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GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

TO INVESTIGATE THE POTENTIAL OF BACILLUS SPP. ON SALT STRESS TOLERANCE OF THE MAIZE (ZEA MAYS L.) SEEDLINGS VIA SEED BIO-PRIMING APPLICATION

TAHA YUNUS KAHRİMAN
A THESIS SUBMITTED FOR THE DEGREE OF
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THESIS SUPERVISOR

ASSOC. PROF. DR. FATMA AYDINOĞLU

GEBZE

2022

T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

BACİLLUS SPP.'LERİN MISIR BİTKİSİ FİDELERİNİN TUZ STRESİ TOLERANSI ÜZERİNDEKİ POTANSİYELİNİN TOHUM BİYO-PRİMİNG UYGULAMASI İLE ARAŞTIRILMASI

TAHA YUNUS KAHRİMAN
YÜKSEK LİSANS TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

DANIŞMANI
DOÇ. DR. FATMA AYDINOĞLU

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2022

GEBZE TEKNİK ÜNİVERSİTESİ	**YÜKSEK LİSANS JÜRİ ONAY FORMU**

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SUMMARY

The main objective of this study was to investigate the growth-promoting potential of *Bacillus spp.* on maize (*Zea mays L.*) plant grown under saline conditions at the seedling stage. Therefore, 7 different maize hybrids from the Maize Research Institute (Sakarya/Turkey) were inoculated with *B. pumilus*, *B. licheniformis* and *B. coagulans* and their salt stress responses as a result of this bio-priming application were screened at morphological, physiological and transcriptional level. Among 7 maize hybrids, Sasa156 was chosen due to its capability of enduring saline conditions and its germination efficiency. The experiments were performed in two environments: a greenhouse and a plant growth chamber. Following the greenhouse experiments, the research was moved inside the plant growth chamber, and 150mM NaCl was decided from among the three salt concentrations. It was observed that *B. licheniformis* (P24) increased the root fresh weight (RFW) and shoot fresh weight (SFW) compared to other treatments. Catalase (CAT) and ascorbate peroxidase (APX) activity were significantly increased with *B. licheniformis* inoculation. All studied miRNAs were upregulated with P24 inoculation, but miR160 was significantly up-regulated in *B. licheniformis* inoculated maize roots.

As a result, the plant growth-promoting potential of *Bacillus spp.* under saline conditions has been shown and regulatory roles of miRNA genes during salt stress response of maize seedlings after seed bio-priming application were elucidated.

Key Words: Maize, *Bacillus spp.*, salt stress, PGPR, miRNA.

ÖZET

Bu çalışmanın amacı, tuz stresi altında yetiştirilen mısır fidesi üzerinde *Bacillus spp*'nin bitki büyümesini teşvik edici potansiyelinin araştırılmasıdır. Bu nedenle Mısır Araştırma Enstitüsü'nden (Sakarya/Türkiye) 7 farklı mısır (*Zea mays L.*) hibriti *B. pumilus*, *B. licheniformis* ve *B. coagulans* ile inoküle edilmiş ve bu bio-priming uygulaması sonucu tuz stresine cevapları morfolojik, fizyolojik ve transkripsiyonel seviyede gözlenmiştir. Yedi mısır hibriti arasından, tuzlu koşullara dayanma kabiliyeti ve çimlenme verimliliği nedeniyle Sasa156 seçildi. Deneyler, sera ve bitki yetiştirme odası olmak üzere iki farklı ortamda gerçekleştirilmiştir. Sera deneylerinin ardından araştırma, bitki yetiştirme odasının içine taşınmış ve üç tuz konsantrasyonu arasından 150mM NaCl'ye karar verilmiştir. *B. licheniformis'in* (P24) diğer uygulamalar olan *B. pumilus* ve *B. coagulans*'a göre yaş kök ağırlığını (RFW) ve yaş gövde ağırlığını (SFW) arttırdığı gözlendi. Katalaz (CAT) ve askorbat peroksidaz (APX) aktivitesi, *B. licheniformis* inokulasyonu ile önemli ölçüde arttı. İncelenen tüm miRNA'ların ekspresyon seviyeleri, P24 ile inoküle edilen bitkilerde artış göstermiştir fakat MiR160 ekspresyonu, *B. licheniformis* inoküle edilmiş mısır köklerinde çok önemli ölçüde artmıştır.

Sonuç olarak, *Bacillus spp.*'lerin bitki büyümesini tuz stresi altındayken teşvik etme potansiyeli gösterilmiş ve miRNA genlerinin mısır tohumlarının bio-priming uygulaması sonucu fidelerin tuz stresine verdikleri cevap sırasında miRNA genlerinin düzenleyici rolleri aydınlatılmıştır.

Anahtar Kelimeler: Mısır, *Bacillus spp.*, tuz stresi, PGPR, miRNA.

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LIST of ABBREVIATIONS and ACRONYMS

Abbreviations and Acronyms		**Explanations**
ºC	:	Celsius degree
g	:	Gram
mg	:	Milligram
µg	:	Microgram
lt	:	Litre
ml	:	Milliliter
µl	:	Microliter
M	:	Molar
mM	:	Millimolar
µM	:	Micromolar
nm	:	Nanometre
mm	:	Millimetre
lx	:	Lux
nt	:	Nucleotide
NaCl	:	Sodium chloride
Ca^{+2}	:	Calcium ion
$MgCl_2$	:	Magnesium chloride
ACC	:	1-aminocyclopropane-1-carboxylic acid
APX	:	Ascorbate peroxidase
ARF	:	Auxin response factor
BSA	:	Bovine serum albumin
CAT	:	Catalase
CC	:	Chlorophyll content
cDNA	:	Complementary DNA
CDS	:	Coding sequence
CFU	:	Colony forming unit
DCL	:	Dicer like protein
ddH_2O	:	Double distilled water
DNA	:	Deoxyribonucleic acid

dNTP	:	Deoxynucleotide triphosphate
EDTA	:	Ethylene Diamine Tetra Acetic Acid
EtOH	:	Ethyl Alcohol
FLL	:	Final leaf length
FW	:	Fresh weight
GR	:	Glutathione reductase
GRF	:	Growth regulating factor
GSSG	:	Glutathione Disulfide
HYL	:	Hyponastic leaves
IAA	:	Indole acetic acid
IST	:	Induced systemic tolerance
K-P buffer	:	Potassium phosphate buffer
LB	:	Luria-Bertani
LER	:	Leaf elongation rate
LL	:	Leaf length
min	:	Minute
miRNA	:	Micro RNA
mRNA	:	Messenger RNA
NADPH	:	Nicotinamide Adenine Dinucleotide Phosphate
NBT	:	Nitroblue tetrazolium
NF_YA	:	Nuclear Transcription Factor Y Subunit Alpha
PGPR	:	Plant Growth Promoting Rhizobacteria
POX	:	Peroxidase
psRNA	:	Plant small RNA
PVP	:	Polyvinylpyrrolidone
q-RT-PCR	:	Quantitative real time polymerase chain reaction
QS	:	Quorum sensing
RDW	:	Root dry weight
RFW	:	Root fresh weight
RISC	:	RNA-induced silencing complex
RL	:	Root length
RT	:	Reverse Transcriptase
rpm	:	Revolutions per minute

SDW	:	Shoot dry weight
SE	:	Serrate
SFW	:	Shoot fresh weight
SL	:	Shoot length
SOD	:	Superoxide dismutase
sRNA	:	Small RNA
TCP	:	Transcription factor proteins
VOC	:	Volatile organic compound

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1. INTRODUCTION

Maize (*Zea mays L.*) is a major annual cereal crop that belongs to the Poaceae family. Zea is an old Greek term which means "life sustainer," and Mays is a word of Taino that means "life giver." The name "maize" comes from the Spanish word "maiz". Various names for the plant include zea, silk maize, makka, barajovar, and others [Kumar and Jhariya, 2013]. It is regarded as a staple food in many regions of the world. It is the world's third most important crop after rice and wheat [Sandhu et al., 2007]. Maize is commonly used as animal feed, cornmeal, grits, starch, flour, tortillas, snacks, and morning cereals are among the many goods made from it [Mehta and Dias 1999]. The genus Zea has 8 species, the most significant of which being *Zea mays L.* and the remaining Zea species are mostly wild grasses endemic to Mexico and Central America. *Zea mays* has a total of 2n = 20 chromosomes.

Increasing agricultural production to fulfil consumer market needs and the world's rising population requires the usage of a considerable number of pesticides and chemical fertilizers, which are frequently exploited in soil [Kumar et al., 2017]. Fertilizers that are chemically produced have the potential to increase crop yield by approximately 50% when compared to non-fertilizer production; but by enhancing nutrient utilization and allocation while diminishing interactions between crops and rhizospheric microorganisms, chemical fertilization approaches disregard the biological features of roots or the rhizosphere. [Meena et al., 2017]. A number of studies have proved that Plant Growth Promoting Rhizobacteria (PGPR) can improve growing conditions while reducing pesticide consumption [Aloo et al., 2019].

PGPR can be an excellent option for farmers facing the new demands of modern agriculture, as severe concerns have arisen as a result of agricultural industrialization caused by the need to provide a huge quantity of nutrients worldwide. At these times, it is critical to maintaining high production while having as little influence on the environment as feasible [Pérez-Montao et al., 2013]. PGPR can use direct or indirect mechanisms to support plant-microbe communities. Each mechanism has various surface-related properties. Plant growth bacteria are found in microbial communities and can be present both at the leaves and in the root systems of plants. PGPR can increase the production of some plant hormones such as auxin, gibberellins and cytokinins and these plant hormones directly related to plant growth.

It was found that auxins have capacity to offer supplementary host activities through the uptake of elements from the soil for plant development, siderophores and phosphorous acquisition or nitrogen fixation [Cassán et al. 2009], [Riggs et al.,2001]. Indirect biological control mechanisms include antagonistic action toward phytopathogenic microbes, eliciting plant systemic resistance responses of plant, interacting with bacterial quorum sensing (QS) systems, and so on. According to some publications, PGPR can employ each of these pathways to improve plant development [Bashan and Holguin, 1997].

Salinity impacts the soil features and balance of the region, as well as crop yield, and so plays a part in lowering financial benefits. Salinity affects plants in a variety of ways, including lower rates of plant development and growth, germination rates, as well as a decrease in spike formation and poor fertility [Munns and Rawson, 1999]. Salinity has a negative influence on differentiation of cells and cell cycles because it decreases the activity of cyclins and the production of cyclin-dependent kinases, resulting in decreased cellular metabolism in the meristem that leads to lessened growth activity [Seckin et al., 2009]. Accumulation of excess Na^+ in parts of plants depresses photosynthetic rate and generates reactive oxygen species (ROS), which leads to damaged membrane, DNA, and protein structure [Islam et al., 2015].

In eukaryotic cells, microRNAs (miRNAs) are a type of noncoding short RNAs with approximately 22 nucleotides. Because of their ability to join with mRNA of the target gene via complementary base pairing and degrade the mRNA, they can regulate the expression of the target gene at the post transcriptional level. By this way, miRNA displays a crucial role in many biological activities such as maintaining stress response via arrangement of metabolic progression. Plants must develop effective-adaptive methods to defend themselves from a range of challenging surrounding environment, and miRNA plays an essential role in plant homeostasis to respond to severe stress conditions [Zhang et al., 2006]. Plants will confront a series of challenges during the development and growth stages, which classified as abiotic stress and biotic stresses. Examples of abiotic stresses can be salinity, drought, high temperature and insufficient nutrient intake [Zhu et al., 2011]. Biological stress might manifest itself as to be exposure to pests, pathogen attack or any microbial infection [Ruiz-Ferrer et al., 2009]. It has been discovered that plant miRNAs play a significant role in overcoming the effects of both biological and abiotic stress [Phillips et al., 2007]. Plants may modify the expression pattern of specific miRNAs

to mitigate the effects of stress, which either increases or decreases the expression of miRNA-targeted genes [Kong et al., 2010]. Salt stress is a common type of abiotic stress which has a detrimental impact on plant development and yield. Saline-alkaline environments cause the expression of a disordered plant transcriptome, which appears as imperfect phenotypes. Recent studies have established the active involvement of miRNAs in several stress scenarios [Cao et al., 2018]. Multiple miRNAs that are specific to a stress factor have also been described in model plants under a variety of biotic and abiotic stresses, such as salinity, cold or chilling, drought, heat, deficiency of nutrients or infections mediated by microbes [Sunkar et al., 2009]. Salt stress-responsive miRNAs have a role in the post-transcriptional regulation of physiological, morphological, and metabolic responses in maize [Ding et al., 2008].

In this context, one of the key goals of this research was to investigate the growth improvement of maize seedlings under salt stress as a result of seed bio-priming application by *Bacillus spp*. and evaluate the molecular mechanisms behind salt stress tolerance after bio-priming application by evaluating the expression of miRNAs and their probable target genes.

