

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

**INVESTIGATING RNA INTERFERENCE, CROSS-KINGDOM
RNA EXCHANGE AND ANTIOXIDANT RESPONSE IN NICOTIANA
BENTHAMIANA-FUSARIUM VERTICILLIOIDES
PHYTOPATHOSYSTEM**

ASLIHAN KULOĞLU
A THESIS SUBMITTED FOR THE DEGREE OF
MASTER OF SCIENCE
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

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THESIS SUPERVISOR
ASSOC. PROF. DR. FATMA AYDINOĞLU

GEBZE

2023

T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

**NİCOTİANA BENTHAMİANA-
FUSARIUM VERTİCİLLİOİDES
FİTOPATOSİSTEMİNDE RNA
İNTERFERANS, TÜRLER ARASI RNA
DEĞİŞİMİ VE ANTİOKSİDAN TEPKİSİNİN
İNCELENMESİ**

ASLIHAN KULOĞLU
YÜKSEK LİSANS TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

DANIŞMANI
DOÇ. DR. FATMA AYDINOĞLU

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2023



YÜKSEK LİSANS JÜRİ ONAY FORMU

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SUMMARY

Fusarium verticillioides is an important mycotoxigenic fungus in phytopathogens. And it is crucial for sustainable agriculture to employ effective and long-term strategies for managing *F. verticillioides*. RNA interference (RNAi) has emerged as plant natural defence mechanism during the plant pathogen interaction. In this research, the *F. verticillioides*-*N. benthamiana* phyto-pathosystem was utilized to investigate the mechanism of RNAi and the molecular control of the plant immune response to *F. verticillioides*. 5-6 leaves stage *N. benthamiana* plants were treated with 10^{10} spores/ml *F. verticillioides* inoculum. The control group plants were treated with water instead of spore inoculum. After 14 days inoculation, fully developed 4th and 5th leaves were harvested for the analysis of the antioxidant enzyme activity, total phenolic content, total protein content, microscopic observations and RNA isolation. For identifying the genes involved in the RNAi process and cross-kingdom RNA exchange during pathogenicity, the sequences of microRNAs, their predicted mRNAs, and long non-coding RNA were obtained from the miRBase and pathogen-host interaction (PHI-base) databases, respectively. The psRNATarget was used to construct the competitive endogenous RNA (ceRNA) regulatory network of miRNA, lncRNA, and mRNA, and qRT-PCR was utilized to evaluate their gene expression levels.

As a result, against *F. verticillioides* in *N. benthamiana*, statistically significant results were obtained in total phenolic and total chlorophyll content, glutathione reductase enzyme activity, microscopic observations, and the expression levels of IPS1, miR172, and AP2 which are part of the ceRNA regulatory network. This work was supported by TUBITAK project number 221O321.

Key Words: RNAi, miRNAs, ceRNA, Plant-Pathogen Interaction, Cross-Kingdom RNA Exchange.

ÖZET

Fusarium verticillioides, fitopatojenler içinde önemli bir mikotoksijenik mantardır. Sürdürülebilir tarım için *F. verticillioides* ile mücadele etmek için etkili ve uzun vadeli stratejiler kullanmak çok önemlidir. RNA interferansı (RNAi), bitki-patojen etkileşimi sırasında bitkinin doğal savunma mekanizması olarak biyotik strese etkili rol oynamaktadır. Bu araştırmada, bitkilerdeki RNAi mekanizmasını ve *F. verticillioides*'e karşı bitki bağışıklık cevabının moleküler seviyede araştırmak için *F. verticillioides*-*N. benthamiana* fito-patosistemi kullanıldı. 5-6 yaprak evresindeki *N. benthamiana* bitkileri, 10^{10} spor/ml *F. verticillioides* inokulumu ile muamele edildi. Kontrol grubu bitkileri, spor inokulumu yerine su ile muamele edildi. İnokulumdan 14 gün sonra, antioksidan enzim aktivitesi, toplam klorofil tayini, toplam fenolik içerik tayini, toplam protein içeriği, mikroskopik gözlemler ve RNA izolasyonu analizi için tam gelişmiş 4. ve 5. yapraklar hasat edildi. Patojenite sırasında RNAi mekanizmasında ve türler arası RNA değişiminde yer alan genlerin belirlenmesi için, mikroRNA(miRNA)' ların ve tahmin edilen hedef genlerinin(mRNA) ve uzun kodlamayan RNA(lncRNA)' ların dizileri miRBase ve patojen-konak etkileşimi (PHI-base) veri tabanlarından elde edildi. miRNA, lncRNA ve mRNA'nın rekabetçi endojen RNA (ceRNA) düzenleyici ağını oluşturmak için psRNATarget kullanıldı ve gen ekspresyon seviyelerini değerlendirmek için qRT-PCR kullanıldı.

Sonuç olarak, *N. benthamiana* bitkisinde *F. verticillioides*'e karşı; toplam fenolik ve toplam klorofil içeriği, glutathione reductase enzim aktivitesinde, mikroskopik incelemelerde ve ekspresyon seviyeleri ölçülen ceRNA düzenleyici ağında yer alan IPS1, miR172, ve AP2'de kontrol grubuna göre istatistiksel önemli bulgular elde edilmiştir. Bu çalışma, TÜBİTAK 221O321 numaralı proje ile desteklenmiştir.

Anahtar Kelimeler: RNAi, miRNAs, Rekabet Endojen Küçük RNA, Bitki-Patojen Etkileşimi, Türler Arası RNA Geçiş

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LIST OF ABBREVIATIONS AND ACRONYMS

<u>Abbreviations</u> <u>and Acronyms</u>	<u>Explanations</u>
°C	: Celsius degree
g	: Gram
ml	: Mililiter
mM	: Milimolar
M	: Molar
APX	: Ascorbate peroxidase
AP2	: Apetala2
BSA	: Bovine serum albumin
CAT	: Catalase
Cdna	: Complementary DNA
Cerna	: Competitive endogenous rna
DCL	: Dicer like protein
ddH ₂ O	: Double distilled water
DNA	: Deoxyribonucleic acid
dNTP	: Deoxynucleotide triphosphate
EDTA	: Ethylene Diamine Tetra Acetic Acid
EtOH	: Ethyl Alcohol
GR	: Glutathione reductase
GSSG	: Glutathione Disulfide
IAA	: Indole acetic acid
IPS1	: Induced phosphate starvation1
L	: Liter
Lx	: Lux
LRKs	: Extracellular leucine-rich repeats
MAMPs	: Microbe-associated molecular patterns
Mg	: Miligram
Min	: Minute
Mirna	: Micro RNA
Mrna	: Messenger RNA

Nt	: Nucleotide
POX	: Peroxidase
PHO2	: Phosphate Overaccumulator2
PTI	: Pathogen-associated molecular pattern-triggered immunity
PVP	: Polyvinylpyrrolidone
qRT-PCR	: Quantitative real time polymerase chain reaction
RISC	: RNA-induced silencing complex
RLKs	: Transmembrane receptor-like kinases
Rpm	: Revolutions per minute
Srna	: Small RNA
SOD	: Superoxide dismutase
WRKY	: Transcriptional reprogramming involving

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1. INTRODUCTION

Plants are stationary because their roots anchor them to the soil, but they have a variety of defense and immune systems to deal with the difficulties they face. And most humans and other living organisms' lives spend on plants somehow. It is important for all living to profit from plants in the best possible ways in the current conditions, where a changing climate and a growing population have a negative impact on agricultural production. While there are already many various plant diseases, researchers are also diagnosing new plant diseases [Almeida et al., 2019]. Plant scientists have shown great interest in studying the mechanisms of plant natural immunity against pests and pathogens in order to get the maximum benefit from plants. However, a greater knowledge of the causes and mechanisms underlying plant-pathogen interactions and the deployment of more effective resistance measures are still required to reduce agricultural losses.

RNA interference (RNAi) has gathered the interest of scientists worldwide for this aim. To silence the genes, the RNAi technique combines the actions of microRNAs (miRNA) and small interfering RNAs (siRNA). In addition to expanding our understanding of how genes are produced and function, the discovery of RNAi has created new potential for cutting-edge plant biotechnology. It has been discovered that RNAi can influence plant growth and development, circadian rhythm, and other crucial physiological functions. RNAi has also been employed successfully to change hormone signal transduction and pathogen attack [Kuo and Falk, 2020]. A paradigm for the synthesis of synthetic RNAi that can suppress the activity of target genes was devised based on these discoveries. RNAi has been successfully applied to the management of diseases and defense against pest infestations [Eamens et al., 2008], in addition to supporting a wide range of developmental processes. There have been identified three natural RNA silencing mechanisms in plants: 1) cytoplasmic siRNA silencing, 2) miRNA-mediated silencing of endogenous mRNA, and 3) Methylation of DNA. These procedures involve the cleavage of double-stranded RNA by Dicer into little (21-26 nucleotide) RNA fragments.

When infecting their hosts, the numerous plant-pathogenic *Fusarium* species produce a huge range of secondary metabolites. Their activities in an infected plant

may result in the inhibition of root or shoot growth, the inhibition of seed germination, leaf colors changes such as chlorosis, cell death, and the repression or activation of plant defense responses. These phytotoxic effects of *Fusarium* metabolites have been seen in a multitude of plant types. The important *Fusarium* species member *Fusarium verticillioides* has a significant impact on agricultural product losses. Fumonisin, which is generated by *Fusarium verticillioides*, induces programmed cell death comparable to apoptosis and may boost the virulence of the fungus on particular plants. Similar to fumonisin, the other mycotoxins, which are also secondary metabolites of *Fusarium* infections, accumulate mostly in cereal grains such as corn, rice, and wheat. Thus, contaminated plants pose a risk to humans and animals who consume them.

1.1. Aim, Contribution, and Content of the Thesis

As the plant's own immune system, RNA interference is the natural and environment-friendly method of plant disease control. In recent years, evidence has accumulated that miRNA and lncRNA play a regulatory role in the plant development and adaptation of plant biotic stress tolerance [Nejat and Mantri, 2018]. Since miRNAs and lncRNA interactions regulate the expression of many genes, RNA-mediated plant immunity researches aim to learn more in the coming years about immune mechanisms in plants against biotic stress. In order to contribute to this objective, the plant-pathosystem of *Nicotiana benthamiana* and *Fusarium verticillioides* was examined for disease infection and fungal development. In this context, a competitive endogenous RNA (ceRNA) regulatory network consisting of miRNAs, mRNAs and lncRNAs was computed to determine how the plant immune system is regulated during plant-pathogen interaction. While most sRNAs have a role endogenously, some of them can move across organismal boundaries between hosts and microbes and silence genes in trans-in-interacting organisms [Huang et al., 2019]. Besides the RNAi role in the pathogenicity, cross-kingdom RNA exchange between *Nicotiana benthamiana* and *Fusarium verticillioides* was investigated in this study.

2. GENERAL INFORMATION

2.1. General Information of Tobacco (*Nicotiana benthamiana*)

As a model organism for plant biology research, tobacco plants have made significant contributions to our knowledge of plant-pathogen interactions. *Nicotiana benthamiana* is a member of the genus *Nicotiana* and the family *Solanaceae*, which contains at least 3,000 known species, the bulk of which belong to the genus *Solanum*. Due to its susceptibility to a broad spectrum of biotic stress agents, such as fungi, bacteria, and viruses, *Nicotiana benthamiana* is used as a model for phytopathological studies to accelerate the study of the molecular mechanisms behind plant pathogenicity.

2.2. General Information of *Fusarium verticillioides*

The *Fusarium* rank was initially described by Link in 1809 and now it is well-known for being a pathogen of humans, animals, and plants as they produce secondary metabolites that lead to toxicity after humans and other animals consume infected food. Numerous plants suffer from at least one *Fusarium*-related disease. The prevalence of *Fusarium verticillioides* that belongs to the fungus genus is widespread throughout the world, especially where maize is cultivated. The nomenclature of this taxon has been the subject of some debate, with some taxonomists referring to it as *F. moniliforme* and others as *F. verticillioides*. It harms cereals such as maize, rice, sorghum, and wheat (Table 2.1).

F. verticillioides life cycles are asexual or include both asexual&sexual stages. The asexual stages of *F. verticillioides* are derived from macroconidia (asexual spores). The sporodochia are created by phialides, which are masses of conidium-producing cells packed together to form cushion-shaped lumps. Macroconidias are generally canoe-shaped, clear (hyaline), and septate (Figure 2.1). The plant infection cycle begins with the development of seedlings from soil-contaminated seeds or when spores are transferred to plants by wind or rain, particularly blooming

structures for head blights and ear rots. Under warm and high humidity conditions, *F. verticillioides* is able to form an anamorphic stage on crop-surface mycelia (Figure 2.2). This occurs when haploid hyphae from the teleomorphic stage combine (anastomose).

Table 2.1: The taxonomy of *Fusarium verticillioides*.

