significant.

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PUBLICATIONS AND PRESENTATIONS FROM THE THESIS

Kızılboğa S., Aydınoglu F., (2024), “Cross Kingdom RNA-Exchange Between *Fusarium verticillioides* and *Zea mays*”, Gebze Technical University Graduate School of Natural and Applied Sciences 8th Graduate Research Symposium, Kocaeli, 30 May - 31 May.