1.1. Aim, Contribution and Content of the Thesis

Plant growth and PGPR interactions are a challenging system that involves various genes that control plant growth and development. Although numerous plant-microbe interactions have been investigated in terms of enzymatic activities, enzymatic regulation mechanisms, and posttranslational regulation, miRNA-mediated gene regulation during maize growth mechanisms are yet to be investigated. As a result, one of the major objectives of this study was to obtain a comprehensive knowledge of how these intricate genetic pathways are regulated. Another key goal was to investigate the ability of various *Bacillus spp.* to promote plant growth and the potential benefits they may provide when exposed to salt stress. It is critical to understand the roles of miRNAs and their predicted target genes in bacterial interactions. In order to achieve this objective, in this study, 7 different maize (*Zea mays L.*) hybrids were subjected to a seed bio-priming application, and the responses of the seedlings were screened at morphological, physiological and molecular levels. These experiments were carried out in both greenhouse conditions

and growth chamber to select the optimal maize hybrid line and to proceed on transcriptional and enzymatic analysis. Since salt stress was studied, the average growing maize line was chosen, not the best growing or worst growing maize line. Otherwise, the intended stress and effects would not be observed when working with salt-resistant lines. Tissue samples were harvested from the roots. The antioxidant enzyme activity of inoculated, non-inoculated and salt stressed plants were measured by spectrophotometry. The bacteria-plant interaction related microRNAs and their targets were identified in silico using bioinformatic tools. For determining transcript levels and analysing data, quantitative-Reverse Transcriptase-PCR (qRT-PCR) method was used. All enzymatic and gene expression data were represented as average of three biological replicates and statistical analyses were performed to calculate significance of the different groups.

In this study, *Bacillus coagulans*, *Bacillus licheniformis*, *Bacillus pumilus* and mixture of these bacteria were used and their interaction with *Zea mays L.* under salt stress were investigated at morphological, physiological, enzymatical and transcriptional levels. Functions of miRNA genes in maize root growth response to salt stress after PGPR treatment were analysed.

2. GENERAL INFORMATION

2.1. General Information of Maize (*Zea mays L.*)

Maize is one of the domesticated food crops initially cultivated by southern Mexicans over 10,000 years ago. Maize (*Zea mays L.*) is a Poaceae family grain that is widely consumed [Gaut et al., 2000]. Maize is an annual diploid plant with 10 chromosomes. The plant has a densely branched and thin root system, and the length of the roots can exceed 1.5 meters under ideal conditions depending on the soil texture and climate. The maize shoot contains about twenty leaves grouped spirally and in opposing rows. The leaf blade is slender, long, wavy at the tip, and hairless. A prominent centre rib runs the length of the leaves, bracing them. Monoecious maize (*Zea mays L.*) has incomplete female florets in the ear and incomplete male (unisexual) florets in the tassel. The formation process of incomplete florets shows difference in male and female. Males achieves this by the abortion of the pistil primordials while females suppress the development of stamens. This means that determining sex is a matter of what governs the stoppage of stamen or pistil development in the ear and tassel florets [Banks et al., 2008].

2.2. Plant Growth Promoting Rhizobacteria (PGPR)

Plant growth promoting bacteria (PGPR) are free-living bacteria that proactively colonize the roots of plants and have a beneficial influence on plant development. PGPR may stimulate plant development and they may utilize their own metabolism by fixing nitrogen, solubilizing phosphate, producing different hormones, as well as influencing the plant metabolism. Plants benefit from PGPR in a variety of ways, including improved nutrient absorption via rhizosphere regulation and increased antioxidant enzyme activity. They can also interact and coexist with other soil microbes to provide a healthier habitat for plants and protect them against pathogens invading their territory. Studies have shown that applying PGPR to maize has multiple benefits [dos Santos et al., 2020]. The usage of PGPR is one potential way to reduce the negative environmental effect of overuse of herbicides, insecticides and chemical fertilizers.

Bacteria from several genera, including *Achromobacter, Azospirillum, Bacillus Enterobacter, Burkholderia, Paenibacillus, Pantoea, Pseudomonas, Variovorax, Rhizobium, Microbacterium* and *Methylobacterium* provide resilience to plants under stressed conditions [Akram et al., 2016]. These bacteria can ease various stressful conditions in agricultural arable lands [Wang et al., 2016]. Several studies have shown that these microorganisms improve the plant's ability to endure stress through a variety of ways; for instance, the production of gibberellins, indole acetic acid and some undiscovered components, which promotes the increase in the surface of the roots, area of root length, tips of the root and significantly increased nutritional content, thus increasing plant survival in the presence of salinity stress [Shahid et al., 2018].

2.3. Salt Stress in Plants

Global warming and shifts in climate have escalated the severity of multiple biotic and abiotic stresses, reducing agricultural production yield. At the same time, increased crop yields are essential in order to fulfil the worldwide food requirement of a developing population, which is estimated to reach 9 billion by 2050 [Rout, 2020]. Excess levels of soluble salts in soil and water cause lower agricultural productivity and, as a result, transform productive fields to marginal lands, leading to their rejection [Ilangumaran and Smith, 2017]. Salinization of the soil is regarded as one of the crucial restrictions limiting agricultural productivity while also jeopardizing the quality of soil [Machado and Serralheiro, 2017]. Increased saline soils are frequently located in areas with high topsoil vaporization, minimal precipitation, and high temperatures [Arora et al., 2018]. Furthermore, the continuous use of high-salt fertilizers, insecticides, and other additives, as well as the use of poor irrigation, are spreading salt accumulation to nearby agricultural areas. [Enebe and Babalola, 2018]. Toxic amounts of soluble salts, mostly NaCl, are found in salt-affected areas, causing osmotic stress, nutritional imbalance, and changes in the plant metabolic system, resulting in reduced plant development [Deinlein et al., 2014]. Furthermore, high NaCl interacts with soil microorganisms and disrupts the balance of their activity [Rath and Rousk, 2015]. Salinity stress plays a role in detrimental effects on biodegradation and nutrient uptake [Yan et al., 2015].

2.4. Role of PGPR in Salt Stress

Up to the present day, increased NaCl levels have harmed more than 20% of arable land. If these conditions continue to rise, arable land might lose half of its viability by 2050 [Din et al., 2019]. Excessive salt levels in soil can cause hyperosmotic and hyper ionic stress, which can reduce plant development, reduce nutrient absorption, and potentially cause plant mortality [Hidri et al., 2019]. To maintain salt-affected soils, alternative approaches are being used, including the use of tube wells for watering, the application of various chemical compounds such as gypsum, acids, and various adjustments, the development of salt stress resistant varieties, and seed treatment with PGPR strains. However, the use of physicochemical approaches is restricted due to its high price and implausibility. PGPR initiatives may modify the development of plant root and help crops to cope with salinity [Azcón et al., 2013].

To endure the outcomes of salt stress and promote growth, PGPR uses two different strategies. Directly by nitrogen fixation, phosphate solubilization, IAA synthesis and indirectly by alarming the antioxidant defence, production of volatile organic compounds (VOC), secreting exopolysaccharides (EPS) and regulating the osmotic balance. Many bacterial components contribute to IST (induced systemic tolerance) enhancement, including IAA (indole acetic acid) synthesis, ACC deaminase capacity, production of EPS, production of VOC and establishment of siderophore [Yang et al., 2009]. An earlier study has suggested that *B. licheniformis* HSW-16 might be used as a bioinoculant to increase plant growth and yield under saline conditions [Singh, 2016]. In another study, it was suggested that *Bacillus pumilus* strain JPVS11 has the ability to improve plant development characteristics, soil enzyme activity, microbial levels, and to mitigate the negative impacts of salt. [Kumar et al., 2020]. High salinity concentration, often sodium chloride, cause osmotic stress by diminishing cell water potential and ionic stress by blocking particular metabolic activities. To compensate for the reduction in water potential, plants respond to salinity by sequestering toxic ions in vacuoles and accumulating compatible solutes in the cytoplasm [Di Martino et al., 2003].

2.5. The *Bacillus* Genus

Bacillus is a widespread bacterium that may be found in a variety of conditions, including rhizosphere. Root associated *Bacillus spp*. generally enhance plant development via a number of processes such as the creation of phytohormone precursors such as indole acetic acid (IAA-auxin), phosphate solubilization, and siderophore formation, or by serving as biocontrol agents, and this is the reason why they call it PGPR. These bacteria can also be found in combination with other bacteria such as *Azotobacter* and *Azospirillum* can be used by co-inoculation to increase fertility of the soil. [Kashyap et al., 2019].

Therefore, the Bacillus genus is of tremendous importance due to its potential applications in biotechnology, healing polluted regions, managing biological agents, and stimulating plant development in agriculture.

Table 2.1: Taxonomy of genus *Bacillus.*

Domain: Bacteria
Phylum: Firmicutes
Class: Bacilli
Order: Bacillales
Family: Bacillaceae
Genus: *Bacillus*

2.6. Plant MicroRNAs

MicroRNAs (miRNAs) are small RNAs (sRNAs) that inhibit transcriptional activity by binding to highly complementary regions in target messenger RNAs (mRNAs). Many miRNAs are ancient, and their sophisticated integration into gene expression patterns has proved their essential role in the life of plants, directing initiatives for growth, and responding to biotic or abiotic signals. Furthermore, a substantial number of conserved miRNAs exist in each plant species, suggesting the likelihood that miRNAs have great influence on nearly every area of plant life [Millar, 2020]. The first perceived experiment to establish whether a miRNA is engaged in a stress response is to examine whether its availability changes under a

given stress. To start with RNA-seq is used to assess the quantity of miRNAs in Arabidopsis under drought, heat, and salinity stressed environments [Pegler et al., 2019]. After maturity, RNAs ranging to 21-25 nucleotides establish a duplex with bordering genomic sequences and convert an RNA duplex from an incomplete to a complete one. These RNAs are known as miRNAs and are frequently obtained from a genomic area other than the expected protein-coding regions. MiRNAs perform important functions in several regulatory pathways and exhibit a wide range of expression patterns in different tissues or developmental stages [Xie et al., 2015]. The plant miRNA mechanism differs significantly from its mammalian counterpart in terms of both synthesis and silencing. In plants, the nuclear RNase Dicer-Like (DCL1) and its supplemental proteins Serrate (SE) and Hyponastic Leaves1 (HYL1) generate miRNAs from an early miRNA transcript (pri-miRNA), that contains a foldback structure. HYL1, DCL1, and SE, which are important factors in the miRNA biogenesis of plants, were identified and served as the groundwork for the initial models of miRNA biogenesis.

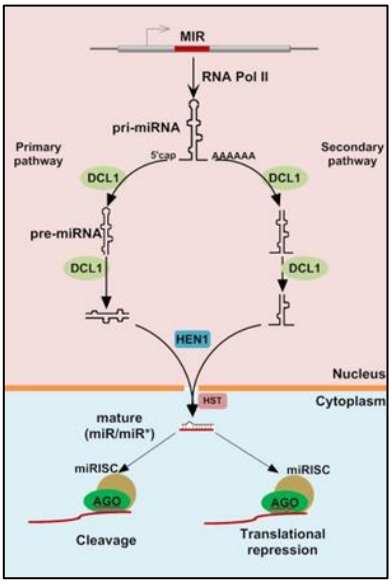

Figure 2.1: Major biogenesis pathways of plant miRNAs.

2.7. Roles of MicroRNAs in Plant-Microbe Interactions

Plants and microorganisms both play important roles in regulating the rhizosphere. They can, in fact, regulate the plant's microenvironment and it can alter

the plant to their favour by the antimicrobials, VOCs, quorum-sensing (QS) molecules and production of phytohormones [Venturi and Keel 2016]. Microbial content of the rhizosphere is also shaped by microbial choice of metabolite substrates and connections among microbial members [Zhalnina et al., 2018]. Microbial interactions can be collaborative, such as QS and exchange of metabolites under poor nutrient availability and antimicrobial compound secretion [Hassani et al., 2018]. Although it is not clear which form of interaction prevails communities. Furthermore, microbial interactions can modify gene expression within communicating microorganisms, implying that microbial interactions influence both the structure and functionality of the microbiota [Sasse et al., 2018], [Uroz et al., 2018].

Formerly, it was considered that miRNAs evolved with pluricellular creatures, and their role was restricted to intercellular connections inside a single organism. Subsequently, miRNAs have been discovered in unicellular species, first exploration was in *C. elegans*, then in oomycetes and finally in bacteria where they are known as miRNA-size small RNA (msRNA) [Lee et al., 1993], [Nelson et al., 2017]. The discovery of miRNAs in single-celled organisms shows that miRNA-based gene control is an old strategy that predates multicellular life [Zhao et al., 2007]. MiRNAs are evolutionarily strongly preserved and expressed in all aspects of life.

Furthermore, miRNAs are not limited to communicating inside a tissue, organ, or organism: they have the capacity to travel great distances, between individuals, and even between species [Brosnan et al., 2021].