Scientific Classification	
Kingdom:	Fungi
Division:	Ascomycota
Class:	Sordariomycetes
Order:	Hypocreales
Family:	Nectriaceae
Genus:	<i>Fusarium</i>
Species:	<i>F. verticillioides</i>

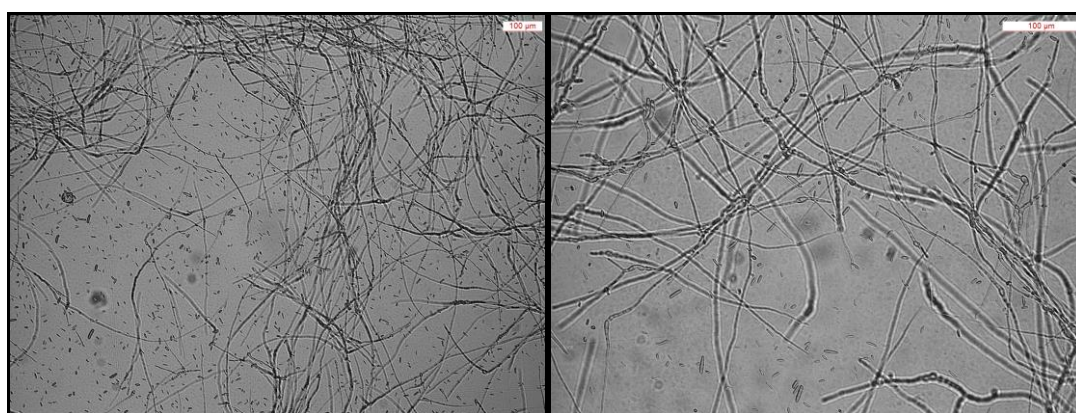


Figure 2.1: The microscopic photo of *F. verticillioides* structures at 10X magnification.

Sexually compatible nuclei brought together by anastomosis between different mycelia do not fuse immediately. Instead, they generate dikaryotic hyphae with pairs of sexually compatible haploid nuclei that couple off during mitosis. This proliferative stage is designed to increase the number of spores produced by a single anastomosis. Around the dikaryotic hyphae, haploid hyphae form an ascus or fruiting structure. Meiosis and sexual nucleus fusion occur in the terminal branches of dikaryotic hyphae to produce haploid ascospores. Ascospores are forcefully ejected

from the ascus and transferred to plant surfaces via wind and precipitation. Both asymptomatic and symptomatic growth are possibilities when it arises as an endophyte in the vegetative and reproductive tissues of maize plants. Symptomatic growth is more common. During asymptomatic growth, *F. verticillioides* can be found in the gaps between plant cells, where it does not cause any damage to the plant cell and produces only trace levels of toxins. When symptomatic growth occurs, the fungus swiftly multiplies and either produces stalk and ear rot later in the season or seedling blight in the early stages of development. Both of these conditions are undesirable. The fungus is able to produce enormous quantities of ascospores at this time because it has transitioned from asexual to sexual reproduction. This is especially true in plants that have wounds in the stalk or ear that were caused by pests.

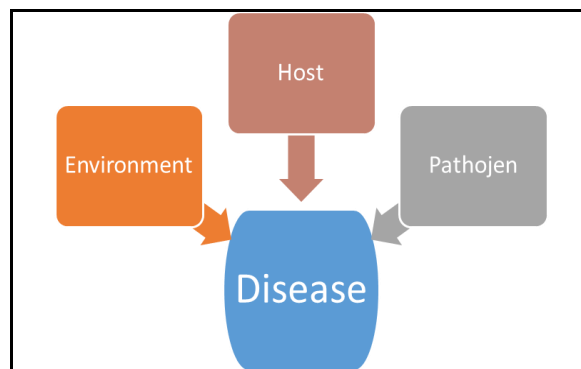


Figure 2.2: Illustrating the interaction of host, pathogen and environment triangle.

2.3. Plant-Pathogen Molecular Interactions

Plants have a basic immune system that is able to recognize conserved elements of bacteria and fight them off, in addition to built-in defense mechanisms against diseases like strengthening plant cell walls. Microbe-associated molecular patterns, also known as MAMPs, are recognized by plants thanks to the activity of transmembrane receptor-like kinases (RLKs) that include extracellular leucine-rich repeats (LRRs). MAMPs are molecules that are present in all microorganisms, such as the chitin or lipopolysaccharides that are found in the cell walls of fungi. MAMPs are known to play an important role in the transmission of infectious diseases. In response to the identification of MAMPs, mitogen-activated protein kinase (MAPK)

cascades and transcriptional reprogramming involving WRKY transcription factors are triggered.

In recent years, the term "basic immune response" has been superseded by the term "immunity activated by pathogen-associated molecular patterns" (PTI). By sending effector proteins to plant cells that block the actions of receptor or signaling host proteins, pathogens can sabotage the basic immune response. A gene-for-gene response that frequently involves intracellular nucleotide-binding LRR (NB-LRR) proteins that sense and either directly or indirectly block the effects of the pathogen effector proteins allows plants to function to effector proteins. Effector-triggered immunity is the name given to this resistance (ETI). Plant pathogens can evolve to change or lose effector proteins or use alternative effectors in the continuing battle between plants and diseases. PTI and ETI may have similar downstream effects, such as ion transport across the plasma membrane, an oxidative burst (OB), the production of nitric oxide (NO), and/or the activation of calcium-dependent and mitogen-activated protein kinases. The formation of an OB is a very speedy (within minutes) respond to a pathogen attack in plants. Reactive oxygen species (ROS) are made by chloroplasts, mitochondria, and the cell surface. The OB can increase cell-wall cross-linking and lignification or directly kill the pathogen to improve resistance. In the hypersensitive-associated defensive response, cell death may also be triggered by ROS. Salicylic acid (SA) synthesis in diseased tissue determines the ability to combat pathogens that feed on living host tissue, also known as biotrophic pathogens. However, signaling pathways mediated by ethylene (ET) and jasmonate (JA) enhance plant defense against necrotrophic diseases that cause cell death in the host.

A large number of plant host genes are activated during a defense response, including genes for pathogenesis-related (PR) proteins and enzymes involved in the production of antimicrobial compounds (phytoalexins). Gene expression is mediated by the activation of transcription factors to bind to the promoter regions of active genes. Plant responses to pathogen infection are typically regulated by DNA-binding proteins with WRKY domains, which have a WRKYGQK sequence followed by a zinc-finger binding motif.

2.4. RNA-Mediated Plant Immunity Response

As many protein regulators were discovered in both prokaryotes and eukaryotes, the possibility that RNA molecules could act as regulators was largely neglected. But investigation on RNA regulators that influence transcription and, more vitally, translation, particularly in eukaryotes, has exploded recently. This new discipline resulted from two discoveries: the early 1990s discovery of miRNAs and the late 1990s discovery of the mechanism known as RNAi.

A miRNA's functional form is typically 21 or 22 nucleotides(nt) in length, but it can also be between 19 and 25 nt in length. From a larger RNA transcript (known as a pri-miRNA, short meaning "primary") that carries a hairpin-shaped secondary structure, these small RNAs are produced via two RNA cleavage processes. Pre-miRNA is created from the stem-loop by the first cleavage, while mature miRNA is formed from the pre-miRNA by the second. The sequence between bases 2 and 9 of the 22 nt miRNA often serves as the base pairing between the miRNA and the target RNA. This region has the highest complementarity, making it the most effective for locating potential target genes. The two cleavage reactions required to generate the miRNA from these primary transcripts are mediated by two distinct RNases. One is Dicer, and the other one is Drosha that specifically required for miRNA processing Figure 2.3 from Saleem et al., 2022.

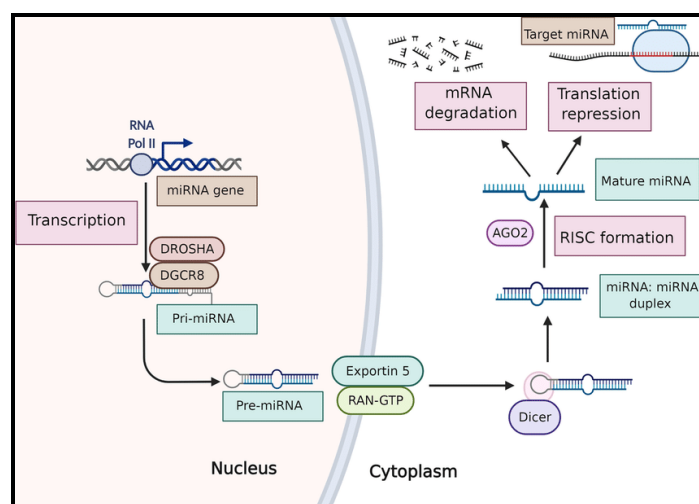


Figure 2.3: Overview of plant endogenous RNAi mechanism which is included miRNA biogenesis and target mRNA degradation.

Knowing where miRNAs are located in tissue will be very helpful in understanding their functions. Since the qualities of plants such as stem elongation, leaf and flowering morphology, physiological and biochemical processes, resistance to biotic and abiotic stresses and crop yield during the growth and development stages of plants throughout their life cycles directly affect agricultural production, it has been the focus of scientific research for sustainable agriculture. In recent years, miRNA-based RNAi mechanism has been started to be used in changing plant structure. And as a result of these studies, many miRNA genes that regulate plant structure have been characterized. These miRNAs are engaged in numerous growth and development-related activities, including cell formation, hormone signaling, leaf, stem, and root growth, organ polarity, and improvement of structural features [Aydınoglu and Aktuğ, 2017; Eldem et al., 2013].

2.5. Competitive Endogenous RNAs Regulatory Network

Recent years have seen various findings that challenge the core dogma of molecular biology because to advancements in genome sequencing. It has become clear that much of the DNA that does not code for proteins really codes for a wide variety of functional RNAs that play crucial regulatory roles. Following the discovery of miRNAs and lncRNA interactions, the complexities of gene regulation grew logarithmically. The formation and synthesis of miRNAs is discussed in the previous chapter and about lncRNAs are defined as any expressed RNA longer than 200 nt that does not encode a protein but according to last studies have shown that miRNAs affect gene expression by interacting with lncRNAs [Seo et al., 2018]. Enhancer sequences can regulate the expression of genes in cis, while genes that encode transcription factors (which can either activate or repress gene expression) or RNA-binding proteins can regulate gene expression in trans. Recent research has shown that both coding and noncoding RNA molecules have the ability to regulate gene expression in trans by acting as sponges for microRNAs [Das et al., 2020; Tao et al., 2019]. These findings were published in a number of scientific journals both for human and plants diseases. The majority of gene regulators are made up of RNA molecules that compete with one another. These molecules are referred to collectively as "competing endogenous RNAs" (ceRNAs). The discovery of this

level of gene control can provide an explanation for the association between the size of the genome and the increase in the complexities of the species. MiRNAs exert their regulatory influence on the genes that they target by binding to complementary sequences in the 3' untranslated (UTR) region. A method that may be taken in general is to construct miRNA sponges or target mimics that contain numerous binding sites for a miRNA of interest. This is one way to acquire a better understanding of the function that these miRNAs play in biological systems. The laboratory run by Phil Sharp was the first to successfully establish the concept of competitive target inhibition of miRNAs within a cell [Lucero et al., 2020]. One is able to generate a loss-of-function phenotype in cell culture by reducing the number of miRNAs that are available to interact with an mRNA of interest using this method. At the same time, it was discovered that miRNA sponges can arise in their natural environment. It was observed that plants had their own endogenous miRNA sponge, which was found to control a miRNA-mediated response to environmental stress [Zorrilla et al., 2007]. The sponge that was discovered was a non-coding RNA called Induced by Phosphate Starvation 1 (IPS1) that had a binding site for miR399, which is a miRNA that is induced by phosphate starvation. Due to the fact that the IPS1 transcript has mismatched nucleotides, binding of the miR399 does not result in the destruction of the IPS1 transcript at the binding site, but rather results in the sequester of miR399 from other target transcripts. Therefore, IPS1 is able to efficiently operate as a sponge, thereby reducing the number of miR399 molecules that are accessible for regulating the Phosphate Overaccumulator 2 (PHO2) mRNA. This mutant carries a mutation in the PHO2 gene, which codes for a ubiquitin-conjugating enzyme (UBC24). This mutation causes a decrease in the number of length transcripts produced by the gene [Zorrilla et al., 2007]. This mutation is responsible for the development of symptoms associated with inorganic phosphate (Pi) poisoning. These symptoms are the result of increased Pi uptake, facilitated Pi translocation from roots to shoots, and impaired Pi remobilization from old to young leaves and PHO2 levels drop at the beginning of phosphate deficiency because of downregulation [Pegler et al., 2020; Baksa et al., 2015]. In the absence of phosphate, RNAs belonging to the IPS1 family become active. The latter can sequester miR399, which then leads to the stability and increased accumulation of PHO2, as well as a concurrent decrease in the amount of phosphate found in the shoots [Franco et al.,

2007]. To describe this method of preventing miRNA from doing its job, the scientists came up with the phrase "target mimicry" to describe it. As the first known example of RNAs naturally regulating themselves, the concept of target mimicry reveals hidden complexity in this system of regulatory interactions, figure: 2.4 [Sharma; 2022].

