SALICYLIC ACID AND *Piriformospora indica* INDUCED MODULATION OF STRESS RESISTANCE MECHANISM IN TOMATO (*Solanum lycopersicum* L.).

by

DEEPTHI MOHAN

(2016-09-021)

B. Sc. - M. Sc. (INTEGRATED) BIOTECHNOLOGY

DEPARTMENT OF PLANT BIOTECHNOLOGY

COLLEGE OF AGRICULTURE

KERALA AGRICULTURAL UNIVERSITY

VELLAYANI, THIRUVANANTHAPURAM-695 522

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THESIS

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DEPARTMENT OF PLANT BIOTECHNOLOGY
COLLEGE OF AGRICULTURE
VELLAYANI, THIRUVANANTHAPURAM - 695 522
KERALA, INDIA
2021

DECLARATION

I hereby declare that the thesis entitled "**Salicylic acid and *Piriformospora indica* induced modulation of stress resistance mechanism in tomato (*Solanum lycopersicum* L.)**" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Place: **Vellayani**

DEEPTHI MOHAN

CERTIFICATE

Certified that this thesis entitled "**Salicylic acid and *Piriformospora indica* induced modulation of stress resistance mechanism in tomato (*Solanum lycopersicum* L.)**" is a record of research work done independently by **Ms. DEEPTHI MOHAN (2016-09-021)** under my guidance and supervision and this has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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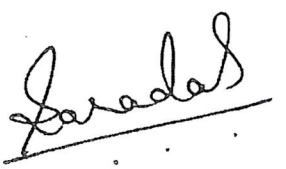

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Deepthi Mohan

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LIST OF ABBREVIATIONS AND SYMBOLS USED

%	Per cent
µg	Microgram
^{o}C	Degree Celsius
CD	Critical difference
cm	Centimeter
ml	Millilitre
M	Molar
EC	Elecrtical conductivity
ppm	Parts per million
CRD	Completely Randomized Design
rpm	Rotations per minute
et al.	and other Co workers
OD	Optical density
Fig.	Figure
g	Gram
i.e.	That is
KAU	Kerala Agricultural University
mm	Millimeter
viz.	Namely
FW	Fresh weight
TW	Turgid weight
RWC	Relative water content
RCI	Relative cell injury
pH	Potential of hydrogen

Cond...

mm	Millimeter
HCl	Hydrochloric acid
µmol	Micromoles
mmol	Millimoles
SOD	Superoxide dismutase
mg	Milligram
nm	Nanometer
s	Seconds
A_{652}	Absorbance at 652nm
A_{420}	Absorbance at 420nm
A_{290}	Absorbance at 290nm
A_{540}	Absorbance at 540nm
A_{450}	Absorbance at 450nm
A_{460}	Absorbance at 460nm
KOH	Potassium hydroxide
JA	Jasmonic acid
ROS	Reactive oxygen species

INTRODUCTION

1. INTRODUCTION

Plants have evolved complex methods to protect themselves from both biotic and abiotic stress. When a plant's defensive state is triggered, it might develop acquired resistance to biotic stimuli or adapt to abiotic stresses. Acquired resistance can spread throughout the plant systemically, which can be classified as induced systemic resistance (ISR) and systemic acquired resistance (SAR). Induced systemic resistance refers to the induction of resistance caused by nonpathogenic microbes in the rhizosphere (van Loon, 1997). The basic phenomenon behind ISR is the process of sensitizing or priming which refers the process of putting a plant in a state of greater attentiveness with no or limited gene activation, (Jakab *et al.,* 2001). Systemic acquired resistance refers to induced resistance that occurs as a result of a natural infection with a pathogen or via the use of chemical inducers (also termed plant resistance inducers (PRI)) (Ryals *et al.,* 1996). The activation of defense-related genes, such as genes coding for pathogenesis-related (PR) proteins, is closely linked to the development of SAR on a local and systemic level. The significance of these two types of acquired resistance is their specific ability of transgenerational induction, which is a change in offspring phenotype triggered by an environmental signal in the parental generation, and it occurs regardless of offspring genetic changes. Such impacts can develop as a result of maternal and/or epigenetic effects, both of which have different mechanisms and outcomes. Holeski *et al*. (2012) describe epigenetic changes that allow learned traits to be transmitted vertically without affecting the underlying DNA sequence.