2.8. Seed Bio-priming and Agricultural Applications

Biological seed treatment, which is also known as bio-priming, is an application of beneficial microorganisms such as bacteria or fungi to seed to stimulate or promote the production of plants while maintaining the environmental balance. Seed bio-priming has recently been acknowledged as a low-cost, eco-friendly approach capable of promoting growth, inducing stress tolerance, and achieving desirable crop yields [Chakraborti et al., 2022]. Seed priming is a method which its application to the seeds is before sowing and it helps to induce a physiological condition which permits seeds to germinate more effectively [Deshmukh et al., 2020]. There are several types of seed priming such as osmo-

priming, hydro priming, hormo-priming, chemo-priming, solid matrix priming and nutri-priming which are commonly used to lower the effects of abiotic stress, but bio-priming is not only providing an assistance to endure abiotic stresses but also helps plants to survive under biotic stress [Lutts et al., 2016]. It has been revealed that bio-priming is directly involved in the plant development by the solubilization of minerals and excretion of compounds [Sukanya et al., 2018]. The bio-priming of seeds with PGPR increases the germination rate, root and shoot biomass by stimulating the growth hormones such as cytokinin, auxin, gibberellin etc. [Glick, 2012], [Noel et al., 1996]. Bio-priming of seeds promotes the production of some antioxidant enzymes which are directly involved in the defence mechanism of plants such as catalase, peroxidase, superoxide dismutase thus helps plants to survive through biotic and abiotic stress [Deshmukh et al., 2020]. Bio-priming of seeds with PGPR increases the performance of plants under stress conditions which leads plants to overcome the negative effects of stress and results in increased crop yield [Dimkpa et al., 2019]. Bio-priming of seeds with PGPR can lead to synergistic occasions where one inoculant functions as a helper for improved performance of another inoculant. This type of synergism is well known between several bacterial genera such as *Pseudomonas*, *Bacillus* and *Rhizobium* which have the ability to promote growth and development of plants [Deshmukh et al., 2020].

Furthermore, under both normal and stressed conditions, bio-priming of seeds with PGPR can greatly enhance seedling establishment, seed germination and emergence, crop development and yield.

3. MATERIAL AND METHODS

3.1. Plant Material and Growth Condition

Through this study, seven maize (*Zea mays L.*) hybrids (Table 3.1) provided by the Maize Research Institute (Sakarya) were investigated. First, a comparative screening analysis was done on seven maize hybrids based on growth response to *Bacillus* spp. At the end of the screening analysis, one maize hybrid out of seven was chosen for further enzymatic and transcriptional analysis to investigate salt stress performance of maize seedlings after bio-priming application.

Table 3.1: The name of maize (*Zea mays L.)* hybrids was used in this study.

No:	**Name**
1	Sasa 155
2	Sasa 156
3	Sasa 159
4	Sasa 196
5	Sasa 197
6	Ada 161
7	Ada 1645

Seeds of maize plant were washed three times in 70% ethanol and then %5 bleach solution by shaking in a shaker for 5 minutes for sterilisation to remove any residual bleach solution, the seeds were rinsed in double distilled H_2O (ddH_2O). The seeds were planted into pots containing autoclaved sterile peat/vermiculite (1:1, w: w) soil mixture. The pots were placed in at green house having the following growth condition; 16-hour at 24 °C/day and 8-hour at 21 °C/night respectively at 70% humidity. The light period and intensity were 16h day and 8h night and 15.000 lux, respectively. The seedling growth was monitored during the V3, V4, and V5 stages. The length of the third leaves of maize plants was recorded daily.

3.2. Growth of Bacteria

Three different strains of *Bacillus spp.* were provided by the MBG department collection. Bacteria were taken from the stock at -80 °C and transferred to Luria-Bertani (LB) growth medium. The inoculums that were transferred to LB medium were grown overnight at 200 rpm at 30 °C. After a night, proliferating cells were obtained by centrifuging at 12,000 for 30 minutes at +4 ºC. The obtained cells were washed twice in a solution of 10 mM $MgCl_2$. Finally, the washed culture was diluted to 10^6 CFU/ml and became ready for inoculation.

3.3. Inoculation of Maize Seeds

In this study, non-bacteria inoculated group named as mock (M) was used as control, bacteria inoculated (I), 150mM NaCl treated (S) and both bacteria inoculated and salt stress treated group (I+S) were experimental groups. For the mock group, sterilized maize seeds were planted in pots and allowed to germinate in the greenhouse under controlled conditions. Mock, bacterial inoculated, salt stressed and both salt stressed and bacteria inoculated groups were cultivated under optimal conditions for comparative analysis For the bacteria inoculated group, the sterilized seeds were inoculated in previously prepared bacteria solution (10^6 CFU/ml) by shaking at 45 rpm for 3 hours at room temperature. Following inoculation, maize seeds were sowed in pots and allowed to germinate in the greenhouse under normal conditions. Sterilized seeds were transferred and grew under conditions following 16-hour at 25 °C/day and 8-hour at 21 °C/night with respectively at 70% humidity. Lx light intensity were 15,000. Plants were cultivated under controlled conditions until their fourth leaves reached full maturity.

3.4. Seed Germination by Paper Towel Method

The paper towel method was used to grow maize seedling (Figure 3.1). Each treatment received 10 seeds with three replications in a totally randomized design and was incubated in a climate room at 25°C. Mock and inoculated maize seeds were separately placed on sterile towel papers at fixed intervals. The towel papers were then moistened with sterile water until they were sufficiently moist. Maize seeds

were gently wrapped into paper towels and placed in glass containers. Finally, they were placed in the plant growth chamber at 25°C for their germination. The number of germinated seeds was counted after 7 days for each treatment.

Figure 3.1: Maize seedlings grown in paper towel method.

3.5. SPAD Analysis of Bacterial Effect on Chlorophyll Content

The chlorophyll content of maize seedlings grown in the greenhouse was measured after 14 days, when the third leaves had fully grown (Figure 3.2). To obtain more healthy results, SPAD measurements were obtained from the three parts of the same leaf by tip, middle and the starting point. Averages of these three measurements were calculated and statistics done in Microsoft Excel 365 Program.

The effect of inoculation with bacteria on the content of photosynthetic pigments was measured using the Chlorophyll meter SPAD-502 Plus (Konica Minolta).

Figure 3.2: Chlorophyll measurement from maize leaf with SPAD-520Plus.

3.6. Measurement of Leaf Elongation Rate

The 3rd leaves of the maize seedlings were measured from their emergence until fully grown. The mean and standard deviation of all data were calculated, and a leaf length (LL) profile graph has been generated. The leaf elongation rate (LER) was estimated using formula 3.1 by calculating the derivative of leaf length. In the formula, x_1 stands for the length measured on the first day, while x_2 represents the length measured on the second day. The time between two measurements is represented by t. The significance of the difference between the groups was determined according to Student's T-test in the Microsoft Excel 365 program.

$$LER = \frac{x_2 - x_1}{t_2 - t_1} \tag{3.1}$$

3.7. Maize (*Zea mays L.*) Growth Conditions

3.7.1. Greenhouse Conditions

In greenhouse conditions the only bacteria studied was *Bacillus pumilus*. The maize seeds were put together with the PGPR solution which contains *Bacillus*

pumilus and left for 3 hours in room temperature in order to increase the adhesion of bacteria to the seed. After bio-priming application the seed were sowed into pots. The maize seeds were kept moist until they germinated. After germination, the water and nutrient requirements of the maize seedlings were met with the help of Hoagland solution. For salt stress applications, Hoagland solution was prepared with 150mM NaCl. After germination, all plants have been watered with Hoagland solution daily. To avoid contamination, the leaf lengths of both mock and bacteria inoculated groups were measured using separate rulers.

Figure 3.3: Maize seedlings grown in greenhouse conditions.

3.7.2. Growth Chamber Conditions

In growth chamber conditions three *Bacillus spp.* (*Bacillus pumilis, Bacillus licheniformis, Bacillus coagulans*) and mixture of these three *Bacillus spp.* were used. In order to grow maize in the growth chamber, paper towels and glass containers which the seeds were placed were autoclaved. To germinate the maize seeds by using the paper towel method, the seeds were placed at periodic intervals. To adhere the seeds, paper towels were moistened as needed and gently rolled into glass containers. To promote better seed germination, the seeds were prevented from full light until rooting initiated. Three concentration of salt stress was tried out to find the optimal level for the future experiments. After the application of 100mM, 150mM and 200mM of salt concentration to the plants, 150mm was chosen to use in our experiments.

Figure 3.4: Maize seedlings grown in growth chamber conditions.

3.8. Hoagland's Solution for Watering of Plants

Hoagland's Solution was prepared to provide required essential nutrients for growth of maize plants (Table 3.2). Maize plants were grown in hydroponic medium containing Hoagland nutrient solution for 14 days.

Table 3.2: The composition of the modified Hoagland Solution.

Macronutrients	Micronutrients
$CaCO_3$ NH_4NO_3 KH_2PO_4 $MgSO_4.7H_2O$	H_3BO_3 $MnSO_4.4H2O$ $CuSO_4.5H_2O$ $C_4H_6O_4Zn2.H_2O$ K_2SO_4 KCl $(NH_4)_6Mo_7O_{24}.4H_2O$
Fe Na EDTA	

3.9. Enzymatic Analysis

3.9.1. Total Soluble Protein Extraction and Determination in Plant Root Tissues

In order to extract total protein, roots of Sasa156 seedlings were harvested after 14 days. The primary roots of maize plants were separated and sampled for 3cm from the root tips. The sampled tissues were immediately transferred to 2 ml tubes and

fixed in liquid nitrogen. Tissue samples were stored at -80°C for future experiments. Under all, 30 samples from M, I, S and I+S groups, including three biological replicates of each sample of seedlings were utilized for comparative and statistical analyses. Root samples were taken from -80 °C to liquid nitrogen. Three metal beads (4 mm in diameter) were inserted in tubes and then the tubes placed into homogenizer blocks. The homogenizer was set at 25 seconds/25 frequencies and executed. After the device was turned off, tubes were quickly immersed in liquid nitrogen. On the samples, 1 ml of extraction buffer containing 50 mM KH_2PO4 (KP) (pH = 7.8), 1mM EDTA, and 2% PVP was added. Tubes were thoroughly and carefully vortexed to avoid overflow. The vortexed samples were centrifuged for 30 minutes at 10,000 rpm at + 4 °C. The supernatant from the centrifuged samples were transferred to new clean tubes without contacting the beads and stored at -20 °C for the future analysis.

The total protein quantity was measured using the Bradford Assay [Bradford, 1976]. The concentration values for the standard curve were changed from 0 mg/ml to 1.2 mg/ml for the Bradford test from 2 mg/ml stock BSA (bovine serum albumin) solution. The sample dilution ratios were 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. Diluted BSAs were placed in the cell culture plate in the order of 5µl BSA, and 250 µl Bradford reagent. For the blank; 250 µl of Bradford reagent (SIGMA) and for protein samples; 5 µl of protein and 250 µl of Bradford reagent were used. The wells were mixed thoroughly by pipetting. Samples were incubated for 10 minutes. The absorbance value of the Spectral scanning multimode reader (Thermo Scientific Varioskan Flash) was set up to 595 nm. Finally, the standard curve was calculated.

3.9.2. Determination of Catalase (CAT) Enzyme Activity

The catalase enzyme activity was measured using the technique provided by Chance and Maehly [1955]. For the experiment, 1.5 mL quartz cuvettes were used. For the reaction in the experiment; 200 mM of 100 µl of H_2O_2, 50 mM 850 µl of K-P buffer (pH: 7.0) and 50 µl of (2-10 µg/ml) of protein extract were used. For the blank; 900 µl of potassium phosphate buffer (KP buffer), 200 mM of 100 µl of H_2O_2

and for working solution; 880 µl of KP buffer, 100 µl of H_2O_2 and 20 µl of protein were used. Absorbance was set to 240 nm, and samples were measured and recorded using a spectrophotometer (Shimadzu, UV1280) for 90 seconds. According to Aebi (1983) catalase activity was calculated. Catalase degrades H_2O_2 to produce water and oxygen. 1 unit of catalase enzyme was introduced as the quantity of enzyme required to degrade 1M of H_2O_2 and release water and oxygen (3.2)

$$Unit\ Activity\ (Units/min/gFW) = \frac{Change\ in\ absorbance/minute \times total\ volume\ (ml)}{Extinction\ coefficient\ \times volume\ of\ sample\ taken\ (ml)}$$

$$Extinction\ coefficient = 6.93 \times 10^{-3} mM^{-1} cm^{-1} \qquad (3.2)$$

The statistical significances were computed by taking the mean of three biological and technical replicates, and the error bars on the graph were marked as standard error. The significance of the difference between the mock and inoculated groups was assessed using the Excel 365 program's Student's t-test. The difference was regarded significant when the P value was lower than 0.05.

3.9.3. Determination of Peroxidase (POX) Enzyme Activity

Peroxidase enzyme activity was calculated according to Quesada et al. [1990]. For the experiment, 1.5 mL quartz cuvettes were used. For the reaction in the experiment, 50mM 880 µl of KP buffer, 20µl of protein extract, 10mM 50 µl of H_2O_2 and 0.02% 50 µl of dianisidine were used. For the blank; 50mM 900 µl KP buffer, 10mM 50 µl of H_2O_2 and 0.02% 50µl dianisidine were used.

The differences in absorbance of the samples were measured in a spectrophotometer (Shimadzu, UV-1280) at 460 nm for 1.5 minutes. The peroxidase enzyme activity is determined according to Quesada et al (1990). The amount of enzyme that required to oxidize 1µM of H_2O_2 described as 1 unit of the enzyme. The analysis was computed by taking the mean of three biological and technical replicates, and the error bars on the graph were marked as standard error.