2.6. Cross-Kingdom RNA Exchange

The mechanism known as "cross-kingdom RNA exchange" allows some sRNAs to move between hosts and cooperating microorganisms or parasites to quiet target genes of the opposing party in trans, despite the fact that many sRNAs work endogenously in both plant and human systems. Plant-fungal interactions provided the first evidence of cross-kingdom RNAi. *B. cinerea*, a gray mold, silences host immune response genes in several hosts, including tomato and arabidopsis, by delivering a variety of sRNAs into plant cells [Islam et al., 2017]. By loading into Arabidopsis AGO1, which silences host genes such as MAPKs, cell-wall associated kinases, and genes involved in the generation of reactive oxygen species, it was experimentally proven that fungal sRNAs use host RNAi machinery (Weiberg et al., 2013). These fungus-specific sRNAs are a brand-new family of effector molecules that suppress host defenses. According to a genetic study, they are reliant on *B. cinerea*'s DCL1 and DCL2 in equal measure. The pathogenicity of the *dcl1/dcl2* double mutant strain has been significantly reduced because it can no longer manufacture sRNA effectors [Huang et al., 2017].

Cross-kingdom sRNA transport from microbes to hosts is not restricted to eukaryotic pathogens that encode RNAi machinery. The symbiotic bacterium *Rhizobium* sends transfer RNA (tRNA)-derived sRNA fragments (tRFs), 18–24 nt cleavage products from mature tRNAs, into soybean cells. Just like in *Botrytis* and *Verticillium*, *Rhizobial* tRFs are also loaded into host AGO1 to induce the silencing of plant nodulation-related genes [Ren et al., 2019]. Additionally, the parasitic plant *Cuscuta campestris* can send miRNAs into host plants to silence host genes involved in defense responses against *C. campestris* [Shahid et al., 2018]. In figure (2.4) [Huang et al., 2019]; (Figure 2.4A) Cross-kingdom RNAi between plants and fungal pathogens works both ways. The secreted EVs, mostly exosomes, carry the host-

made sRNAs to the pathogens, where they stop the expression of virulence genes in the fungal pathogen. Pathogen-made sRNA molecules can act as effectors that move into host cells and use the host's AGO protein to turn off the host's defense genes, making it possible for the pathogen to invade and take over. (Figure 2.4B–E) Host-made sRNAs or dsRNAs can be moved into the cells of parasitic nematodes (B), insect pests (Figure 2.4C), parasitic plants (Figure 2.4D), or symbionts (Figure 2.4E) and cause gene silencing in trans. Even though it is not known how sRNA gets from one kingdom to another, it was thought that extracellular vesicles may be a key part of cross-kingdom sRNA trafficking and that cross-kingdom RNAi may work both ways between plants and organisms that interact with them.

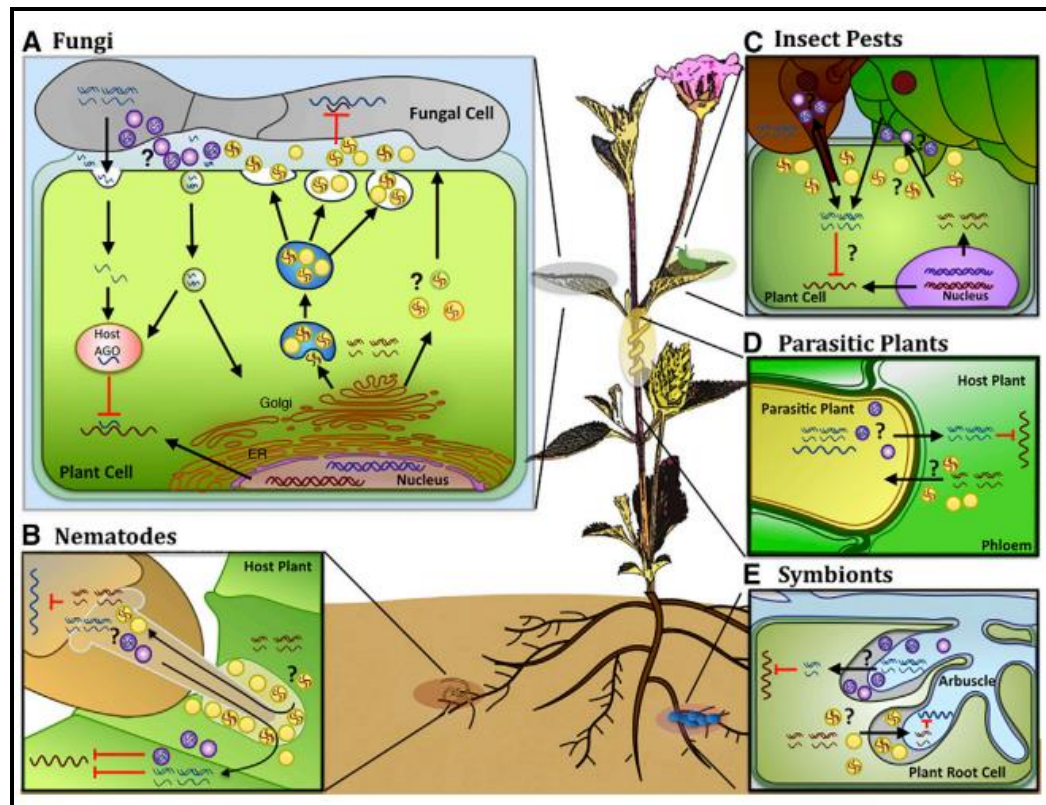


Figure 2.4: Cross-kingdom sRNA trafficking between plants and other organisms.

3. MATERIAL AND METHODS

3.1. Plant Material and Growth Conditions

3.1.1 Plant Material and Growth Conditions

The *Nicotiana benthamiana* were used as plant material in this thesis's experiment. To sterilize the *Nicotiana benthamiana* seeds, 70% ethanol was used three times shaking the seeds for five minutes in an eppendorf tube, and they were rinsed three times with double-distilled water. The pods where the seeds would be planted were cleaned by washing with hydrogen chloride and rinsed with double distilled water. Soils were weighed and filled in autoclave bags and then sterilized at 121°C for 20 minutes in the autoclave. The sterilization of all materials to be used in the experiment was meticulously taken care of and then sterile seeds were placed on the sterile soils with the help of a micropipette with cut end tips (Figure 3.1) (Figure 3.2).



Figure 3.1: *Nicotiana benthamiana* seeds were sowed into pots that include sterile soil and sealed with plastic film to prevent loss of soil moisture.



Figure 3.2: The germinated *Nicotiana benthamiana* seeds after 4-5 days of sowing.

3.2. Fungal Isolate and Growth Conditions

Fungal isolates used in this study were provided from Republic of Turkey Ministry of Agriculture and Forestry Protection Director Center Research Institute (Ankara). The diagnosis of isolates was performed using molecular and biological methods and prepared for monospore culture. *Fusarium verticillioides* is the fungal phytopathogen of this study. Fungal isolate used in these experiments has been sub-cultured to synthetic nutrient agar (SNA) medium in the petri dishes in order to increase the number of spores from monospore culture of stock *F. verticillioides* and incubated at 24-28 °C for two weeks. Why we use the culture of a single spore is because to maintain purity and pathogenicity and to permit consistent production of spore. And also to growth mycelium of the *F. verticillioides* we sub-cultured to PDA/B periodically. The recipe for PDB and PDA (Table 3.1):

Table 3.1: The PDB and PDA recipe.

Potato Dextrose Broth (PDB) 1L	Potato Extract Agar (PDA) 1L
200 gr peeled and sliced potatoes	200 gr peeled and sliced potatoes
20 gr dextrose	20 gr agar
1 L distille water	20 gr dextrose
	1 L distilled water

3.3. Inoculation of *Nicotiana benthamiana*

When *N. benthamiana* seedlings reached to 5-6 leaves approximately 4-5 in weeks, *F. verticillioides* inoculum was sprayed on plants for disease infection. The spores of two-week-old 1st sub-culture of *F. verticillioides* cultivated in SNA medium were collected from the surface of the petri dish by adding 2 ml ddH₂O and filtered by sterile filter paper and then transferred into an eppendorf tube. The density of conidial suspensions were counted using haemocytometer under the microscope

connected to a camera (Leica DM250 Fluorescence microscope) [Gregory, 1983; Purwati et al., 2008]. The spores were counted with the help of ImageJ software (www.imagej.nih.gov) and inoculum was diluted 10^6 conidia/ml and stored in 15% glycerol until further experiments. For spray inoculation treatment, 1-2 ml of spore stock inoculum from -80 °C were added into PDB erlenmeyer flasks and incubated at 28 °C at 100 rpm overnight. The fungal mycelia were removed using a sterile filter paper and spores density was adjusted to 10^{10} conidia/ml. All materials, except fungal culture, were sterilized or thoroughly cleaned with ethanol before use and the total experiments were conducted in the laminar flow cabin under sterile conditions. The plant was treated to spray inoculation according to Lichtenzveig (2006). The total volume of the inoculum was 24 ml and 2ml was sprayed under and above every leaf of each plant of inoculated group abaxial of each leaf was sprayed with 2ml inoculum. The plant was placed into water prior to inoculation to open the stomatas for the facilitation entrance of spores throughout to leaf. For the comparative analysis, the control (mock) group was sprayed with autoclaved water instead of inoculum. The non-inoculated group named as mock was used as a control, and the inoculated group named *F. verticillioides*-inoculated (FV) was the experimental group in this study. Each group had 12 plants. The plants were transferred to a plastic nylon greenhouse separately which had 90% humidity which helps to create a suitable environment for successful infection and placed in a growth room under conditions at 26°C (light) for 16 h and at 21 °C (dark) for 8 h with 70% relative humidity and 15.000 lux. The morphological response of the plants against to *F. verticillioides* case was observed every day for 14 days after post-inoculation.

3.4. Physiological Measurements

3.4.1. Determining Chlorophyll Content

The chlorophyll content of *Nicotiana benthamiana* seedlings in mock and Fv-inoculated groups grown in the plastic greenhouse was measured 14 days after post-inoculation. The SPAD-502 Plus (Konica Minolta) chlorophyll meter was used to measure the chlorophyll content. The measurements were obtained from the three parts of the same leaf and averaged. The mean value of each group was calculated

and the statistical significance between groups was determined by Student t-test by using Microsoft Excel 365 Program.

3.4.2. Measurement of Leaf Area and Petiole Length by ImageJ & Fiji Software

For measurement of the leaf area of *Nicotiana benthamina* seedlings grown in control and mock condition, ImageJ&Fiji software was used (last version from (<https://imagej.net/software/fiji/downloads>)). The instructions (Figure 3.3); A: File » Open » Select the image » Select the straight and measure a reference certain length to adjust the measurement scale » Analyse » B: Set scale clicked and known distance and unit of length were filled » Select Free Hand to click by one by all around the leaf and close the circle » Click ‘M’ for measurement results. All leaf area measurements were calculated based on ‘area’ column results. C: For the measurement of leaf petiole length (yellow highlighted part) the same way was followed but ‘straight line’ was used instead of polygon selection tool and calculated in programme.

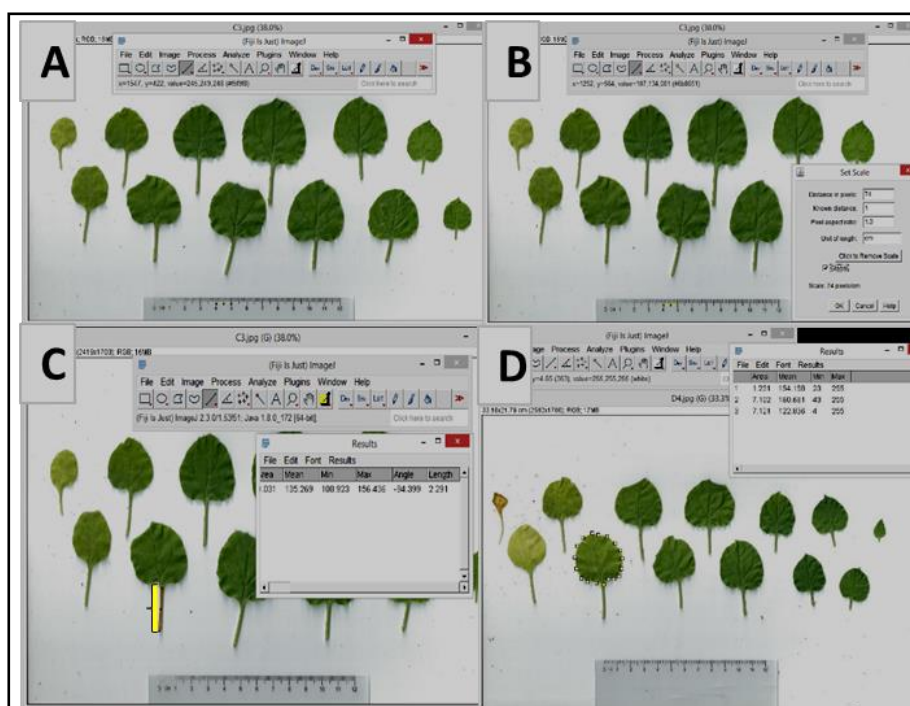


Figure 3.3: The instructions for the measurement of leaf area and petiole length by ImageJ&Fiji software originally.