Different approaches are used for triggering defensive mechanisms in plants. Plant resistance inducers (PRIs) are chemicals that help plants defend themselves against infections by triggering their own defensive mechanisms. (Alexandersson *et al.,* 2016; Nobutoshi *et al.,* 2012). Unlike the widespread use of antimicrobial chemicals, which can lead to the growth of drug-resistant pathogens, the use of PRIs, which target plants rather than pathogens, poses no risk. They frequently confer broad-spectrum resistance, lowering the risk of infection by a variety of pathogens. A number of PRIs have been developed and are now being used to protect crops in the field. One such prominently used PRI is salicylic acid (SA), whose exogenous application induces

resistivity. SA stimulates the production of PATHOGENESIS-RELATED (PR) genes, which prepares plants to respond more quickly and effectively to pathogen infection (Ward *et al.*, 1991). Another environmentally friendly approach is the use of beneficial microbes to induce resistance. *P. indica* , a root colonising endophytic basidiomycete fungi, induces resistance in plants via induced systemic resistance (ISR) (Moliter *et al.*, 2009). A molecular level of study is strictly recommended to analyse the mode of action of induced resistance and the methodology of transgenerational expression. The *NONEXPRESSOR OF PATHOGENESIS-RELATED GENES* 1 (*NPR1*) gene acts as the positive regulator for the expression of PR proteins in both SAR and ISR. Furthermore, *NPR1* is a critical signalling molecule in salicylic acid and *P. indica* -mediated defence signalling (Backer *et al.*, 2019, Stein *et al.*, 2008). Luna *et al.* (2012) observed that progeny from the defence regulatory mutant nonexpressor of PR1 (*NPR1*) failed to produce transgenerational defence phenotypes in *Arabidopsis*, suggesting that *NPR1* plays a vital role in the production of transgenerational acquired resistance. So, by analysing the induction of resistance through salicylic acid and *P. indica* mediated mechanisms and the persistence and transfer of this induced resistance, we will provide future prospective regarding the acquired resistance and transgenerational expression of acquired resistance in plants.

The study entitled 'Salicylic acid and *Piriformospora indica* induced modulation of stress resistance mechanism in tomato (*Solanum lycopersicum* L.)' was conducted at College of Agriculture Vellayani for analysing the impact of *NPR1* transgenerational expression and tolerance acquired against biotic and abiotic stress factors through adopting modulation of biochemical, physiological and molecular parameters.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 *Solanum lycopersicum* L.

Tomatoes are well suited to nearly all climatic zones in the world, as they are a tropical plant. One of the most important horticultural crops is *S. lycopersicum* (tomato), which belongs to the Solanaceae family. Zhan *et al.*, 2018 suggested that, it has been used as a model system for the growth of fruits. The plants usually reach a height of one to three metres. Originating in Central and South America, its domestication and use as a cultivated food, on the other hand, may have begun with the indigenous peoples of Southern Mexico. Tomato plants are currently grown in many different kinds all over the world. It is a perennial plant in its natural habitat, however it is frequently cultivated outside in temperate climates.

2.1.1 Taxonomic profile of *S. lycopersicum*

Kingdom	: Plantae
Phylum	: Spermatophyta
Subphylum	: Angiospermae
Class	: Dicotyledonae
Order	: Solanales
Family	: Solanaceae
Genus	: *Solanum*
Species	: *Solanum lycopersicum*

There are indeed 16 wild tomato species, including *S. habrochaites*, *S. pennellii*, *S. pimpinellifolium*, *S. cheesmaniae*, *S. galapagense*, *S. peruvianum*, *S. corneliomulleri*, *S. chilense*, *S. chmielewskii*, *S. arcanum*, *S. neorickii*, *S. huaylasense*, *S. lycopersicoides*, *S. ochranthum*, *S. jugandifolium*, and *S. sitiens.*

All of the tomato clade species are diploid, with 2n = 24 as the basic chromosomal number (Zhou *et al.*, 2015). The tomato genome was just published, and

researchers discovered that it contains around 35,000 genes, making it a useful resource for research (Sato *et al.,* 2012).