3.9.4. Determination of Ascorbate Peroxidase (APX) Enzyme Activity

The activity of APX enzyme was measured using the technique provided by Nakano and Asada [1981]. For the experiment, 1.5 mL quartz cuvettes were used. For the reaction in the experiment, 830 µl of KP buffer, 50 µl of H_2O_2, 50 µl of 0.5mM ascorbic acid, 50 µl of 0.1mM EDTA and 20 µl of protein were used. For the blank, 850 µl of 50mM KP buffer, 50 µl of 0.1mM EDTA, 50 µl 0.5mM ascorbic acid and 50 µl 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 µM of ascorbate. The analysis was computed by taking the mean of three biological and technical replicates, and the error bars on the graph were marked as standard error.

3.9.5. Determination of Glutathione Reductase (GR) Enzyme Activity

Glutathione reductase catalyses the oxidation of NADPH to reduce glutathione disulfide (GSSG). Glutathione reductase enzyme activity method performed according to Çakmak et al. [2006]. For the experiment, 1.5 mL quartz cuvettes were used. For the reaction in the experiment, 50 mM K-P buffer and 0.1 mM 880 µl of EDTA K-P buffer solution, 0.5mM 50 µl GSSG, 0.12 mM 50 µl NADPH, 20 µl protein extract were prepared to use. For the blank, 850 µl of 50Mm KP buffer, 50 µl of 0.1mM EDTA, 0.5mM 50 µl of GSSG and 0.12 mM 50 µl of NADPH were added. Samples were placed on a spectrophotometer (Shimadzu, UV-1280) for 3 min to measure absorbance changes at 340 nm.

The activity of the glutathione reductase enzyme was calculated according to Carlberg and Mannervik (1975). The amount of enzyme that required to oxidize 1 µM of NADPH/min/gFW defined as 1 unit of the enzyme. The analysis was computed by taking the mean of three biological and technical replicates, and the error bars on the graph were marked as standard error.

3.9.6. Determination of Superoxide Dismutase (SOD) Enzyme Activity

Superoxide dismutase (SOD) enzyme assay was performed according to the procedure provided by Banowertz et al., [2004]. The procedure is based on the inhibitory impact of SOD on nitro blue tetrazolium (NBT) dye by superoxide radicals generated by hydroxylamine hydrochloride auto-oxidation. The decrease in NBT was followed by an increase in absorbance at 540 nm. For this assay, a 96-well TPP cell culture plate was used. In reaction, 40 µl of 250mM K-P buffer (pH = 7.8), 26 µl of 100 mM methionine, 4 µl of 5mM EDTA, 20 µl of 750 mm of NBT, 90 µl of ddH_2O, 20 µl of protein extract and 0.002 g of riboflavin were added into the wells.

For the blank, 40 µl of 250mM K-P buffer (pH: 7.8), 20 µl of 750 mm NBT, 26 µl of 100 mM methionine, 90 µl of ddH_2O, 4 µl of 5mM EDTA and 0.002 g of riboflavin were added. The cell culture plate was shaken for 1 minute in a Varioskan spectrophotometer (Thermo Scientific Varioskan Flash). After the shaking, the samples were measured at 560 nm absorbance. Following measurement, the cell culture plate was exposed to fluorescent light for 10 minutes. The samples were shaken again on the spectrophotometer for 1 min and the absorbance values measured at 560 nm. The activity of the SOD was calculated according to Kono [1978]. Under the experimental conditions, 1 unit enzyme is the amount of enzyme needed to suppress the absorbance difference from chromogen synthesis at 540 nm by 50% in one minute. The analysis was computed by taking the mean of three biological and technical replicates, and the error bars on the graph were marked as standard deviation.

3.10. Transcriptional Analysis

3.10.1. Investigation of Salt Stress-Responsive MicroRNAs and Their Predictable Target Genes

The salt stress-responsive miRNAs were determined based on literature search. mir396, mir319, mir169, and mir160 were identified as potential salt stress-responsive as being growth regulators. The coding sequences of the genes (CDS)

were retrieved from the maize genetics and genomics database (MaizeGDB; www.maizegdb.org), whereas the sequences of maize miRNA genes were taken from the miRbase (http://www.mirbase.org/). The chosen miRNAs' potential target genes; *Teosinte branched1 cycloidea transcription factors 5 (TCP5), Auxin response factor 17(ARF17), Growth regulation factor15(GRF15)* and *nuclear transcription factor Y subunit A-1 (NF_YA1)* were predicted by psRNATarget (A Plant Small RNA Target Analysis Server, 2014) web service [Dai and Zhao, 2011].

3.10.2. Primer Designing

The sequence information for specific primers for miRNA target genes was received from the Maize GDB database (https://www.maizegdb.org/) and designed using SCiTools Oligo-Analyzer 3.1 (https://eu.idtdna.com). Using SCiTools OligoAnalyzer 3.1, the designed primers were evaluated for melting temperature (Tm) at 58-61 °C, hair hairpin creation at 50 °C with ΔG> -3 kcal/mol, and self and counter dimer formation at 50 °C with ΔG> -6 kcal/mol. The BLASTn program was used to determine primer specificity. The conversion of miRNAs to cDNA were accomplished by the usage of universal reverse primer [Varkonyi-Gasic et al. 2007]. The list of primers used in thus study were listed in Table 3.3.

Table 3.3: qRT-PCR primers used to evaluate the transcription levels of miRNAs and their target genes

miR396c	miRNA sequence	5'-UUCCACAGGCUUUCUUGAACUG-3'
	Stem–loop RT Forward Primer	5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC **CAGTTC**-3' 5'- **TGC G**TT CCA CAG GCT TTC TT -3'
GRF15 (Growth Regulation Factor15)	Forward Reverse	5'-CGGTGCTCTAGAGATGCTGTC-3' 5'-TTGCCTTTCGGCCTTCCA-3'
miR319a-3p	Reverse	5'- AGA CCA CGA CGT CGA TAA -3'
	Stem–loop RT Forward	5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC **GGGAGC**-3' 5'- **GCG AC**T TGG ACT GAA GGG T -3'

Table 3.3: Continued

TCP5 *(TEOSINTE BRANCHED1, CYCLOIDEA transcription factors 5*)	Forward Reverse	5'- CAGCCTCACCACGCAATC -3' 5'- AGGCACGCATGCTGAGA -3'
miR169f-5p	miRNA sequence	5'-UAGCCAAGGAUGACUUGCCUA-3'
	Stem–loop RT Forward	5'-TCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC **TAGGCA**-3' 5'- **CGG GT**T AGC CAA GGA TGA CTT -3'
NF_YA1 (Nuclear transcription factor Y subunit A-1)	Forward Reverse	5′-ATGCAGCAGCGTGTTTC-3′ 5′-CAACAGCCAAGGATGAGTTG−3′
miR160d-5p	miRNA sequence	5'-UGCCUGGCUCCCUGUAUGCCA-3
	Stem–loop RT Forward	5' GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC **TGGCAT**-3' 5'- **CCT CT**T GCC TGG CTC CC -3'
Arftf17 (Auxin Response Factor 17)	Forward Reverse	5'-AGCCCACTTCGCTGTTTCT-3' 5'-GCTGGTTAAGCTGGTAGTCTGT-3'
Universal	Reverse 2	5' CA GGG TCC GAG GTA TTC G 3'
β-TUB (Beta Tubulin)	Forward Reverse	5’-CTACCTCACGGCATCTGCTATGT-3 5’-GTCACACACACTCGACTTCACG-3’

3.10.3. RNA Isolation

The TRIzol technique was used to extract the total RNA. The primary roots of maize plants were separated and sampled for 3cm from the root tips. The sampled tissues were immediately transferred to 2 ml tubes and fixed in liquid nitrogen and stored at -80 °C until analysis. Root samples were taken from -80 °C to liquid nitrogen before starting to extraction. Three metal beads (4 mm in diameter) were inserted into each tube and the tubes were placed into homogenizer blocks. The homogenizer was set at 25 seconds/25 frequencies and executed. After the device was turned off, the tubes were quickly immersed in liquid nitrogen. 1 ml of TRIzol (Invitrogen) solution added into each tube. Following that, the tubes were vortexed

well and left to incubate at room temperature for 5 minutes. When the incubation period ends, 200 µl of chloroform was added to the tubes corresponding to 1ml TRIzol, and the mixture was allowed to incubate for 3 minutes. The maize root samples were centrifuged for 15 minutes at + 4 °C at 11,000 rpm., and the supernatant (liquid phase) was transferred to clean tubes. 450 µl of isopropanol was added onto supernatant which was taken to the new tubes (for 450 µl of supernatant). Samples were allowed to incubate at room temperature for 10 minutes. Following incubation, the samples were centrifuged at 11,000 rpm for 10 minutes at 4 °C. After centrifugation, the supernatant was discarded. 1ml of 75 % EtOH (for 1ml TRIzol) was used to wash the pellets. The washed pellets were re-centrifuged at 11,000 rpm for 5 minutes at 4 °C. When the centrifugation was completed, EtOH was removed from tubes. The pellet in the tubes were left to dry. Finally, 20 µl of ddH_2O was added into each tube and the samples were stored at -20 °C. Concentration and purity of isolated RNAs were determined by Nano-Drop (Thermo Scientific, NanoDrop Lite Spectrophotometer). The ratio of absorbance at 260/230 nm was used to assess purity of RNAs, and for RNA; a ratio of 2.0 is approximately accepted as pure and those samples with approximately 2.0 ratio were used in cDNA synthesis.

3.10.4. cDNA Synthesis and Stem-loop Reverse Transcription

cDNAs of miRNA target genes were synthesized using the protocol described in the SuperScript III First-Strand Synthesis Kit (Invitrogen). mRNAs were transformed to cDNA by reverse transcriptase enzyme (RNA dependent DNA polymerase) and oligo (dT) primers used as templates. Total RNAs specifically synthesized in previous experiments were used for cDNA synthesis. For each reaction, it was planned to use 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) (50u/ml) and 2 µl total RNA. However, due to different RNA concentrations, the required RNA amounts were calculated separately for each group. The PCR conditions were set as shown in Table 3.3. After the reaction was finished, the samples were stored at -20 °C for later use.

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	∞

The miRNA cDNA was synthesized using the stem-loop RT procedure proposed by Varkonyi-Gasic et al [2007] (Figure 3.5). For each reaction; 2 µl RT buffer(10x), 0.2 µl dNTP (100mM), 2 µl stem-loop primer (5µM), 4 µl of RNA (1000 µg/µl),0.8 µl of RTase (50u/µl) and 11 µl of ddH_2O (double distilled water) were used. Due to different RNA concentrations, the required RNA and ddH_2O amounts were calculated separately for each group. Stem-loop primers were prepared at 65 °C for 5 min and left to incubation on ice for 5 min. The PCR machine (PTC-200, MJ Research) was programmed to complete 60 cycles, with each cycle lasting 30 minutes at 16 °C, 30 seconds at 30 °C, 30 seconds at 42 °C, and 1 second at 50 °C, with a final stage lasting 5 minutes at 85 °C (Table 3.4). Following the operation, the samples were withdrawn from the device and stored at -20 °C for future usage.

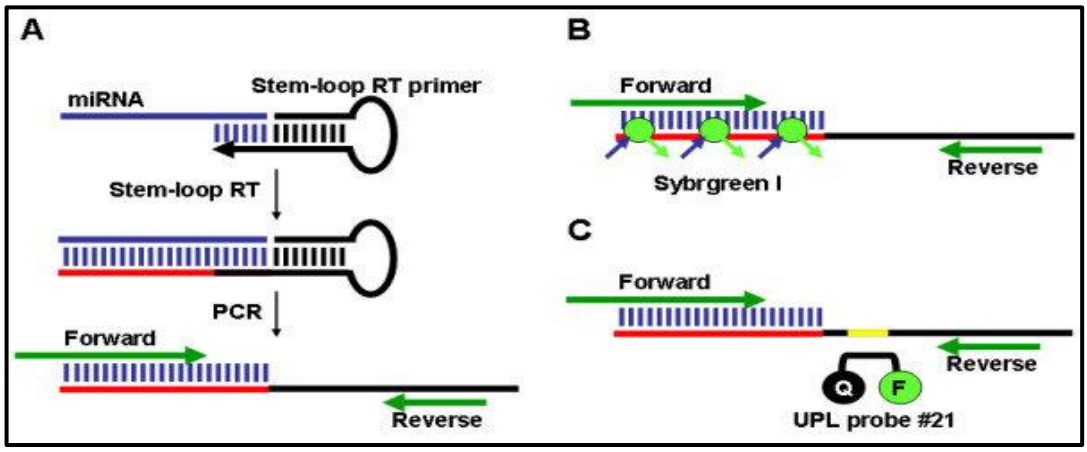

Figure 3.5: Schematic representation of SYBR green I miRNA assays. Real time quantification of miRNAs using SYBR green I includes two steps; A; stem–loop RT and B; SYBR green I real-time PCR assay.