3.5. Biochemical Measurements

3.5.1. Total Soluble Protein Extraction

For the total protein extraction, leaves of *N. benthamiana* seedlings grown in inoculated and mock condition were harvested 14 days later after inoculation. As a sample for the experiment, 3 cm² of leaf tissue was taken and immediately placed into 2 ml eppendorf tubes and fixed by liquid nitrogen. The tissue samples were stored at -80°C until analysis. Comparative and statistical analyses were conducted on a total of 10 samples from the inoculated and mock groups, comprising five biological and three technical replicates of seedlings. For the extraction, the leaf samples were taken from -80 °C and immediately placed into liquid nitrogen until homogenization. Three metal beads with a diameter of 4 mm were put into tubes, which was then placed in cold homogenizer blocks. The homogenizer machine was set at 25 seconds/25 frequencies and ran. When the homogenization was complete properly, the tubes were immediately placed back into liquid nitrogen. 1 ml of a 50 mM potassium phosphate extraction buffer (pH = 7.8), 1 mM EDTA, 2% PVP were added on the homogenized tissues. They were vortexed thoroughly and then they were centrifuged for 30 minutes at 10,000 rpm at +4 °C. Caring not to touch the beads, the supernatants were transferred to new sterile tubes and stored at -20 °C for future experiments. The total protein amount was determined according to the Bradford Assay [Bradford, 1976]. The concentration values for the standard curve for the Bradford test were modified from 0 mg/ml-1.2 mg/ml using a 2 mg/ml BSA (bovine serum albumin) stock solution. The sample dilution ratios ordered as 0 mg/ml, 0.2 mg/ml, 0.4 mg/ml, 0.6 mg/ml, 0.8 mg/ml, 1 mg/ml, and 1.2 mg/ml. On the cell culture plate were inserted 5 µl of diluted BSA solutions and 250 µl of Bradford reagent. For the blank measurement, 250 µl of Bradford reagent (SIGMA) was utilized, whereas 5 µl of sample and 250 µl of Bradford reagent were used for the protein assay. Each well was well stirred using a pipette. Ten minutes were spent incubating the samples. The standard curve was calculated after the absorbance value of the spectral scanning multimode reader (Thermo Scientific Varioskan Flash) was set to 595 nm.

3.5.2. Catalase (CAT) Enzyme Activity

Chance et al. [1955] devised a method for measuring catalase activity. For the experiment, 1.5 mL quartz cuvettes were used. 100 μ l of 200 mM H_2O_2 , 850 μ l of 50 mM phosphate buffer (pH:7), and 50 μ l of protein extract (2-10 g/ml) were used for the test. 900 μ l potassium phosphate extraction buffer and 100 μ l 200 mM H_2O_2 were used for the blank measurement. Spectrophotometer (Shimadzu, UV1280) was used to measure absorbance at 240 nanometer for 90 seconds. Aebi [1983] estimated the values calculation. 1 unit of catalase enzyme destroys 1M H_2O_2 to create water and oxygen. Statistical differences were evaluated by taking the mean of five biological and three technical replicates. Graph error bars were labeled as standard error. Excel 365's Student's t-test was used to compare the Mock and FV groups. The difference between the Mock and FV groups was significant when $P < 0.05$.

3.5.3. Peroxidase (POX) Enzyme Activity

The enzyme activity of peroxidase was measured using a method developed by Quesada et al. [1990]. Quartz cuvettes of 1.5 ml volume were utilized in the experiment. 880 μ l of 50mM Potassium phosphate, 20 μ l of leaf sample, 10 mM of H_2O_2 (50 μ l), and 0.02 % of dianisidine (50 μ l) were employed in each reaction. The blank included 50 mM (900 μ l) potassium phosphate, 10 mM (50 μ l) hydrogen peroxide, and 0.02% (50 μ l) dianisidine. In a spectrophotometer (Shimadzu, UV-1280), we monitored the quartz cuvettes' absorbance shifts at 460 nanometer for 1.5 minutes to see how different solutions affected their transparency. To measure POX enzyme activity, we followed Quesada et al, [1990]'s protocol. The amount of enzyme needed to oxidize 1 M of H_2O_2 is defined as 1 unit of the enzyme. Error bars on the graph represent the standard error, and the statistical differences were analyzed by averaging data from five biological and three technical replicates. The difference between the Mock and FV groups was analyzed using an Excel 365 Student's t-test, and the results were reviewed. If the P value for the difference between the Mock and FV groups was less than 0.05, the difference was deemed to be statistically significant.

3.5.4. Ascorbate Peroxidase (APX) Enzyme Activity

The APX enzyme activity was measured using the technique provided by Nakano and Asada [1981]. For the experiment, 1.5 mL quartz cuvettes were used. For the assay, 830 μ l of KP buffer, 50 μ l of H_2O_2 , 50 μ l of 0.5mM ascorbic acid, 50 μ l of 0.1mM EDTA and 20 μ l of protein were used. For the blank, 850 μ l of 50mM KP buffer, 50 μ l of 0.1mM EDTA, 50 μ l 0.5mM ascorbic acid and 50 μ l of 0.1mM H_2O_2 were used. The absorbance change of the samples was measured at 290 nm for 90 seconds. The enzyme activity was determined using method in Nakano and Asada [1981]. Ascorbate peroxidase uses substrate to catalyse the reduction of H_2O_2 . 1 unit of enzyme is described as the amount of enzyme required to oxidize 1 μ M of ascorbate. Error bars on the graph represent the standard error, and the statistical differences were analyzed by averaging data from five biological and three technical replicates. A Student's t-test conducted in Excel 365 was used to analyze the difference between the Mock and FV groups, and the results were discussed. If the P value for the difference between the Mock and FV groups was less than 0.05, then the difference was considered significant.

3.5.5. Glutathione Reductase (GR) Enzyme Activity

Glutathione reductase (GR) is a flavoprotein oxidoreductase that uses NADPH as a reducing agent to convert GSSG to GSH. GR enzyme activity measurement was tested according to protocol published by Çakmak et al. [2006]. For the experiment, quartz cuvettes (1.5 ml) were used. Assay medium included, 50 mM potassium phosphate and 0.1 mM EDTA buffer solution (880 μ l), 0.5mM GSSG (50 μ l), 0.12 mM NADPH (50 μ l), 20 μ l sample were prepared to use. For the blank measurement, 50 mM Potassium phosphate (850 μ l), 0.1mM EDTA (50 μ l), 0.5mM GSSG (50 μ l) and 0.12 mM NADPH (50 μ l) were prepared. Quartz cuvettes were placed on a spectrophotometer (Shimadzu, UV-1280) absorbance values were monitored for three minute to measure absorbance changes at 340 nm of each sample. According to Carlberg et al. [1975], the activity of the glutathione reductase enzyme was computed. 1 unit of the enzyme is defined as the amount of enzyme necessary to oxidize 1 M of NADPH in one min per g fresh weigh (min/gFW). The

statistical differences were computed by averaging five biological & technical replicates, and the error bars on the graph were labeled as standard error. The significance of the difference between the Mock and FV groups was calculated by using the Student's t-test in Excel 365. The difference between the Mock and FV groups was considered significant when the P value was less than 0.05.

3.5.6. Superoxide Dismutase (SOD) Enzyme Activity

The superoxide dismutase (SOD) enzyme experiment was conducted in accordance with Banowertz et al. [2004]. The approach was based on the inhibition of nitro blue tetrazolium (NBT) dye by superoxide radicals generated by hydroxylamine hydrochloride auto-oxidation by SOD. Absorbance at 540 nm increased in response to the drop in NBT. A 96 well plate was utilized for the experiment. In the wells containing the assay media, 250 mM potassium phosphate buffer (40 μ l) (pH = 7.8), 100 mM methionine (26 μ l), 5 mM EDTA (4 μ l), 750 mM of NBT (20 μ l), ddH₂O (90 μ l), 20 μ l of sample, and 0.002 g of riboflavin were prepared. 250 mM potassium phosphate buffer (40 μ l) (pH: 7.8), 750 mM NBT (20 μ l), 100 mM methionine (26 μ l), ddH₂O (90 μ l), 5 mM EDTA (4 μ l), and 0.002 g of riboflavin were prepared for the blank measurement. The cell culture plate was shaken in a Varioskan spectrophotometer for one minute (Thermo Scientific Varioskan Flash). After 10 seconds of shaking, the absorbance of the samples was measured at 560 nm. The cell culture plate was exposed to fluorescent light for 10 minutes immediately following the measurement. The samples were then shaken for 10 seconds on the spectrophotometer again, and the absorbance values were measured at 560 nm. The SOD activity was computed according to Kono [1978]. The statistical differences were analyzed by averaging five biological and three technical replicates, and the error bars on the graph were labeled as standard error. The significance of the difference between the Mock and FV groups was discussed using the Student's t-test in Excel 365. When $P < 0.05$, the difference between the Mock and FV groups was deemed statistically significant.

3.5.7. Measurement of Total Phenolic Content of Leaf Tissue

In this investigation, the total phenolic content was evaluated using Folin-Ciocalteu (F-C) reagent, a microplate-adapted colorimetric method for measuring total phenolics, as described by Ainsworth [2007]. The F-C assay was performed by spectroscopically determining the 765 nanometer absorption of phenolic compounds formed in an alkaline media. The experiment was conducted in microcentrifuge tubes, and spectrometric measurements were obtained using a 96-well plate reader. The 1 cm² leaf sample approximately 20 mg, was collected from the fifth fully grown leaves of mock and FV plants and immediately placed in 2 ml ependorf tubes containing liquid nitrogen. For the homogenizing the tissue, three sterile steel beads were put in tubes with adding 2 ml of ice-cold (stored at -20) 95% (v/v) methanol, followed by the placement of the samples in pre-cooled (stored at -20) blocks. The leaf tissues were homogenized at 30 hz for 5 minutes. They were then incubated for two hours at room temperature and in the dark. The samples were centrifuged at 13,000 rpm for 5 minutes at room temperature, and 100 µl of the supernatants were transferred to new 2 ml tubes for each sample. For the gallic acid standard curve and blank, empty tubes were used, and 200 microliters of 10% (vol/vol) F-C reagent was added to the sample tubes and blank tubes, which were then vigorously vortexed. Before adding the alkali-Na₂CO₃, wait 5 to 10 minutes to prevent the air-oxidation of phenols. 700 mM Na₂CO₃ (800 µl) were added to each tube, and the assay tubes were incubated at room temperature for 2 h. Transfer 200 µl of sample, standards, and blank from the ependorf tube to the 96-well microplate and measure the absorbance of each well at 765 nm using the Varioskan spectrophotometer (Thermo Scientific Varioskan Flash). For the determination of total phenolics, a standard curve was derived from the blank-corrected A₇₆₅ values of gallic acid standards. Using the regression equation between gallic acid standards and A₇₆₅, calculate total phenolics as gallic acid equivalents. The statistical differences were analyzed by averaging five biological and three technical replicates, and the error bars on the graph were labeled as standard error. The significance of the difference between the Mock and FV groups was determined by using the Student's t-test in Excel 365. The difference between the Mock and FV groups was deemed statistically significant when the P value was < 0.01.

3.5.8. Spectrophotometric Measurement of Total Chlorophyll Content

The spectrophotometric measurement of total chlorophyll content experiment was applied according to Lichtenthaler [2001]. The acetone solvent is utilized for the quantitative assessment of chlorophyll a (Chl a), Chlorophyll b (Chl b), and carotenoids in plant leaf tissue by UV-VIS spectroscopy (Shimadzu, UV-1280). These plant pigments absorb light in spectral areas that overlap. Chl a and Chl b have narrow absorption bands (maxima) in the blue (near 428 and 453 nm) and red (near 661 and 642) spectral regions. The isolated yellow carotenoids show a wide absorption with three maxima or shoulders between 400 and 500 nm in the blue spectral band. For the study of total chlorophyll, the fifth fully developed leaves of the seedlings were analyzed. To see a fully transparent leaf, plant leaves were treated with 1 ml of pure ethanol and left for 24 hours after harvesting. 1 ml of samples were taken to 1.5 ml quartz cuvette and after three different wavelength was set up, each sample was measured. The data were calculated according to formula (3.1). The statistical differences were analyzed by averaging five biological and three technical replicates, and the error bars on the graph were labeled as standard error. The significance of the difference between the Mock and FV groups was discussed using the Student's t-test in Excel 365. When $P < 0.05$, the difference between the groups was deemed statistically significant.

$$\begin{aligned}c_a (\mu\text{g/ml}) &= 11.24 A_{661.6} - 2.04 A_{644.8} \\c_b (\mu\text{g/ml}) &= 20.13 A_{644.8} - 4.19 A_{661.6} \\c_{(x+c)} (\mu\text{g/ml}) &= (1000 A_{470} - 1.90 c_a - 63.14 c_b) / 214\end{aligned}\tag{3.1}$$

3.6. Microscopic Observation and Plant Epidermis Cell, Stomata, and Trichome Density

The samples were taken from the fifth fully developed leaves of mock and FV plants in a mini glass bottle. The leaf blade was cut into 3 cm² with the main leaf veins to be clear and placed into 100% ethanol in order to decolorize. After 24 hours

of waiting in 100% EtOH, all samples were looking colorless. Then, the ethanol was dropped out and 1 ml lactic acid was added to each sample to obtain a transparent leaf blade sample before observation under the light microscopy. The samples were transparent enough to be observed directly under the light microscope and they were cut in 2 cm² without taking the vein. We observed the both the adaxial and abaxial epidermises by applying the microscope sets but we count the stomata only on the abaxial epidermis as the dicotyledon plants have more stomata under their leaves. In our study, the images of samples were taken under light microscope at 10X/20X/40X magnification and the .jpg files were opened with ImageJ&Fiji Software to count thricoms, stomata, epidermis cells respectively (Figure:3.5).

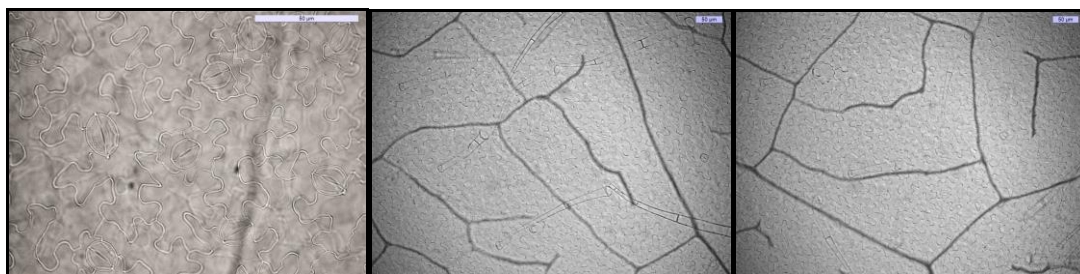


Figure 3.5: The abaxial epidermis cells, the thricomes and the stomatas densitiy were measured with ImageJ&Fiji software respectively.

The statistical differences were analyzed by averaging five biological and three technical replicates, and the error bars on the graph were labeled as standard error. The significance of the difference between the Mock and FV groups was discussed using the Student's t-test in Excel 365. When $P < 0.05$, the difference between the groups was deemed statistically significant.

3.7. Transcriptional Analysis

3.7.1. Investigation of ceRNA Regulatory Pathway

The sequence of lncRNA, mRNAs, and microRNAs (miRNA) of *N. benthamiana* and the *F. verticillioides* gene named 'Vesicle-fusing ATPase' were obtained from the pathogen-host interaction database (PHI-base) and the miRBase databases, respectively, for the gene expression analysis to identify the genes

involved in the RNAi mechanism during pathogenicity. For this aim, Nb_lncRNA called IPS1 compete with PHO2 which is the gene targeted by miR399 and AP2 which is the gene targeted by miR172 were determined via bioinformatical analyses and literature search. The interaction between these competitive endogenous RNAs and their target was predicted with the psRNATarget web tool (Figure 3.6).

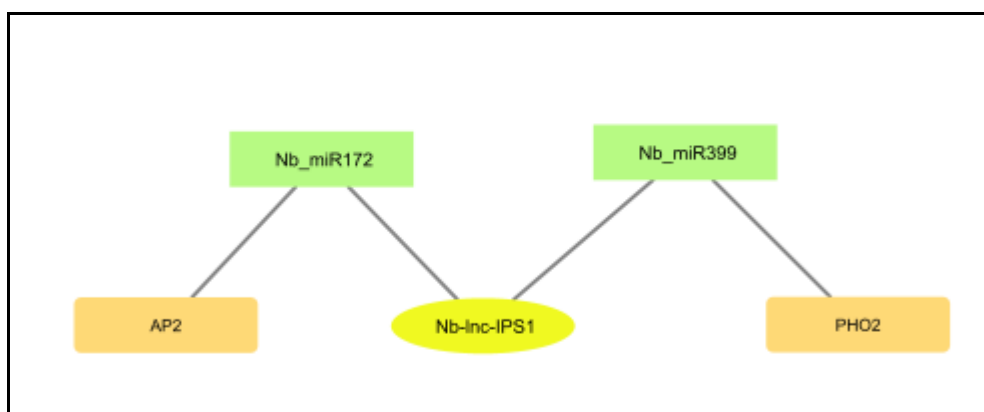


Figure 3.6: ceRNA regulatory network in *N. benthamiana*, figure prepared by cytoscape.

3.7.2. Primer Designing

The sequences of the miRNA and lncRNA target genes of *N. benthamiana* and *F. verticillioides* were obtained from solgenomics database (www.solgenomics.net). Primer design was conducted with OligoAnalyzer 3.1 (<https://eu.idtdna.com>). The parameters were assessed for melting temperature (T_m) at 58-61 °C, hairpin formation at 50 °C with $G > -3$ kcal/mol, and self and counter dimer formation at 50 °C with $G > -6$ kcal/mol. The BLASTn (www.blast.ncbi.nlm.nih.gov) tool was used to examine the specificity of primers (ref Altschul, S.F., Gish, W., Miller, W., Myers, E.W. Lipman, D.J. (1990) "Basic local alignment search tool." J. Mol. Biol. 215:403-410). The stem-loop RT primers to convert miRNAs into cDNA and universal reverse primer and miRNA specific forward primers were used in qRT-PCR [Varkonyi-Gasic et al., 2007]. The primers used in this experiment were listed in Table: 3.2.

Table 3.2: The primers used in the thesis.

Nb_miR399	5'-TGCCAAAGGAGAGTT GCCCTG -3'
Nb_miR399 Stem-loop RT	5'-GTCGTATCCAGTGCAGGGTCCGAGGTA TTCGCACTGGATACGACC AGGGC -3'
Nb_miR399 F	5'-CGGCGGTGCCAAAGGAGAGTT-3'
Nb_miR172	5'-AGAATCTTGATGATG CTGCAT -3'
Nb_miR172 Stem-loop RT	5'GTCGTATCCAGTGCAGGGTCCGA GGTATTTCGCACTGGATACGACAT GCAG -3'
Nb_miR172-F	5'-CGGCGGAGAATCTTGATGATG-3'
Nb_Uni-R	5'-GTGCAGGGTCCGAGGT-3'
Nb-PHO2 F	5'-TCCTTTCATGTGAGAGCATCTT-3'
Nb-PHO2 R	5'-CCACGATAAAGAACCACCAAAC-3'
Nb-AP2 F	5'-CTTCACAATGGCAATCCAATTA-3'
Nb-AP2 R	5'-TTTGAGGTCTTACTATCTGCTG-3'
Nb-IPS1 F	5'-GAAGACTGCAGAAGGCTGATT-3'
Nb-IPS1 R	5'-CGAAGCTTGCCAAAGGATAGA-3'
Fv-Vesicle-fusing ATPase (FVEG_11582T0) F	5'-CGTCTAGAAGTCCAGCTAGAGA-3'
Fv-Vesicle-fusing ATPase (FVEG_11582T0) R	5'-TCATGGTCCAAGATGTTCGTTTC-3'

3.7.3. RNA Isolation

Total RNA was isolated according to TRIzol method. The fully expended 4th leaaves of *N. benthamiana* grown in mock and FV plants were harvested and 3 cm² leaf tissue approximately 60 mg were cutted. The leaf tissues were transferred immediately into 2 ml ependorf tubes and fixed in liquid nitrogen. The samples were stored at -80 °C until analysis. For the extraction, leaf samples were taken out from -80°C and immediately placed into liquid nitrogen box. Each tube was filled with three sterile metal beads (4 mm in diameter) and then placed into cold homogenizer blocks. Execution began once the homogenizer was adjusted at 25 seconds/25 frequencies. Immediately following tissue homogenization, the tubes were submerged in liquid nitrogen. Rapid addition of 1 ml of TRIzol (Invitrogen) solution to each tube. The tubes were then vortexed and allowed to incubate for 5 minutes at room temperature. After incubation, 0.02 ml of chloroform was added to each tubes containing 1 ml of TRIzol, and the mixture was incubated for three minutes. The samples were centrifuged at +4 °C at 11,000 rpm for 15 min, and the supernatant was transferred to a new steril tubes. 455 µl of isopropanol was added to the supernatant

in proportion to the volume of the supernatant. The samples were incubated for 10 minutes at room temperature. After incubation, the samples were centrifuged for 10 minutes at 11,000 rpm and 4 °C. Following centrifugation, the supernatant was disposed of. 1 ml of 75% EtOH (per 1 ml of TRIzol) was used to gently wash the pellets. The pellets were centrifuged for 5 min at 4 °C and 11,000 rpm. When the process was complete, EtOH was expelled from the tubes. The pellet within the tubes was allowed to dry out. Then, 20 µl of nuclease-free water was added to each tube, and the samples were frozen at -20 °C. Utilizing NanoDrop, the concentration and purity of extracted RNAs were assessed (Thermo Scientific, NanoDrop Lite Spectrophotometer). The ratio of absorbance at 260/280 nm was used to determine the purity of RNAs, and for RNA, a ratio of roughly 2.0 was considered acceptable, and samples with a ratio of approximately 2.0 were utilized in cDNA synthesis. RNA was converted to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, USA) according to the manufacturer's instructions following quantification and quality control.

3.7.4. cDNA Synthesis and miRNA Stem-loop Reverse Transcription PCR

cDNAs of miR399 and miR172, lncIPS1, and *Fusarium verticillioides* Vesicle binding ATPase were generated using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher). By using reverse transcriptase enzyme, miRNA specific stem loop RT primer and total RNAs as templates, miRNAs were converted to cDNA. For cDNA synthesis from mRNA, total RNAs converted to cDNA by reverse transcriptase enzyme and random primers.

cDNA synthesis for mRNA, reaction mixture included 12.2 µl ddH₂O, 2 µl 10x RT buffer, 0.8 µl 25x dNTP mix (100mM), 2 µl 10x random primer, 1 µl Reverse transcriptase (RTase) (50 u/ml) (Table: 3.3), and 1 µl total RNA are required for a 20 µl reaction. Due to variable RNA concentrations, the required RNA amounts for each group were determined independently. The PCR conditions were established according to Table:3.3 and Table:3.4. After the completion of the reaction, all samples were stored at -20 °C for future analysis.

Table 3.3: The components and their required amounts for cDNA synthesis.

Components (Reaction for 20 μ l)	Volume
10X RT BUFFER	2.0 μ l
25X DNTP MIX (100mM)	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 μ l

Table 3.4: PCR program used to synthesize cDNA.

Step 1	Step 2	Step 3	Step 4
25°C	37°C	85°C	+4°C
10 min	120 min	5 min	∞

cDNA synthesis for miRNA, stem-loop RT method suggested by Varkonyi-Gasic et al. was followed (Table: 3.4) (Table: 3.5). Each reaction required 2 μ l of 10X RT buffer, 0.2 μ l of 100 mM dNTP, 2 μ l of 5 mM stem-loop primer, 0.8 μ l of 50 U/ μ l RTase, and 11 μ l of ddH₂O. Due to varying RNA quantities, the needed RNA and ddH₂O levels for each sample were determined independently using 1000 ng/ μ l. The stem-loop primers were incubated at 65 °C for 5 minutes and then placed on ice for 5 minutes. The pulsed RT PCR was conducted as following: 60 cycles, with each cycle lasting 30 min at 16 °C, 30 sc at 30 °C, 30 sc at 42 °C, and 1 sc at 50 °C, followed by 5 min at 85 °C (Table 3.4). After application, samples were stored at -20 °C until use.

Table 3.5: miRNA cDNA synthesis by pulsed reverse transcriptase PCR conditions.

Step 1	Step 2			Step 3
16°C	30°C	42°C	50°C	85°C
30 min	30 sec	30 sec	1 sec	5 min
Pre-heating	60 cycles			Incubation

3.7.4. Determination of Transcript Amount by Quantitative Reverse Transcriptase PCR (qRT-PCR)

Expression levels of miRNAs, lncRNAs and target mRNA representing a competitive RNA regulatory network associated with biotic stress were measured using qRT-PCR analysis. The qPCR Green Mix Hi-ROX (Ampigene) kit and the kit's protocol were utilized for qRT-PCR studies (Figure: 3.6). 10 µl of 2X Buffer, 1 µl of 10 mM forward primer, 1 µl of 10 mM universal reverse, 2 µl of cDNA, and 6 µl of ddH₂O were added to a tube to amplify miRNAs and lncRNAs and mRNAs. To make the growth-targeting miRNA, the reaction mixture was as following; 7,5 µl 2X Buffer with 0.6 µl of 10 mM forward primer, 0.6 µl of 10 mM reverse primer (R), 1 µl of cDNA, and 5,3 µl of ddH₂O. The qRT-PCR settings were set as follows: initial denaturation at 95 °C for 5 min, then 40 cycles at 95 °C for 5 sc, 60 °C for 10 sc, and 72 °C for 1 sc. A melting curve analysis was conducted at 95 °C for 15 seconds, 60 °C for one min, and 95 °C for another 15 sc. Delta-delta Ct ($2^{-\Delta\Delta C_t}$) was used to determine the relative gene expression level. The β -Tubulin gene was used for normalization gene. Three biological replicates and two technical replicates were used for the analysis.

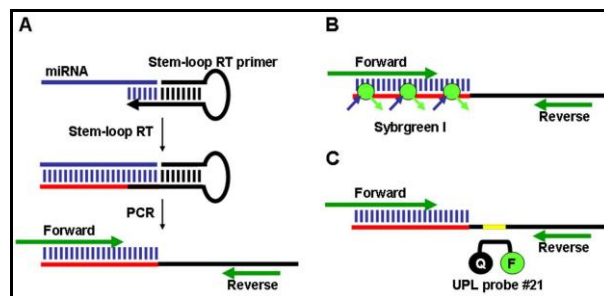


Figure 3.6: The schematic visualizing of stem loop miRNA cDNA synthesis (A) and qRT real time quantification of miRNAs using SYBR green (B).

3.8. Statistical Analysis

The statistical differences of control and FV groups were analyzed by averaging five biological and three technical replicates, and the error bars on the graph were labeled as standard error. The significance of the difference between the Mock and FV groups was determined using the Student's t-test in Excel 365. When $P < 0.05$, the difference between the groups was deemed statistically significant.