Table 3.5: miRNA stem-loop Real Time-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	60cycle			incubation

3.10.5. Determination of Transcript Amount by Quantitative RT-PCR (Quantitative Reverse Transcriptase-PCR) Method

The quantitative RT-PCR (qRT-PCR) (LightCycler® 480, Roche) analysis was used to measure the expression levels of miRNAs that are related to salt stress and their predicted target genes. For qRT-PCR analyses, Platinum® SYBR® Green qPCR SuperMix UDG (Invitrogen) was used. For miRNAs, 10 µl of Syber Green, 1 µl of forward primer (F) (10 µM), 1 µl of universal reverse (UR) (10 µM), 2 µl of c-DNA and 6 µl of ddH_2O were added. For the miRNA that targets growth; 7,5 µl of Syber green, 0,6 µl of forward primer (10 mM), 0,6 µl of reverse primer (R) (10 mM), 1 µl of cDNA and 5,3 µl of ddH_2O were added. qRT-PCR conditions were set up to as, pre-denaturation at 95 °C for 5 min was followed by 40 cycles of 95 °C for 5 s, 60 °C for 10 s, and 72 °C for 0.01 s. The melting curve was adjusted as 95 °C for 15s, 60 °C for 1 min and 95 °C for 15 s (Table 3.5). Relative amount of the expression was determined according to delta-delta Ct ($2^{-\Delta\Delta Ct}$) method. Normalization was performed with the β-tubulin gene (Housekeeping gene). Analysis was operated as 3 biological and 2 technical replicates. The expression amount of the transcripts was represented as relative to mock group [Varkonyi-Gasic et al., 2007].

Table 3.6: miRNA Sybr Green qRT PCR conditions.

Pre-denaturation	Cycling Stage			Melt Curve Stage		
95°C	95°C	60°C	72°C	95°C	60°C	95°C
5 min	5 sec	10 sec	1 sec	15 sec	1 min	15 sec

3.11. Statistical Analysis

Each experiment was repeated twice, and each data set contained the outcomes of three biological replicates. The results were represented as means and error bars were shown as standard error in the graphs. The Microsoft Excel Student's t-test was used to evaluate the significance of the difference between the groups. Results indicating $P<0.05$ were considered to have significant difference between groups.

4. RESULTS

4.1. Morphological Comparisons Between Hybrid Maize Lines at Greenhouse Conditions

To compare mock and inoculated seven maize hybrids under salts stress at morphologically, the seeds were planted into soil and grown at greenhouse until their third leaves were fully grown. The germination capability and growth abilities of seedlings were observed. Leaf elongation rates (LER), the length of 3rd leaves of the seedlings were measured daily. When the 3rd leaves were fully matured, the seedlings were harvested and dry root and dry shoot weights, fresh root and fresh shoot weights were evaluated (Table 4.1). After the third leaves of maize emerged and started to elongate, the chlorophyll content of the leaves was measured every day by a Chlorophyll meter SPAD-502 Plus (Konica Minolta). Furthermore, the tolerant and sensitive lines to salt stress were determined. Seeds inoculated with bacteria were also screened and evaluated at green house condition. Following morphological comparisons between all these groups, Sasa139 and Sasa152 maize hybrids were chosen to pursue the experiment. To obtain more accurate results, the experiment repeated several times to see if our previously selected hybrid lines are sensitive or tolerant to salt stress.

4.1.1. Physiological Assessment of Maize Hybrid Lines

Maize hybrid lines including Adasa16, Sasa139, Sasa152, Sasa166 and Sasa186 were evaluated based on their Root Fresh Weight (RFW), Root Dry Weight (RDW), Shoot Fresh Weight (SFW), Shoot Dry Weight (SDW) and Chlorophyll Content (Table 4.1).

Table 4.1: The chlorophyll content (CC), root dry/fresh weights (RD/RFW) and shoot dry/fresh weights (SD/SFW) of the third leaf of maize hybrids grown at greenhouse conditions under mock (M), inoculated (I), 150mM NaCl applied salt stress (S) and inoculated + salt stressed (I+S) conditions.

		CC(SPAD)	RFW (g)	RDW (g)	SFW (g)	SDW (g)
Adasa16	M	29 ±3	1.95 ±0.17	0.14 ±0.01	2.26 ±0.34	0.15 ±0.02
	I	27 ±3	1.47 ±0.01	0.11 ±0.01	1.62 ±0.12	0.11 ±0.02
	%M-I	NS	(-24) *	(-21) *	NS	(-28) *
	S	25 ±5	1.86 ±0.34	0.16 ±0.01	2.26 ±0.38	0.12 ±0.01
	%M-S	(-11) *	NS	NS	NS	NS
	I+S	27 ±4	1.70 ±0.02	0.14 ±0.02	1.71 ±0.02	0.13 ±0.03
	%M-(I+S)	NS	NS	NS	NS	NS
Sasa139	M	29 ±4	1.87 ±0.23	0.16 ±0.04	2.03 ±0.21	0.14 ±0.02
	I	33 ±2	1.88 ±0.04	0.16 ±0.06	1.85 ±0.37	0.14 ±0.04
	%M-I	(+14) **	NS	NS	NS	NS
	S	22 ±4	1.78 ±0.23	0.19 ±0.04	1.76 ±0.23	0.12 ±0.02
	%M-S	(-24) **	NS	NS	NS	NS
	I+S	24 ±4	1.82 ±0.03	0.21 ±0.06	1.74 ±0.04	0.11 ±0.03
	%M-(I+S)	(-15) *	NS	NS	NS	NS
Sasa152	M	28 ±4	1.75 ±0.26	0.13 ±0.02	1.55 ±0.18	0.10 ±0.01
	I	29 ±4	1.60 ±0.02	0.12 ±0.02	1.52 ±0.34	0.12 ±0.03
	%M-I	NS	NS	NS	NS	NS
	S	26 ±3	1.81 ±0.02	0.16 ±0.02	1.70 ±0.21	0.11 ±0.02
	%M-S	(-7) *	NS	NS	NS	NS
	I+S	29 ±3	1.99 ±0.03	0.16 ±0.02	2.14 ±0.11	0.15 ±0.01
	%M-(I+S)	NS	(+14) *	(+23) *	(+39) **	(+36) *
Sasa166	M	28 ±4	1.49 ±0.18	0.13 ±0.05	1.47 ±0.25	0.10 ±0.03
	I	28 ±5	1.34 ±0.02	0.09 ±0.03	1.37 ±0.20	0.10 ±0.02
	%M-I	NS	NS	NS	NS	NS
	S	24 ±3	1.41 ±0.12	0.10 ±0.02	1.50 ±0.11	0.11 ±0.04
	%M-S	(-14) **	NS	NS	NS	NS
	I+S	25 ±3	1.16 ±0.04	0.08 ±0.03	1.31 ±0.10	0.09 ±0.01
	%M-(I+S)	NS	NS	NS	NS	NS
Sasa186	M	30 ±4	1.53 ±0.13	0.12 ±0.03	1.97 ±0.39	0.13 ±0.03
	I	31 ±6	1.56 ±0.03	0.12 ±0.02	1.78 ±0.16	0.12 ±0.03
	%M-I	NS	NS	NS	NS	NS

Table 4.1: Continued.

S	25 ±3	1.40 ±0.22	0.13 ±0.03	1.64 ±0.20	0.11 ±0.02
%M-S	(-16) **	NS	NS	NS	NS
I+S	29 ±3	1.5 ±0.04	0.15 ±0.02	1.77 ±0.40	0.13 ±0.04
%M-(I+S)	NS	NS	NS	NS	NS

CC: Chlorophyll content, M: Mock; I: Inoculated; S: Salt Stressed; I+S: Inoculation and Salt Stress applied; ave ±sd; n=5; NS: Not Significant; %: The percentage difference between mock against other treatments (-% indicates percent reduction against mock, +% indicates percent increased against mock).
Significant at $p<0.05$ = *, $p<0.01$= **.

Chlorophyll content (CC) of all inoculated maize hybrids showed increase except Adasa16. However, only Sasa139's chlorophyll content increase was significant against the mock by %14 ($p<0.01$). Root fresh weight (RFW) of the Sasa152 maize hybrid line was significantly increased against the mock by %14 ($p<0.05$). RFW of the three lines with I+S treatment showed increase against the salt treatments which was Sasa139, Sasa152 and Sasa186 but none of them was significant based on p value. Root Dry Weight (RDW) of the Sasa152 with I+S treatment showed significant increase against the mock by %23 ($p<0.05$). Only Sasa152 and Sasa186 showed an increased RDW against salt stress treatments but there was no significance.

Only the I+S treated Sasa152 showed significant increase against the mock at Shoot Fresh Weight (SFW) and Shoot Dry Weight (SDW). I+S treated Sasa152 increased the SFW by 39% against mock ($p<0.01$). This I+S treated group also showed an increase against both S and I treated groups but there was no significance. I+S treated Sasa152 showed a significant increase against the mock group by 36% ($p<0.05$) in the SDW. Only I+S treated Sasa152 increased the SDW against the salt treated maize hybrids, but no significance seen.

Salt treatment reduced the CC significantly in all the maize hybrids. CC were reduced 11% ($p<0.05$) in Adasa16, 24% ($p<0.01$) in Sasa139, 7% ($p<0.05$) in Sasa152, 14% ($p<0.01$) in Sasa166, 16% ($p<0.01$) in Sasa186.

4.1.2. Physiological Analyses of Selected Maize Hybrids

4.1.2.1 Leaf Elongation Rate of The Third Leaves

According to outcomes from screening investigations, Sasa139 and Sasa152 was chosen among five hybrids due to their advantages on both plant growth and survival ability under saline conditions. To obtain a proper overview of the growth of the Sasa139 and Sasa152 leaf, phenotypic, molecular, and enzymatic analysis was performed. The length of the third leaf of the mock and inoculated maize seedlings was measured every 24 hours to the tip of the leaf using the soil level as a reference point. Leaf elongation rate (LER) (mm/h) of the third leaf began to be measured from the emergence of the third leaf of maize. The third leaves of the inoculated and mock groups including Sasa139 and Sasa152 grew at a steady pace for four days, then the rate of growth slowed and stopped on the tenth day. However, the third leaves of the salt stressed, and inoculated salt stressed Sasa139 and Sasa152 could accelerate their leaf elongation rate for only the first two days and started to decline on the remaining days. As a result, no significant differences observed between inoculated and mock groups but the groups that faced salt stress showed considerable differences against the other treatments ($p<0.05$) (Figute 4.1 and Figure 4.2).

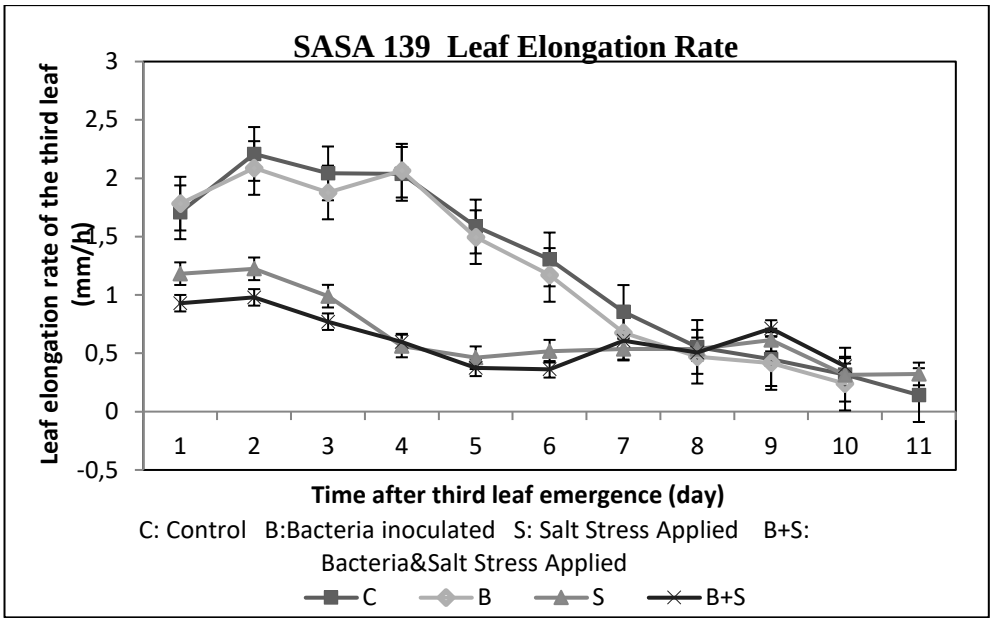

Figure 4.1: Leaf elongation rate (LER) of the Sasa139 maize seedlings grown under mock (M), inoculated (B), salinity stressed (S) and both bacteria inoculated and salt stress applied (B+S) conditions. LER (mmh^{-1}) was displayed over time period. Error bars represents the standard deviation ($P < 0.05$).