4. RESULTS

4.1. Morphological Assessment of *Fusarium verticillioides*

Environmental factors, such as the culture media, have an impact on the proliferation and sporulation of *Fusarium* mycelia [Moura et al., 2020]. For the culture of *F. verticillioides*, synthetic nutrient agar (SNA) and potato dextrose agar (PDA), are frequently utilized (Figure 4.1). However, it has been demonstrated that these media promote the development of spores with asymmetrical dimensions and shapes. The generation of spores differs significantly between PDA and SNA, and PDA has the potential to be degenerative. Besides, the SNA and PDA media, PDB was used as a liquid broth media for increase the quantification of sporulation and mycelium.



Figure 4.1: Growing *F. verticillioides* 1 day after sub-culture in SNA medium and 5 days after subculture of *F. verticillioides* in SNA medium.

4.2. Physiological Response of *N. benthamiana* inoculated by *F. verticillioides*

Growth and developmental differences between the mock and FV groups were observed and photographed daily. In terms of metrics such as number of leaves, leaf area, and stem elongation, germinated plants exhibited uniform growth and development characteristics. 2-3 days after inoculation, there were barely some differences between control and inoculated plants (Figure 4.2). Inoculated group has shown disease symptoms such as chlorosis, leaf wilting and brownish. It may not be true but when we look at carefully in every angle, the inoculated group seem to be leaning not straight as much as control groups and their leaf and stem angle was

more wider than control group (Figure 4.3). 3-4 days after inoculum, in the edge of leaves and under leaf started to covered by fungus (Figure 4.4).



Figure 4.2: 2 days after 10^{10} spore/ml spray inoculation of *N.benthamiana* plants (left) and water sprayed *N. benthamiana* plants (right).



Figure 4.3: 14 days after 10^{10} spore/ml spray inoculation of *N. benthamiana* plants (left) and water sprayed *N. benthamiana* plants (right).



Figure 4.4: The growing mycelia structure of *F. verticillioides* on plant surface an anamorphic stage under warm, humid conditions.

4.2.1. Evaluation of Leaf Area and Petiole Length

Considering the disease affect on growth and development in plant, plant petiole and leaf area were measured and calculated with the ImageJ&Fiji program. In spite of the fact that the p value indicated that there was no significant difference between the FV and MOCK groups for the leaf petiole length measurement, the FV group had a 10% slow rate of growth and development in comparison to the mock group(Figure 4.5). In spite of the fact that the p-value indicated that there was no significant difference between the FV and mock groups for the leaf area measurement, the FV group had a decrease 4,5 % of growth and development in comparison to the mock group.

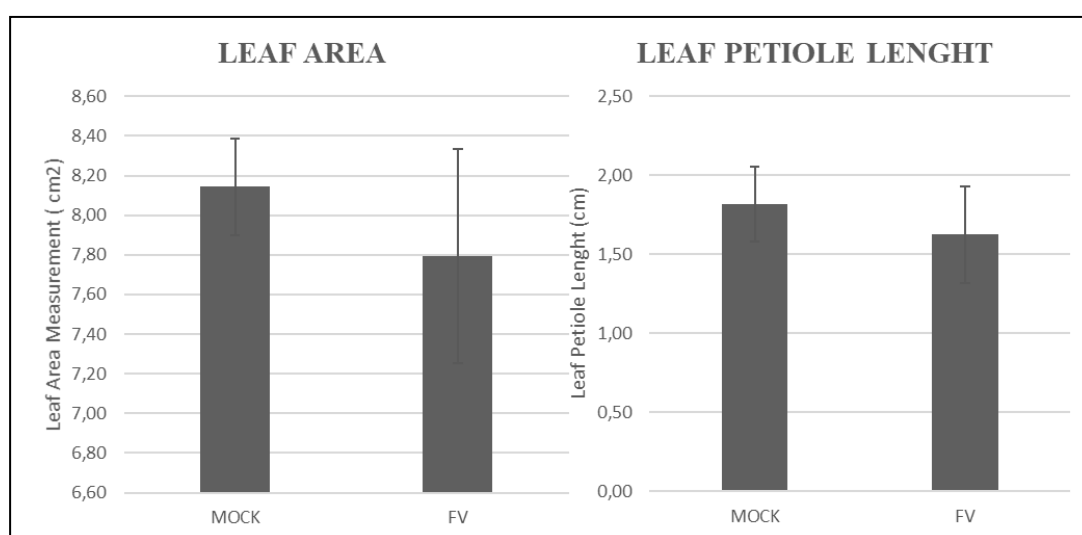


Figure 4.5: The leaf area and leaf petiole length measurement of Mock and FV (10^{10} spore/ml *F. verticillioides* inoculated) groups. The data indicates as mean, and error bars indicated $\pm$ standard error ($n=5$, $\text{ave} \pm \text{se}$, $p < 0.05$).

4.3. Antioxidant Enzyme Activity of *Nicotiana benthamiana* in Response to *Fusarium verticillioides*

4.3.1. Results of Catalase (CAT) Enzyme Activity Analysis

Catalase is an enzyme which purpose to convert hydrogen peroxide into oxygen gas and water molecules. Hydrogen peroxide can be detrimental to plant cells

during metabolic activities. The cells then use catalase to accelerate the process of converting the damaging hydrogen peroxide into oxygen gas and water molecules. Catalase enzyme activity in tobacco plants was calculated in Mock and FV group (Figure 4.6). The activity of catalase enzyme of the FV group increased by 84 % against to the control group. The lowest catalase enzyme activity was observed in the control group anyway there was no significance between the FV and Mock groups ($p < 0.05$).

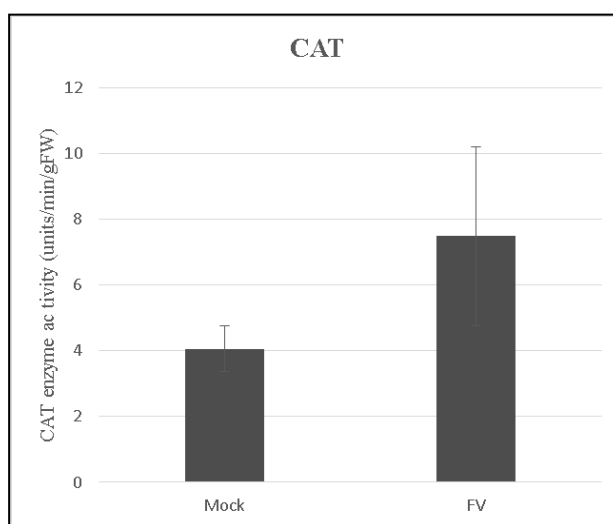


Figure 4.6: The catalase enzyme activity of Mock and FV (10^{10} spore/ml *F. verticillioides* inoculated) groups. The data indicates as mean, and error bars indicated $\pm$ standard error ($n=5$, $p < 0.05$).

4.3.2. Results of Peroxidase (POX) Enzyme Activity Analysis

Peroxidase enzyme activity in tobacco plants was calculated in control (Mock) and inoculated (FV) group. The peroxidase enzyme activity of the FV group increased by 319 % against to the control group. There was no significance between the FV and Mock groups ($p < 0.05$)(Figure 4.7).

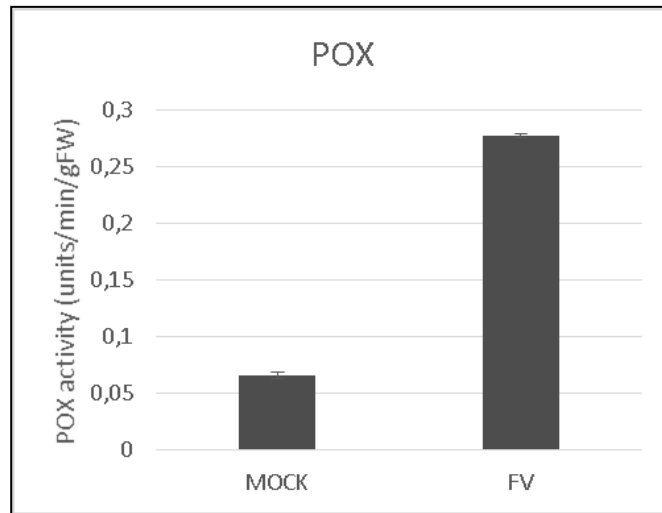


Figure 4.7: The POX enzyme activity of Mock and FV (10^{10} spore/ml *F.verticillioides* inoculated) groups. The data indicates as mean, and error bars indicated $\pm$ standard error (n=5, $p<0.05$).

4.3.3. Results of Ascorbate Peroxidase (APX) Enzyme Activity Analysis

Ascorbate peroxidase enzyme activity in tobacco plants was calculated in mock and inoculated groups. The peroxidase enzyme activity of the FV group increased by 8,3 % against to the control group. There was no significance between the FV and mock groups ($p<0.05$) (Figure 4.8).

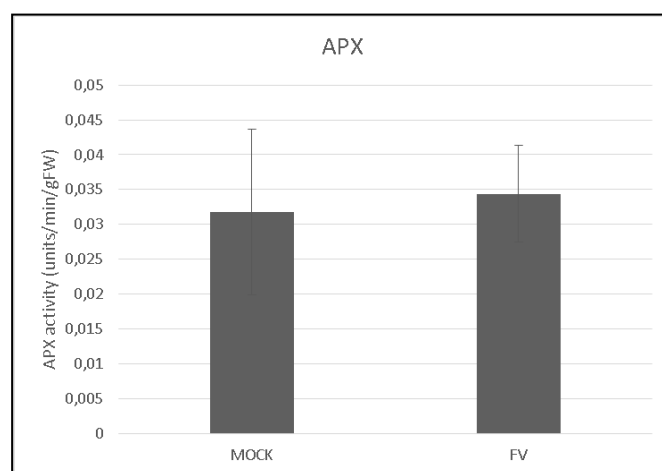


Figure 4.8: The APX enzyme activity of Mock and FV (10^{10} spore/ml *F.verticillioides* inoculated) groups. The data indicates as mean, and error bars indicated $\pm$ standard error (n=5, $p<0.05$).

4.3.4. Results of Glutathione Reductase (GR) Enzyme Activity Analysis

Glutathione Reductase (GR) enzyme activity in tobacco plants was calculated in control (Mock) and inoculated (FV) groups. The GR enzyme activity of the FV group increased by 56,8% against to the control group. The FV group has a significant increase against to Mock group ($P < 0.05$) (Figure 4.9).

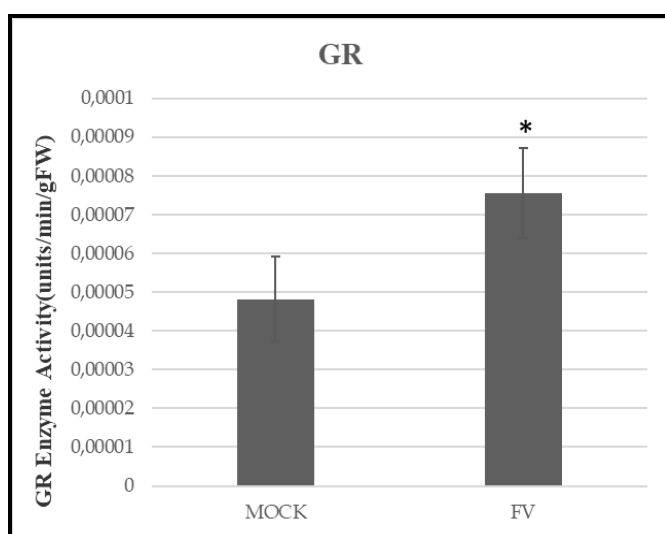


Figure 4.9: The Glutathione Reductase (GR) enzyme activity of Mock and FV (10^{10} spore/ml *F. verticillioides* inoculated) groups. The data indicates as mean, and error bars indicated $\pm$ standard error ($n=5$, $p < 0.05$).

4.3.5. Results of Superoxide Dismutase (SOD) Enzyme Activity Analysis

Superoxide Dismutase (SOD) enzyme activity in tobacco plants was calculated in control (Mock) and inoculated (FV) groups. The SOD enzyme activity of the FV group decreased by 5.8 % against to the control group. There was no significant difference between Mock and Fv groups ($P > 0.05$) (Figure 4.10).

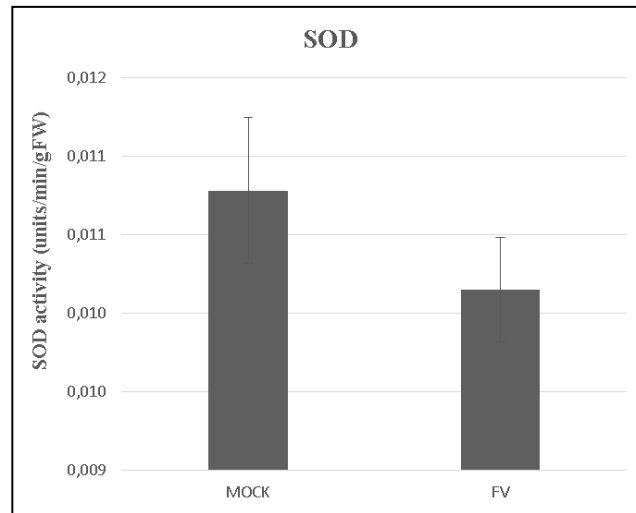


Figure 4.10: The SOD enzyme activity of Mock and FV (10^{10} spore/ml *F. verticillioides* inoculated) groups. The data indicates as mean, and error bars indicated $\pm$ standard error (n=5, p<0.05).

4.4. Evaluation of Microscopic Observation of Plant Epidermis Cell, Stomata, and Trichomes Density

The pathogen first comes into contact with the plant's outside, so this is the first line of defense against biotic pathogens. So, epidermal tissue is known as a structure with many different parts. Different parts of the skin, like trichomes, are known to be defensive [Koch et al., 2009]. Mechanisms behind the protective functions of trichomes in the Solanaceae family, which includes *N. benthamiana*, include trapping insects and stopping them from eating (mostly by non-glandular trichomes), making volatile compounds and proteinases toxic to insects, fungi, and bacteria (which is what glandular trichomes do) and turning on the plant's defense pathways. Stomata and other naturally occurring openings on the leaf surface provide a pathway for diseases to enter the plant. Plants have developed a system that allows them to close their stomata when they detect molecular patterns linked with microbes (MAMPs). Stomatal defense is the name given to this particular system. As the result of growth and development, cell numbers can vary by cell division. We counted the epidermis cells in the leaf samples to see whether or not there was a significant difference in the total number of cells between the mock plant and the pathogen-inoculated plant. As a result of microscopic counting of *N. benthamiana* trichomes, inoculum group has shown 38.7% increase to control group plant. The result of stomata measurement of the inoculated group has shown 3.4% increase to the control group. And the cell

number of the inoculated group has shown a 16.4% decrease. The microscopic results of this study support each disease's symptoms affecting the plants (Figure 4.11).

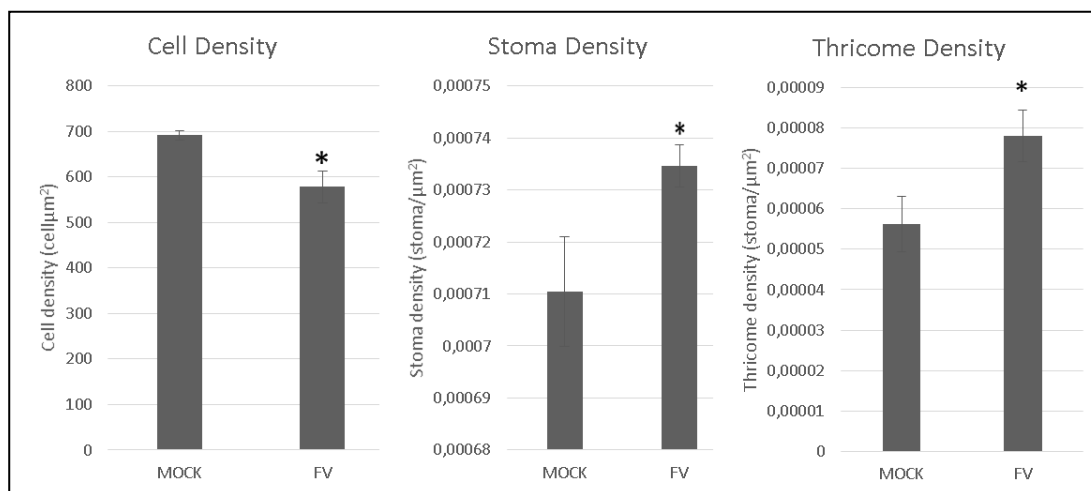


Figure 4.11: The cell number, stoma density and trichome density of Mock and FV (10^{10} spore/ml *F. verticillioides* inoculated) groups. The data indicates as mean, and error bars indicated $\pm$ standard error (n=5, ave $\pm$ se, *=p<0.05).

4.5. Evaluation of Total Phenolic Content of *N. benthamiana* to *F. verticillioides*

Upon disease attack, the phenolic chemicals can act as antioxidants to protect the plant. As stated by Ainsworth [2007], the F-C assay is based on the transfer of electrons in an alkaline medium from phenolic chemicals to phosphomolybdic acid complexes. The inoculated group had a 45 % higher total phenolic content than the mock group (p<0.01). The blank-corrected A_{765} of the gallic acid standards was used to create a standard curve for the total phenolics calculation. Using the regression equation between gallic acid standards and A_{765} , determine total phenolics as gallic acid equivalents (Figure 4.12). Error bars on the graph represent the standard error, and the statistical differences were analyzed by averaging data from three separate technical and biological replicates. A Student's t-test conducted in Excel 365 was used to analyze the difference between the mock and FV groups, and the results were discussed. When the $P < 0.01$, the results were accepted a significant difference between the groups (Figure 4.13).

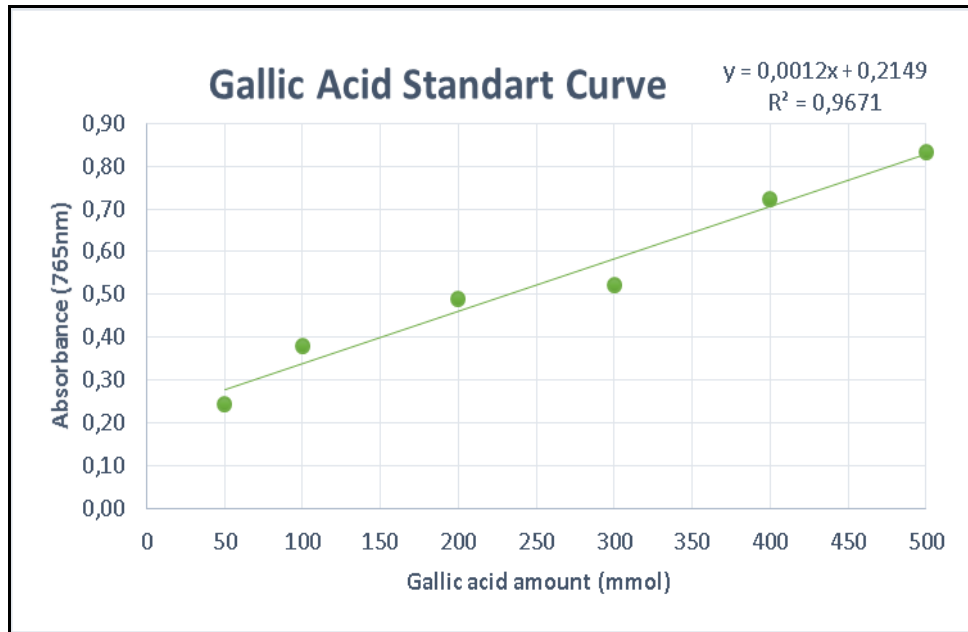


Figure 4.12: The Gallic acid standart curve for the calculation of total phenolic assay.

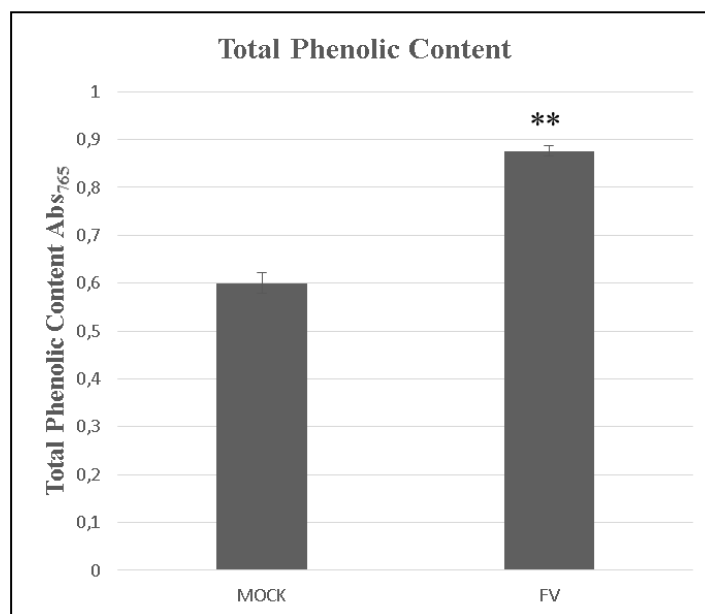


Figure 4.13: The total phenolic content of Control (Mock) and FV (10^{10} spore/ml *F. verticillioides* inoculated) groups. The data indicates as mean, and error bars indicated $\pm$ standard error (n=5, ave $\pm$ se, **=p<0.01).

4.6. Evaluation of Total Chlorophyll Content of *N. benthamiana* inoculated by *F. verticillioides*

Leaf chlorosis, or yellowing of leaf tissue, happens when leaf spots or other infected areas turn yellow due to disease infection. This caused decreasing chlorophyll. In this study, inoculated plants have shown a significant decrease in total chlorophyll content. Chlorophyll a content of inoculated group has shown a decrease against to control plants by 23.25 % ($p<0.01$). Chlorophyll b content of inoculated group has shown a decrease against to control plants by 27.28 % ($p<0.01$). And the caretenoid showed a decrease against to control plants by 25.19 % ($p<0.01$) (Figure 4.14).

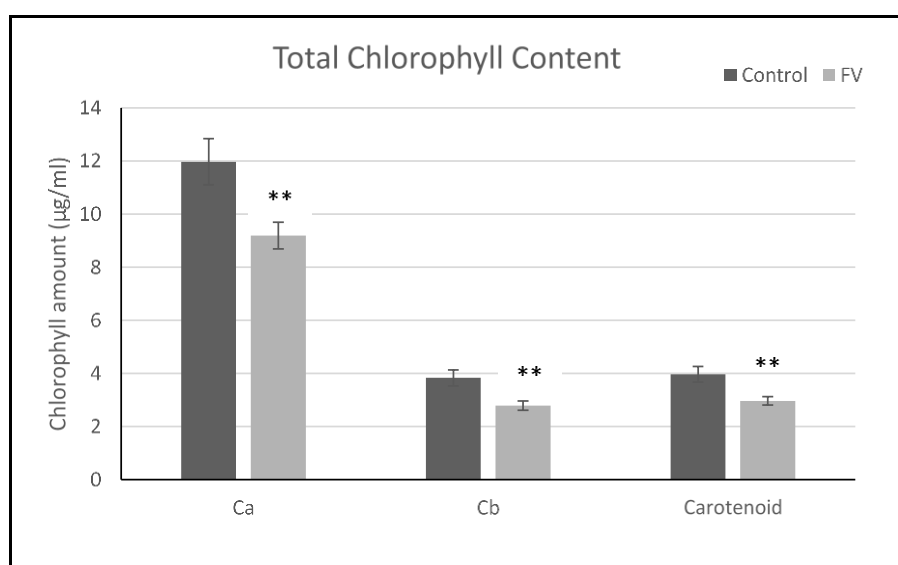


Figure 4.14: Chlorophyll content of *N. benthamiana* inoculated by pathogenic fungus *F. verticillioides*. Ca: Chlorophyll a, Cb: Chlorophyll b. $n=5$, $\text{ave}\pm\text{se}$, $**=P<0,01$.

4.7. The Expression Analysis of Biotic Stress-Related miRNAs and Their Predicted Target mRNA and IPS1 in *N. Benthamiana* Response to *F. verticillioides*

The expression analysis of biotic stress-related miRNAs and their predicted target mRNAs using the *N. benthamiana* leaf samples, quantitative real-time PCR was used to confirm the results of computational analysis. The Nb-IPS1 (Induced Phosphate Starvation1) is *N. benthamiana* long non-coding RNA that plays a role in regulating phosphate balance and uptake by competing for binding the PHO2

mRNA. PHO2 negatively regulated phosphate transporters and is downregulated by miR399 cleavage of its mRNA. Through the bioinformatic analysis, IPS1 was computed as interacting with Nb-mir172 in addition to Nb-miR399. The Phosphate over accumulator (PHO2) which was predicted a target gene of mir399, as well as APETALA2 (AP2) predicted as a target of Nb-miR172, were used for validation. The findings showed that statistically significant downregulation of IPS1, PHO2, and miR172 expression in *F. verticillioides*-inoculated plants compared to mock plants ($P<0.05$) (Figure 4.15).