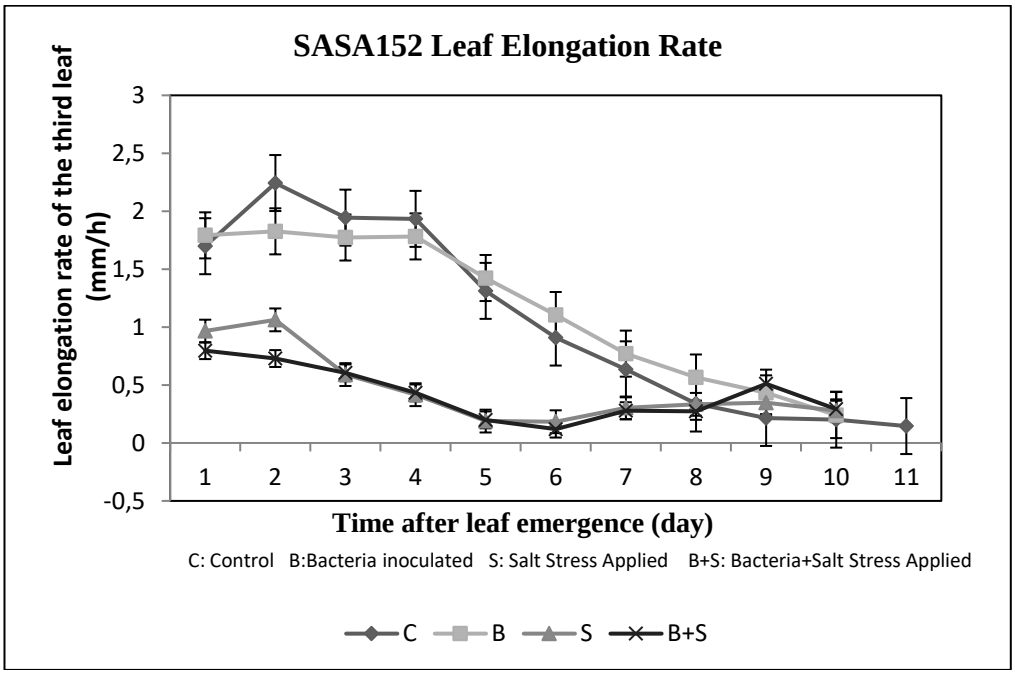

Figure 4.2: Leaf elongation rate (LER) of the Sasa139 maize seedlings grown under mock (M), inoculated (B), salinity stressed (S) and both bacteria inoculated and salt stress applied (B+S) conditions. LER (mmh^{-1}) was displayed over time. Error bars represents the standard deviation ($P < 0.05$).

4.2. Evaluation of Maize Hybrids at Growth Chamber Conditions

In order to optimize the concentration of salt stress and which concentration is suitable enough to proceed the experiment, three conditions (100mM, 150mM, 200mM) were planned to apply to the maize hybrids. Twenty maize seeds were used for each group. Maize hybrids grown under growth chamber conditions for 10 days and their germination rates were screened (Table 4.2).

Table 4.2: Germination rates of the seven maize hybrids grown under different concentrations of NaCl.

Germination Rate %				
Varieties	Control	100mM NaCl	150mM NaCl	200mM NaCl
Sasa155	87	90	70**	60**
Sasa156	95	85	80**	55**
Sasa159	87	85	80**	50**
Sasa196	85	90	70**	50**
Sasa197	95	90	85**	70**
Ada161	87	80	65**	50**
Ada1645	82	75	70**	50**

($p<0.05$=*, $p<0.01$=**)

According to our previous experiment on greenhouse, maize hybrids shown that they can tolerate NaCl at low concentrations. Sasa156 and Sasa197 had the highest germination rate at 100mM NaCl. Sasa155 and Sasa196 germination rates increased at 100mM NaCl. In no other case NaCl treatment produced better outcomes than the control plants. When maize hybrids were treated with 200mM NaCl, their germination rates dropped significantly. Sasa197 was the best germinating maize hybrid under both 150mM and 200mM NaCl treatment ($p<0.01$). However, the aim of the study was not to choose the most tolerant maize hybrid line. To investigate the effects of salt stress on plants, the maize hybrid Sasa156 was moderately responsive to all other concentrations so Sasa156 was selected for further enzymatic and transcriptional analysis.

4.2.1. Physiological and Morphological Assessments of Sasa156 and Effects of *Bacillus spp*. on Maize Plant Growth under Salt Stress

Root Length (RL) of all groups decreased significantly under 150mM NaCl application ranging from 26% to 63% ($p<0.01$ and $p<0.05$). Among other *Bacillus spp*., L23 (*Bacillus pumilus*) made the least contribution to root length. When the influence of *Bacillus spp.* on shoot length (SL) was studied, it was shown that P24 obtained better results among other *Bacillus spp.* and was more effective in enduring salt stress.

In terms of Root Fresh Weight (RFW), P24 (*Bacillus licheniformis)* achieved the best results (NS), although L23 alleviated the salt stress better in NaCl treatments. Mix (L23+P14+P24) treatment was the least contributing among other *Bacillus spp.* treatments at RFW. Even though P24 performed better RFW among other *Bacillus spp*. L23 outperformed P24 in NaCl applications in terms of RFW ($p<0.01$). The group that increased RDW the most was the Mix group (NS). NaCl treatments decreased the RDW in all treatments but P24 alleviated the effects of stress the most.

In terms of SFW, P24 obtained the closest results to the control group. In NaCl treatments, L23 inoculated maize plants had the highest SFW. In terms of shoot dry weight statistics, P14 (*Bacillus coagulans*) obtained the highest result. However, the P24 and mix treatments produced similar results. L23 inoculated maize plants were shown to have the optimal bacteria for increasing shoot dry weight and managing salt stress in NaCl treatments (Table 4.3).

Table 4.3: Physiological and morphological measurements of Sasa156 with inoculation of different *Bacillus spp.* under 150mM NaCl treatment.

Treatments	RL (mm)	%	RFW(g)	%	RDW(g)	%	SL(mm)	%	SFW(g)	%	SDW(g)	%
Control	180.6		0.67		0.05	NS	119.6		0.59		0.051	
L23	118.8	(-34)**	0.48	(-28)*	0.042	NS	88.9	(-26)**	0.45	NS	0.04	NS
P14	122.8	(-32)**	0.45	(-33)*	0.046	NS	96	NS	0.52	NS	0.056	NS
P24	160.7	NS	0.57	NS	0.05	NS	118	NS	0.54	NS	0.054	NS
Mix	151.3	(-16)*	0.44	(-34)**	0.053	NS	93.2	(-22)**	0.45	(-22)*	0.052	NS
NaCl	65.33	(-63)**	0.32	(-52)**	0.03	(-40)*	61.3	(-49)**	0.34	(-43)**	0.026	(-46)**
L23-NaCl	129.5	(-28)**	0.52	NS	0.047	NS	73.8	(-38)*	0.41	(-30)**	0.056	NS
P14-NaCl	127.1	(-35)**	0.39	(-42)**	0.032	(-36)*	70.6	(-41)**	0.34	(-43)**	0.044	NS
P24-NaCl	117.1	(-30)**	0.43	(-35)**	0.051	NS	80.9	(-32)**	0.38	(-36)**	0.043	NS
Mix-NaCl	133.9	(-26)**	0.44	(-34)**	0.049	NS	70.5	(-41)**	0,38	(-36)**	0.041	NS

RL: Root length; SL: Shoot length; RFW: Root fresh weight; RDW: Root dry weight; SFW: Shoot fresh weight; SDW: Shoot dry weight. Control: No inoculation; L23: *Bacillus pumilus*; P14: *Bacillus coagulans*; P24: *Bacillus licheniformis*; NaCl: 150mM NaCl application. ($p<0.05$ =*, $p<0.01$=**, NS= not significant).

4.3. Antioxidant Enzyme Activity of Sasa156 in Response to *Bacillus spp.* and Salt Stress

4.3.1. Results of Catalase (CAT) Enzyme Activity Analysis

Catalase enzyme activity in maize plants was calculated in control (C), salt stress (S), bacterial inoculation (L23, P14, P24, Mix), bacteria and applied salt stress (L23/, P14/S, P24/S, Mix/S) groups. The P24 inoculated (*Bacillus licheniformis*) group had the greatest catalase activity among all other groups and increased the catalase enzyme activity by 150% against to control group. The lowest catalase enzyme activity was observed in the control group. In the inoculation applications, it was observed that the catalase enzyme activity increased in a range from 7% to 79% compared to the group that was treated with only 150mM NaCl (S). There was no significance between the groups ($p<0.05$).

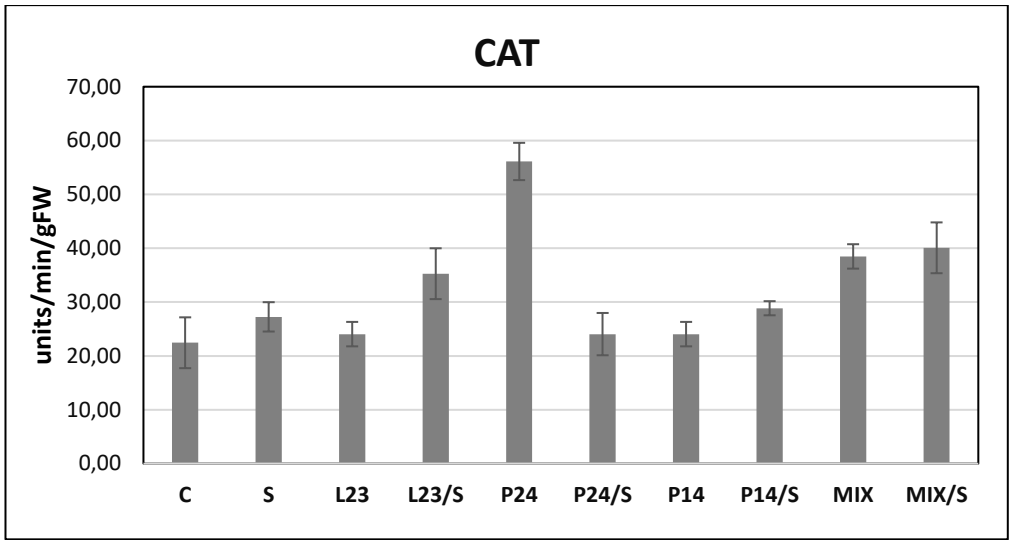

Figure 4.3: Enzyme activity of catalase in roots of Sasa156 response to inoculation of *Bacillus spp*. under 150mM NaCl application. C: Control; S: NaCl application; L23: *Bacillus pumilus*; P14: *Bacillus coagulans*; P24: *Bacillus licheniformis*; Mix: Bacterial consortium of L23, P14 and P24. The data indicates as mean, and error bars indicated ± standard error. (n=3, $p<0.05$ and $p<0.01$).

4.3.2. Results of Peroxidase (POX) Enzyme Activity Analysis

The lowest peroxidase enzyme activity was observed in control group. The biggest increase in peroxidase enzyme activity was recorded on maize hybrids inoculated with L23 (*B. pumilus*). When compared to the control plants, the group inoculated with L23 shown a significant increase in the peroxidase enzyme activity by 128%. ($p<0.05$).

Peroxidase enzyme activity of P24 (*B. licheniformis*) was the closest treatment to the control group by only 14% difference. After P24 the Mix groups was the second-best treatment in the case of alleviating the increased peroxidase enzyme activity by 14% to 23%. Following increased rates of POX results was; S by 53% increase, P14 101% increase, P14/S by 96% increase, P24/S by 26% increase, L23/S by 96% increase.

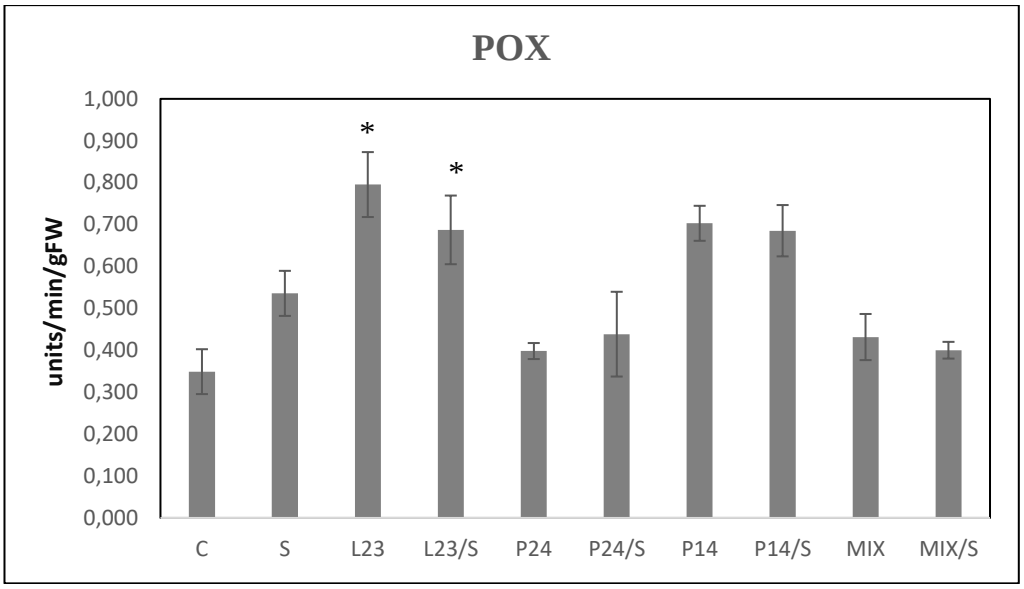

Figure 4.4: Enzyme activity of peroxidase in roots of Sasa156 response to inoculation of *Bacillus spp*. under 150mM NaCl application. C: Control; S: NaCl application; L23: *Bacillus pumilus*; P14: *Bacillus coagulans*; P24: *Bacillus licheniformis*; Mix: Bacterial consortium of L23, P14 and P24. The data indicates as mean, and error bars indicated ± standard error (n=3, $p<0.05$ and $p<0.01$).

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

The lowest ascorbate peroxidase (APX) enzyme activity was measured in P14/S group which includes inoculation of P14 (*B. coagulans*) with 150 mM NaCl treatment. P14/S group decreased the APX enzyme activity by %55 compared to control group. The highest APX enzyme activity was measured in P24/S group which includes inoculation of P24 (*B. licheniformis*) with 150 mM NaCl treatment. P24/S group significantly increased the APX enzyme activity by %311 compared to control group ($p<0.01$). Among the bacteria inoculated but not NaCl treated groups, L23 showed a significant %200 increase in APX enzyme activity compared to control group ($p<0.05$). In the other side, mix group decreased the APX enzyme activity by 44% which no significance recorded according to p value.

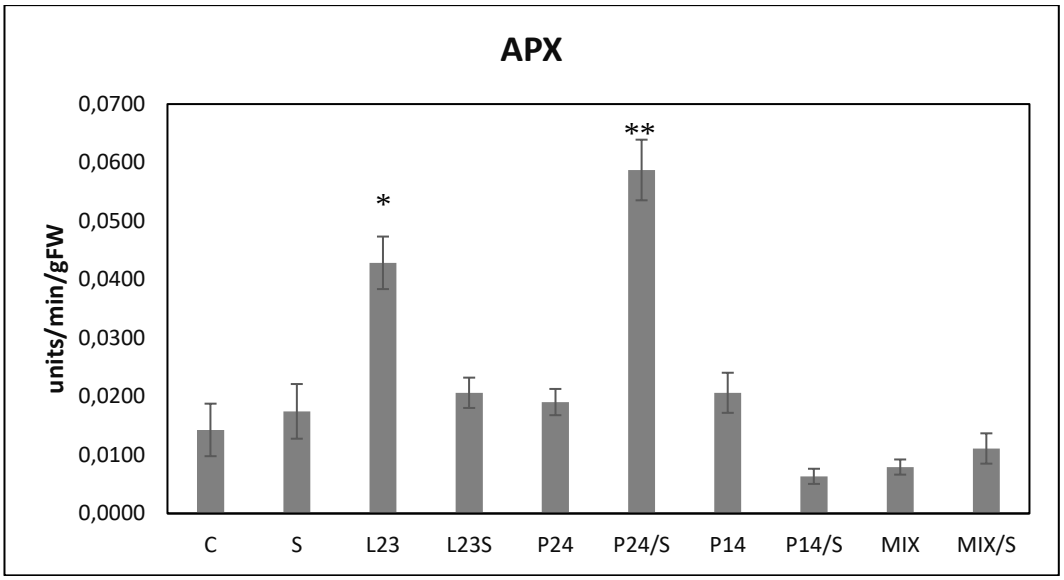

Figure 4.5: Enzyme activity of ascorbate peroxidase in roots of Sasa156 response to inoculation of *Bacillus spp*. under 150mM NaCl application. C: Control; S: NaCl application; L23: *Bacillus pumilus*; P14: *Bacillus coagulans*; P24: *Bacillus licheniformis*; Mix: Bacterial consortium of L23, P14 and P24. The data indicates as mean, and error bars indicated ± standard error. (n=3, $p<0.05$ and $p<0.01$).

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

The highest glutathione reductase (GR) enzyme activity was measured in control group. The lowest GR enzyme activity was measured in L23/S group by significant 60% decrease ($p<0.01$). The closest treatment to control group was P14 (*B. coagulans*) by only %16 decrease ($p<0.01$). Among the NaCl treated groups, only the P24/S group showed an increased amount of GR enzyme activity compared to P24 (*B. licheniformis*) inoculation. Among the inoculated treatments, L23 GR enzyme activity was 1.9 fold higher compared to L23/S treatment (Figure 4.6). The percentage decrease compared to the control group was as follows; P14 16%, L23 24%, S 24%, P24/S 24%, Mix 28%, P14/S 40%, P24 48%, Mix/S 48%, L23/S 60%.

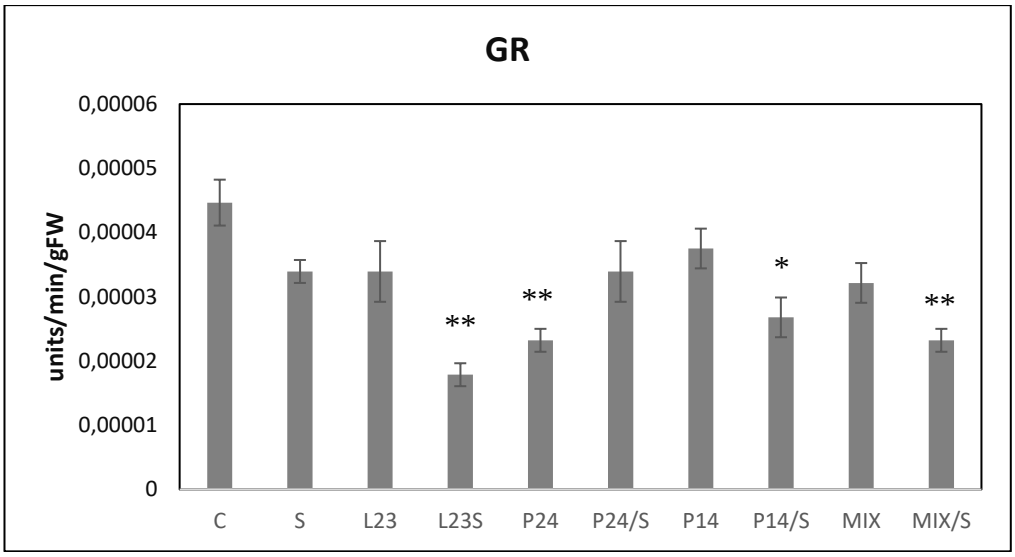

Figure 4.6: Enzyme activity of glutathione reductase in roots of Sasa156 response to inoculation of *Bacillus spp*. under 150mM NaCl application. C: Control; S: NaCl application; L23: *Bacillus pumilus*; P14: *Bacillus coagulans*; P24: *Bacillus licheniformis*; Mix: Bacterial consortium of L23, P14 and P24. The data indicates as mean, and error bars indicated ± standard error. (n=3, $p<0.05$ and $p<0.01$).

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

The highest superoxide dismutase (SOD) enzyme activity was measured in P14 (*B. coagulans*) inoculated group. P14 inoculation significantly increased the SOD enzyme activity by 310% compared to control group ($p<0.01$). The lowest amount of SOD enzyme activity was measured in Mix (L23, P14 and P24 bacterial consortium) inoculated group and this treatment showed a 8% reduced SOD enzyme activity compared to control group, but data was not considered significant. Among all treatments, only three of them showed a reduced SOD enzyme activity against control and they follow as; P14/S by 1% decrease, P24/S by 6% decrease and mix group by 8% decrease.

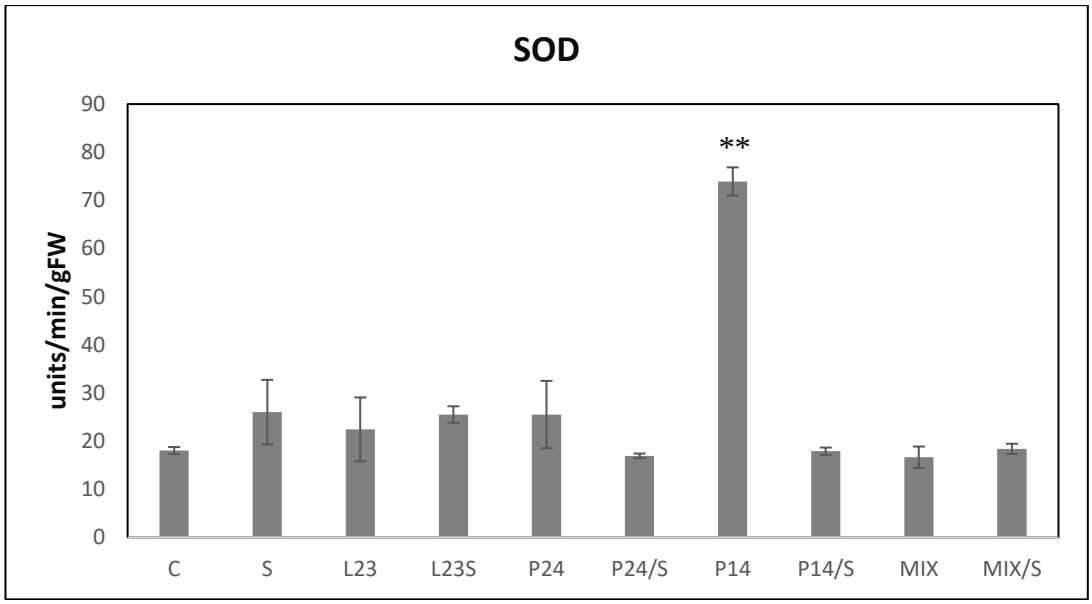

Figure 4.7: Enzyme activity of superoxide dismutase in roots of Sasa156 response to inoculation of *Bacillus spp*. under 150mM NaCl application. C: Control; S: NaCl application; L23: *Bacillus pumilus*; P14: *Bacillus coagulans*; P24: *Bacillus licheniformis*; Mix: Bacterial consortium of L23, P14 and P24. The data indicates as mean, and error bars indicated ± standard error. (n=3, $p<0.05$ and $p<0.01$).

4.4. The Expression Analysis of Putative Growth-Related miRNAs and Response of Their Predicted Targets Against *Bacillus spp.*

4.4.1. Expression Levels of miR160 and *ARF17*

To assess the correlation between growth-related miRNAs and their predicted target gene, expression levels of these two parameters were measured with qRT-PCR. The expression analyses were conducted on mock (M), salt stressed (S), P24 (*B. licheniformis* inoculation) and P24 inoculation under saline conditions (P24/S). The expression level of miR160 was highest in P24/S group and lowest in control group. Transcript levels of miR160 in inoculated groups (P24 and P24/S) were 5 to 9.5 folds higher compared to control groups ($p<0.01$). The highest ARF17 expression was recorded at NaCl treated group (Figure 4.8). ARF17 levels of S group was %78 higher than the control group ($p<0.05$). P24/S group which was treated with both bacterial inoculation and salt stress showed a higher ARF17 upregulation.

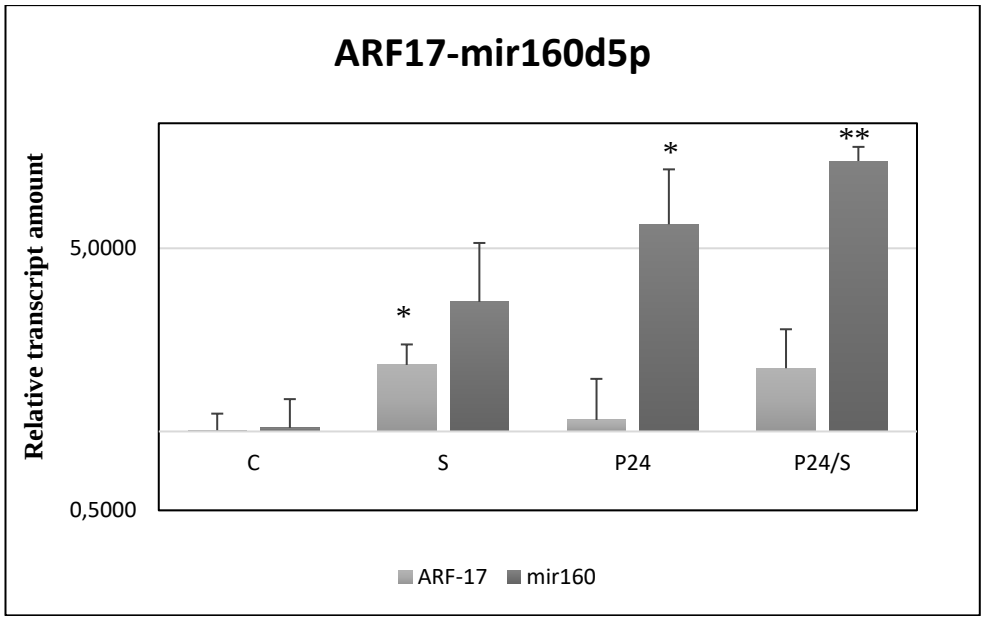

Figure 4.8: The expression analysis of mir160 and its predicted target gene *ARF17* in root of maize seedlings grown under the control (C), salinity stress (S), inoculated (P24) and inoculated and salinity stress (P24/S) applied conditions. (n=3, $p<0.05$ and $p<0.01$).

4.4.2. Expression Levels of miR169 and *NF_YA*

The expression level of mir169 was highest in P24 inoculated group and it increased the relative transcript amount 276% compared to control group which had the lowest relative transcript amount. Transcription level of NF_YA gene was very low in all groups (Figure 4.9). P24/S group showed 105% increase in relative transcript amount compared to S group but the data showed no significance ($p<0.05$). Transcript levels of miR169 in inoculated groups (P24 and P24/S) were 28 to 120 folds higher compared to control groups ($p<0.01$).