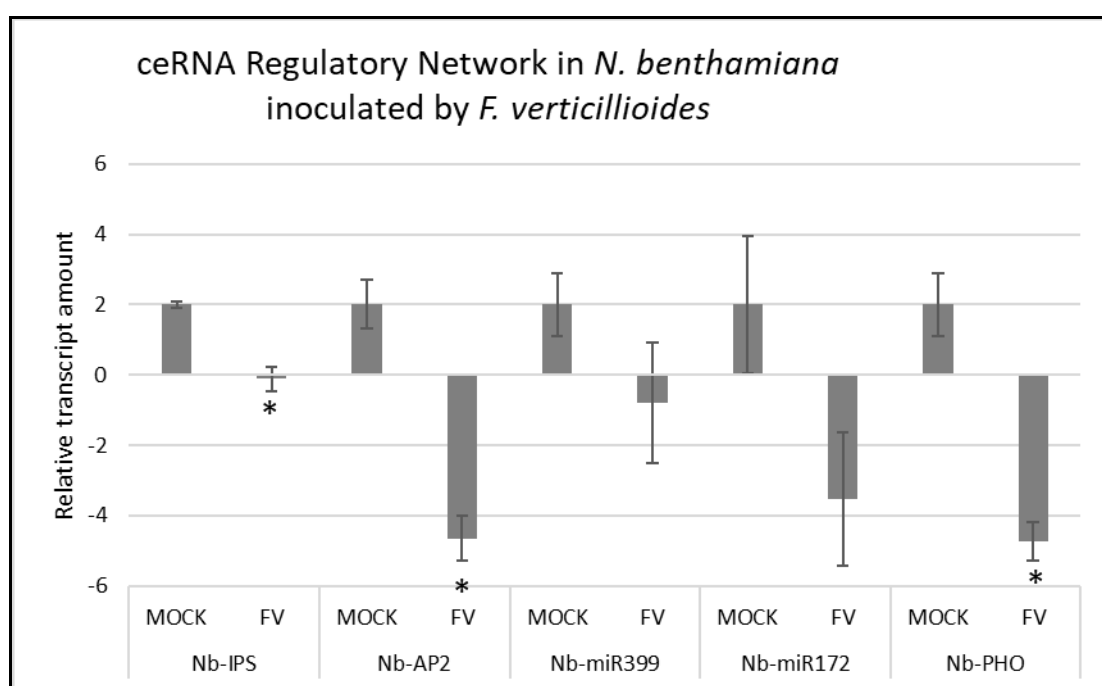


Figure 4.15: The ceRNA regulatory network of Control (Mock) and FV (10^{10} spore/ml *F. verticillioides* inoculated) groups. The data indicates as mean, and error bars indicated $\pm$ standard error ($n=5$, $*=p<0.05$).

4.8. Investigation Cross-Kingdom RNA Exchange

Some small RNAs can travel between hosts and interacting fungal pathogens to silence target genes with a mechanism termed “cross-kingdom RNAi”. In our study, we predicted Nb-miR172 of *N. benthamiana* and Vesicle-fusing ATPase (FVEG_11582T0) of *F. verticillioides* was interacted through bioinformatical analysis. In this study, we analysed that *F. verticillioides*’s gene were expressed in *N. benthamiana*. The findings pointed out that *F. verticillioides* sent its sRNAs to *N.*

benthamiana in order to suppress the defense during infection via mechanism cross-kingdom RNAi. This can be investigated by the further analysis. This findings will help to understand how fungal pathogens transport their small RNAs and repress the host plant defense molecules during pathogenicity (Figure 4.16).

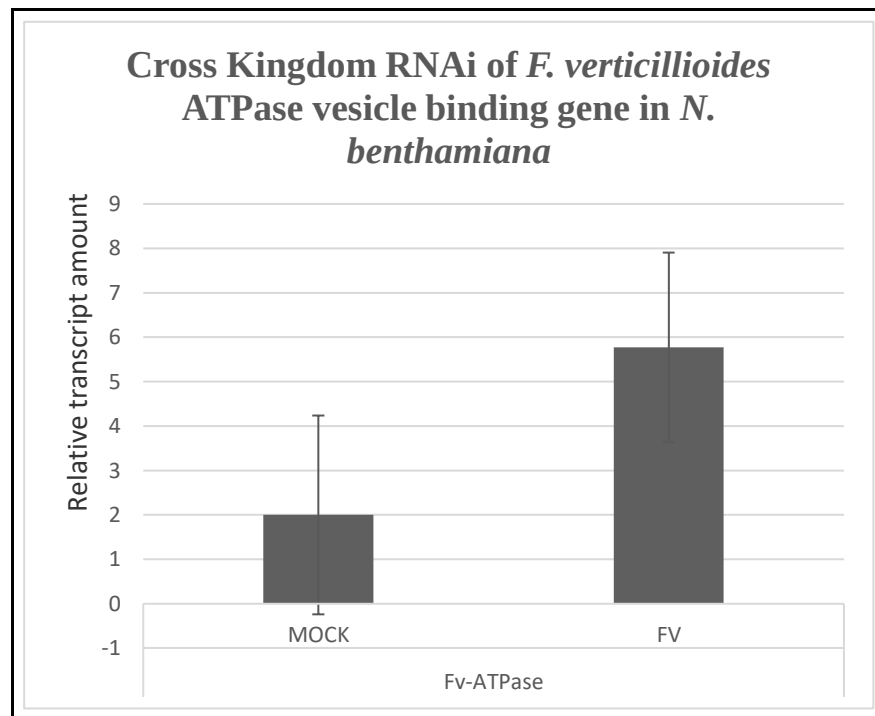


Figure 4.16: The expression of *F. verticillioides* ATPase vesicle binding via cross kingdom RNAi. The data indicates as mean, and error bars indicated $\pm$ standard error (n=5, p<0.05).

5. CONCLUSION and DISCUSSION

Currently, agriculture faces issues such as climate change, a growing population, the misuse of agricultural lands, and excessive fertilizer use. Additionally, the health of plants subjected to biotic and abiotic challenges, as well as the health of organisms that feed on or indirectly depend on these plants, is in danger. As many living beings depend on plants for nutrition and growth, protecting the health of plants and making them resistant to diseases and other dangers is one of the finest actions to take. When aiming to apply these strategies, it is essential to have a deep understanding of the associated risks and difficulties. It is important to consider and evaluate the defense mechanism of the plant as a whole, at physiological, microscopic, molecular, biochemical, and transcriptional levels when exposed to an infection.

F. verticillioides, which produces mainly known fumonisin mycotoxins, is one of the leading agricultural pathogens. *F. verticillioides* mainly damaged agricultural crops such as maize, wheat, rice and other cereal crops, banana. The fight against this pathogen is crucial since these plants represent the primary food source in many countries across multiple continents. In this study, the changes in the immune response of the tobacco plant infected with *F. verticillioides* during disease formation and development were investigated through disease-related parameters. What it was wanted to emphasize in this study is the immunity of plants against the disease by RNAi interference mechanism through the ceRNA regulatory network. After identifying the miRNAs and lncRNAs involved in the RNA interference mechanism, using bioinformatics tools such as psRNATarget, the genetical regulatory effect of these ceRNAs was investigated against the pathogen. For this study, *N. benthamiana* was used to be infected by *F. verticillioides* 10^{10} spore/ml inoculum as a model organism for observing plant-pathogen interaction.

The first disease symptom observed after inoculum was chlorosis which was confirmed by the data with a significant difference as a result of the total chlorophyll content analysis. As the chlorophyll concentration affects the color changes in the leaf, 2-3 days after inoculum, FV group plants had more yellow leaf area than mock plants and this situation continued throughout two weeks of the disease development, progressing to necrosis, a brown or dark area formed by cell death, in the infected leaf areas.

AP2, SNZ, TOE1, TOE2, TOE3, and SMZ are some of the transcription factor genes that are targeted by miR172 in Arabidopsis [Mlotshwa et al., 2006]. These genes act as floral repressors and they are silenced by miR172 by translational inhibition or transcript cleavage. Previous research has demonstrated that miR172 and the target interactions play important roles in the regulation of plant development such as meristem size, trichome initiation, stem elongation, shoot branching, and floral competence. These transitions are essential for the development of the plant. In light of these functions of miR172, we made measurements using a microscope to investigate the impact of the disease on the cell density, trichome density, and stoma density. The biomechanical function of the non-glandular trichomes that tobacco plants have emerges as a vital part of another one of the plant's defensive strategies [Maya, 2019; Yuan et al., 2020] and based on the findings, we determined that the FV group had a noticeably higher density of trichomes in comparison to the mock group. We counted the epidermis cells in the leaf samples to see whether or not there was a significant difference in the total number of cells between the mock and the pathogen-inoculated plants. Cell densities of the FV group were found to be significantly different from those of the mock group when compared with the results of precise microscopic counting. And stomata density of the FV group which is also another opening for the pathogen to enter in plant cells has shown significantly different from those of the mock group.

In addition to the analysis of the effect of the disease on plant growth and development, plant petiole and leaf area were measured and calculated with the ImageJ&Fiji programme. When we analyze our experimental data, we find that our findings are consistent with earlier studies' aspect of disease causes to decrease the growth and development rate. Although there is no significant difference, the disease caused by *F. verticillioides* reduced the growth and development in the leaf and petiole structure of the plant.

Reactive oxygen species (ROS) plays a crucial signaling role in plants, influencing activities such as growth, development, and response to biotic and abiotic stress. To ensure their survival, plants have evolved an antioxidant enzyme strategy that consists of enzymatic components such as superoxide, dismutase, catalase, ascorbate peroxidase, and glutathione reductase. In our analysis of each of SOD, APX, GR, CAT and POX has shown varied amount of increasing even though there is no significant difference in antioxidant enzyme levels in tobacco plants against *F.*

verticillioides.. But research on the pea pathogen *Mycosphaerella pinodes* revealed that infection enhanced vulnerability of the plants to several typically nonpathogenic fungi, such as *Alternaria alternata*, providing initial evidence that fungi create suppressors of plant defense responses [Mbengue et al., 2016]. Some fungi appear to actively transport metabolites and proteins into the plant in order to reduce the plant's innate defense mechanisms, such as the formation of reactive oxygen species and programmed cell death, in response to a pathogen invasion. Enzymes like catalase and superoxide dismutase (SOD) can be released to neutralize reactive oxygen species. To dampen the oxidative burst, *Sclerotinia sclerotiorum* produces oxalic acid, which is thought to disrupt plant signal transduction pathways. Similar to how the human pathogenic fungus *Cryptococcus neoformans* inhibits animal defense responses by producing and secreting mannitol, *A. alternata* produces and secretes mannitol in response to host stimuli to inhibit reactive oxygen species-mediated defenses [Mbengue et al., 2016]. So, the reason why there is no significant difference may be that the *F. verticillioides* has repression molecules against plant defensive antioxidant enzyme molecules.

In general, when a plant is infected, its phenolic content rises as a defense mechanism against the infection, according to Dicko et al. [2005]. As shown by our results, phenolics are not the only substances that contribute to tobacco plants' resistance to *F. verticillioides*, although their synthesis is boosted by the infection with a significant difference.

In post-transcriptional regulation, miRNA399 has been found to play a crucial role in maintaining Pi homeostasis [Tian et al., 2018]. Decreases in external Pi levels strongly stimulate miR399 expression in shoots and roots. PHOSPHATE 2, which is encoding for a ubiquitin E2 conjugase (UBC24), is a target of miR399 cleavage. Through a process known as 'target mimicry,' two Pi starvation-induced long non-coding RNAs, IPS1 and AT4, further regulate the activity of miRNA399 and miR172 [Franco-Zorrilla, 2007]. Pi poisoning symptoms including necrosis or chlorosis on the edges of mature leaves have been linked to overexpression of miR399 and loss-of-function of PHO2. Increased Pi uptake was discovered to be the cause of Pi saturation [Tian et al., 2018]. Together, our findings imply that miR399 and miR172 and their predicted target genes, have an effect on both Pi homeostasis and blooming in response to ambient temperature changes [Kim et al., 2011]. Our findings has shown that statistically significant downregulation of IPS1, PHO2, and

miR172 expression according to $P < 0.05$ had occurred in leaf. Chlorotic and necrotic tissue formation in the leaves of Arabidopsis plants molecularly modified to over-accumulate miR399, or in the PHO2 mutant, has been previously reported and shown to be the result of toxic P over-concentration in the shoot tissues of these Arabidopsis lines when grown in a P-rich environment. If we combine the results of our study with those of previous research, we may conclude that there is a direct and positive correlation with our work's results including physiological, biochemical and transcriptional analysis.

However, due to the fact that research on plant lncRNAs is still in its early stages, the behavior of plant lncRNAs is something that has not yet been well examined. Recent studies have shown that lncRNAs are complex regulatory components that play important roles in plant immunological responses to biotic and abiotic challenges in tissue-dependent and stress-specific and these roles are highlighted by the fact that lncRNAs are involved in sophisticated regulatory networks [Sharma et al., 2022]. However, their precise function within the signaling pathways of the defense system as well as the crosstalk between lncRNAs and the other components of the defense network are not yet completely characterized. The interactions of lncRNAs and miRNAs are something that has continued to be investigated in order to develop sustainable plant protection strategies. Each year, an increasing number of studies show us the critical roles played by sRNAs, RNAi mechanism and cross-kingdom RNAi in host-pathogen interactions and plant immunity. Recent discoveries have provided new tools for crop protection and disease management. The development of effective disease management is more critical than ever. The discovery of extracellular vesicle-mediated trafficking of sRNAs from plant hosts to fungal pathogens has given rise to many exciting questions. Though it has been shown that plants package sRNAs into exosomes for transport into fungal pathogens, it remains to be understood how specific sets of sRNAs are sorted into vesicles for transport, how these vesicles are then targeted to infection sites for secretion by the host, and how vesicles cross both cell walls of plants and pathogens, although pathogens often secrete cell-wall degradation enzymes at the beginning of the infection process. Yet another unanswered question is how the fungal pathogens secrete and deliver sRNA effectors to the plant host. Meanwhile wondering how fungal pathogens send their sRNAs to host plant we observed that Vesicle-fusing ATPase of *F. verticillioides* was expressed in *N.*

benthamiana and some of the findings especially which are not shown significant differences like antioxidant enzymes may be the result of the effect of suppression fungal sRNAs in the host plant. Answering these key remaining questions is critical in continuing to develop an understanding of the communication mechanisms and in producing applied RNAi technologies in crop protection. As a result of the study, it was suggested that further studies can make clear the expression level of fungal sRNAs in the host plant.

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