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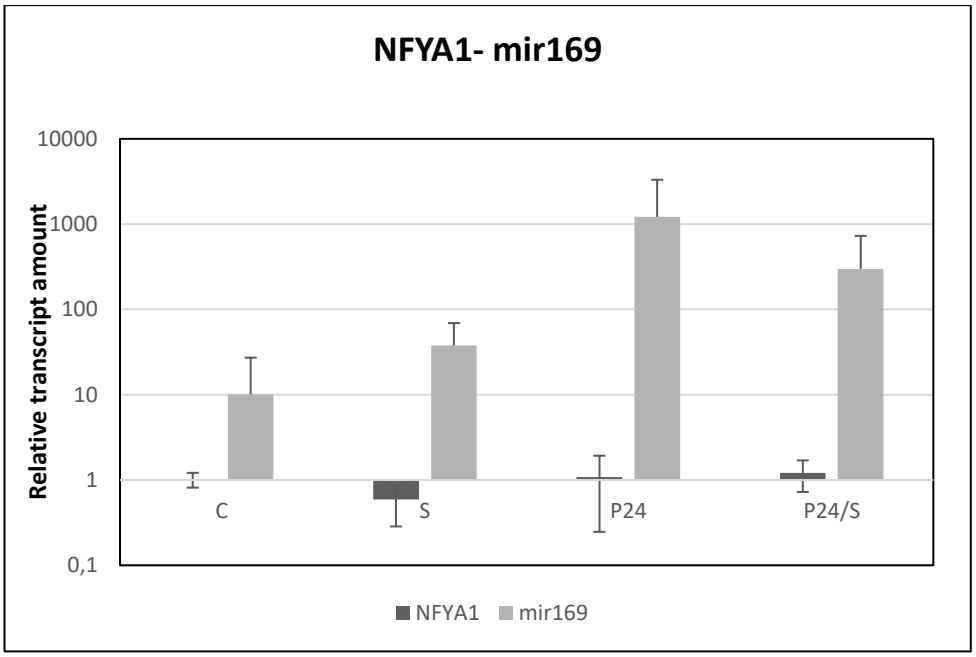

Figure 4.9: The expression analysis of mir169 and its predicted target gene *NF_YA1* in root of maize seedlings grown under control (C), salinity stress (S), inoculated (P24) and inoculated and salinity stress (P24/S) applied conditions. (n=3, $p<0.05$ and $p<0.01$).

4.4.3. Expression Levels of miR319 and TCP5

The expression levels of miR319 were highest in P24 and lowest in control group. The relative transcript amount of mir319 in P24 group was 570% higher compared to control group but no significance recorded ($p<0.05$). The expression levels of TCP5 gene in all group were significantly low compared to miR319. The highest TCP5 gene activity was measured in P24/S group which was 70% higher than the control group (Figure 4.10). The only group that has increased the expression levels of TCP5 against to control group was NaCl treated S group by 61% but the data showed no significance ($p>0.05$).

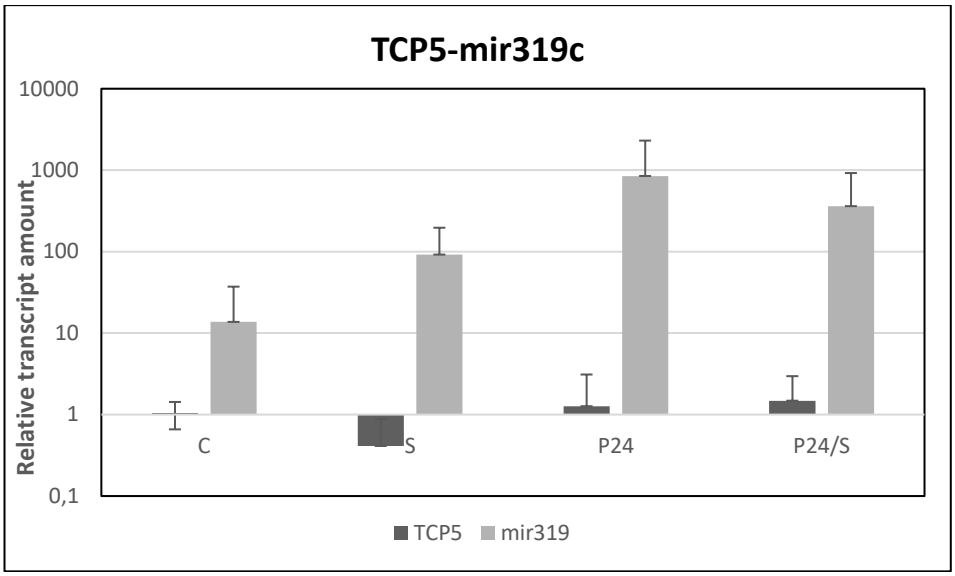

Figure 4.10: The expression analysis of miR319 and its predicted target gene *TCP5* in root of maize seedlings grown under control (C), salt stress (S), inoculated (P24) and inoculated and salinity stress (P24/S) applied conditions. (n=3, $p<0.05$ and $p<0.01$).

4.4.4. Expression Levels of miR396c and *GRF5*

The expression levels of miR396 was highest in P24 inoculated group and lowest in control group. P24 inoculated maize roots showed higher expression levels of miR396 and *GRF5* respectively. There was two group treated with salt and the *B. licheniformis* inoculated P24/S group showed a significant increase in miR396 by 92% compared to no inoculated S group.

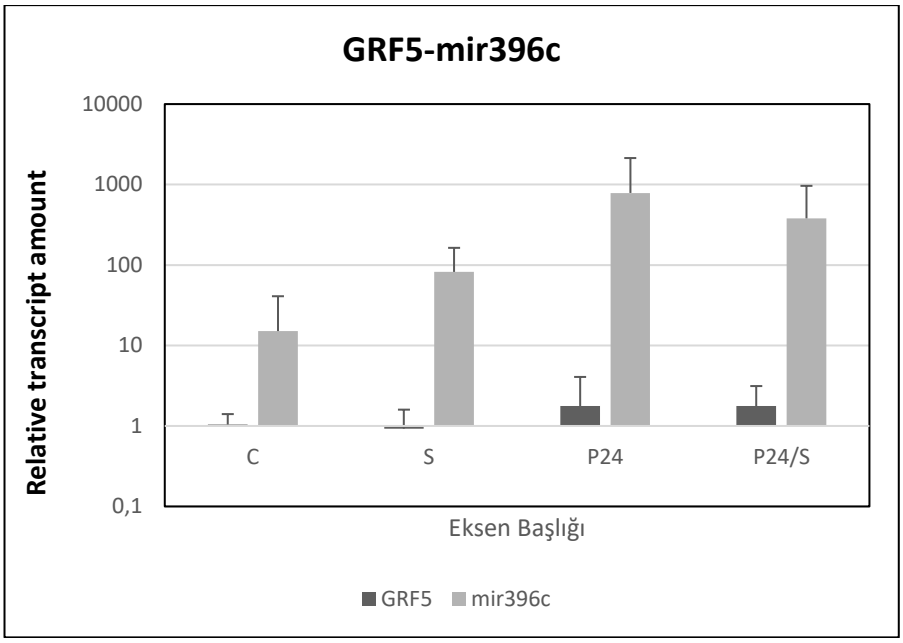

Figure 4.11: The expression analysis of miR396 and its predicted target gene *GRF5* in root of maize seedlings control (C), salinity stress (S), inoculated (P24) and inoculated and salt stress (P24/S) applied conditions. (n=3, $p<0.05$ and $p<0.01$).

5. CONCLUSION and DISCUSSION

Chemical fertilizer demand is increasing in developing countries on a daily basis and Turkey is one of them. Chemical fertilizers not only damage soil microflora and fauna, but they also create land pollution, water pollution, health issues, and boost production costs. As a result, there is great necessity to establish an alternative to these toxic products. In this context, PGPR applications can be used as an alternate path to secure both the land and the food. Many studies have shown that a variety of PGPR species including *Bacillus*, *Pseudomonas*, *Rhizobium*, *Azotobacter*, *Enterobacter* and *Azospirillum* have high resistance to biotic and abiotic conditions [Kashyap et al., 2019]. We would expect to see environmentally sustainable PGPR products that are more reliable than chemicals if we understand the actual mechanism of PGPR and the influence of abiotic stressors on the relationship of host and PGPR.

Morphological, physiological, and molecular studies were conducted to determine if *Bacillus spp*. that we have actually exhibit PGPR activity. An experiment was designed to choose one maize hybrid line to carrying all other experiments. Among seven maize hybrids Sasa156 was chosen due to its ability to tolerate salt stress in an optimal level. Three concentrations of salt stress were tested through our greenhouse and growth chamber experiments and 150mM NaCl was the suitable condition to carry out the experiments. When 100mM NaCl applied to soil, maize plants tolerated the outcomes of salt stress and grow even better than normal. This experiment has led us to try more concentrated salt treatments to see the effects of stress on maize leaves and roots. 200mM NaCl was detrimental enough to plants to not even emerge in their seventh day and lots of plants stopped to elongate their third leave under 200mM NaCl. Sasa156 showed 80% germination rate under 150mM NaCl and Sasa197 was %70. Sasa197 showed a great germination rate and chlorophyll content under salt stress, but our aim was to see the effects of salt stress more than its tolerating capability. In the near future, the salt tolerant PGPR can be used as a biofertilizer to reduce salt stress and promote agricultural yields in a financially viable way.

Plants maintain homeostasis under biotic and abiotic stress via physiological alteration resulting in morphological and cellular modification for proper growth and

development. In this context, RDW, RFW, SDW, SFW and CC were marked as possible physiological parameters for the growth of maize.

The bacterial inoculation of *B. pumilus* with maize plants significantly increased the RFW (+14%), RDW (+23%), SFW (+39%) and SDW (+36%) on maize in greenhouse conditions. Also, inoculation with *B. pumilus* increased the CC of Sasa139 respectively. In growth chamber analysis, *B. coagulans, B. licheniformis* and Mixture of three *Bacillus spp*. increased the SDW, respectively.

B. licheniformis was the best performing bacteria in terms of increasing the RL, SL, RFW and SFW. Among two salt stress applied groups (S and P24/S), salt stress application with bacterial inoculation (P24/S) showed better results than non-inoculated group (S) in terms of enhancing the RL.

In a study it has been considered that inoculation of such stressed plants with PGPR alleviates their stress [Han and Lee, 2005].

Several studies have found that these microbes improve plant abiotic stresses through numerous mechanisms, such as the synthesis of gibberellins, IAAs, and some unexplained component, which results in enhanced physiological outcomes and increased nutrient status, thereby providing a better survival for plants undergo salinity stress [Shahid et al., 2018a-2018b].

ROS is usually generated in smaller quantities during plant cellular metabolism. In the case of abiotic stress conditions, ROS levels rise, causing changes in redox balance, damaging the DNA, denaturation of membrane proteins, changes in protein synthesis, cell homeostasis and disruption of enzyme activity and ultimately cell mortality [Halo et al., 2015]. When plants are suffering from excess amount of salts, primary target of ROS is likely to be lipids, which disrupt membrane phospholipids (polyunsaturated fatty acids) and initiate lipid peroxidation [Miller et al., 2010]. Enzymatic antioxidants such as monodehydroascorbate (MDA) reductase, APX, SOD, GR, CAT and antioxidants that are non-enzymatic such as glutathione, ascorbate and tocopherols are associated with ROS degradation and enhance oxidative stress alleviation [Kim et al., 2014]. Several number of PGPR have been shown to be able to mitigate oxidative stress with the assistance of antioxidant enzymes. It was reported that *Enterobacter spp*. increased the APX activity in tomato seedlings [Sandhya et al., 2010].

In our studies, it is shown that P24 *(B. licheniformis)* increased CAT activity by 150%. L23 (*B. pumilus*) significantly increased the POX activity by 128%

compared to control group ($p<0.05$). P24/S (*B. licheniformis*) showed a significant increase in APX activity by 311%. P14 (*B. coagulans*) significantly increased the SOD activity by 310% when compared to control group.

A study showed that when PGPRs were inoculated into the gladiolus plant, the CAT and SOD levels were higher than in the control [Damodaran et al., 2013].

Another study showed that overexpression of miR160 safeguards the development stage of seedlings against the negative effects of ROS when they were under salt stress [Tang et al., 2020].

Plants have developed other adaptation processes that have yet to be completely investigated. One of the known critical mechanisms possessed by miRNAs is the post-transcriptional regulatory process, the modification of gene expression via microRNA (miRNA) and improves the quality of life of plants struggling with abiotic stress [Sunkar et al., 2012].

In our studies, plants inoculated with *B. licheniformis* significantly increased the miR160 transcript levels up to 9.5-fold ($p<0.05$), miR169 transcript levels up to 119-fold, miR319 transcript levels up to 25-fold and miR396 transcript levels up to 51-fold.

The survival of beneficial bacteria in rhizoplanes and the success of these products will be determined by our ability to control rhizospheric soil. Cultural techniques, in combination with PGPR formulations, will benefit in increasing the amount of PGPR inoculum in soils.

The results of this study are important in terms of the possible use of *Bacillus spp.* as a biofertilizer. It will propose an alternate technique to avoiding existing improper fertilization in agriculture. The use of these bacteria as a stress regulator in salty soils has shown significant benefits. It will be used as a model study in monocots and will give insight into future biotechnological uses of plant microbe interactions.

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