2.1.2 Relevance of Tomato as a food crop

It is a short-lived fruit crop with a high nutritional value that is extensively used. Lycopene, beta-carotene, flavonoids, Vitamins A, B, C, and E, as well as hydroxycinnamic acid derivatives, are prominent in them. Potassium, phosphorus, iron, and calcium are also abundant in them. It has developed in prominence in recent years as a result of its anti-oxidant properties (Kashyap *et al.,* 2020).

2.1.3 Cultivation of Tomato

Tomato output in India topped 21 million metric tonnes in 2020, according to statistics released by the Statista Research Department. The three leading producers in the country were Andhra Pradesh, Karnataka, and Madhya Pradesh. After potatoes, they are the second most popular vegetable crop (Kashyap *et al.,* 2020). In 2017, the worldwide tomato output surpassed 170.8 million tonnes. China is the world's top tomato grower, accounting for one-third of international output in 2017, according to the Food and Agriculture Organization Corporate Statistical Database, followed by India and Turkey. Apart from being ingested raw, it has been used as a culinary vegetable in meals. After potatoes, they are the second most popular vegetable crop.

2.1.4 Diseases affecting Tomato cultivation.

The tomato plant is frequently used as a model plant for experimentation into different aspects of plant biology, including host-pathogen interactions. Although it may be attacked by a variety of diseases, including viruses, viroids, bacteria, nematodes, fungi, and oomycetes, the tomato pathosystem is a good model for researching plant-pathogen interactions. Pathogens lead to infections that not only

harm tomato crop output but also cause significant post-harvest losses, putting the tomato processing sector at risk (Naveed and Ali., 2018).

2.2 Bacterial wilt disease

Bacterial wilt is a damaging crop disease caused by *Ralstonia solanacearum* that affects many economically significant crops in the warm humid tropics and temperate parts of the world. *R. solanacearum* is a soil-borne pathogen that enters plant roots through wounds or natural openings. The bacterium colonises the intercellular space of the root cortex and vascular parenchyma while inside the host, rapidly multiplies, and finally enters the xylem vessel, spreading into the stem, leaves, and fruits. The pathogen travels up the vascular system and into the infected xylem, obstructing water transfer and causing wilting. As a result of the deterioration of the xylem vessels and nearby tissues, the plant dies. Infected plants usually die in 3–4 days (Nion and Toyota., 2015). After the plant dies, the bacterium returns to the soil from the infected root. It stays in the soil until it comes into contact with a new host plant. Bacterial wilt disease has caused a considerable drop in yield in several solanaceous crops around the world, ranging from 30 to 80 per cent (Sharma, 2018). It has impacted practically every tomato-producing country in the world.

2.2.1 Dissemination of the pathogen and symptoms

The causal agent, *Ralstonia solanacearum* species complex (RSSC), is dispersed in many ways. It has largely been aided by the spread of latently contaminated planting materials.

R. solanacearum can be found in infected crop wastes in the field, infested moist soil and weeds, polluted irrigation water, contaminated farm equipment, agricultural processing waste from industries, and latently infected crops like tomato seeds and potato tubers. Insects also play a role in the disease's transmission as vectors. The bacterium can live for two to three years without

producing infection. Environmental variables, on the other hand, play a critical influence in the genesis and spread of disease. When examining illness development, weather variables such as temperature and humidity are taken into account (Mamphogoro *et al.*,2020).

Symptoms might appear early in the course of an infection. They are generally seen on the leaves of plants. Even when dried leaves remain green, the entire plant may wilt and desiccate as the illness progresses, resulting in general withering and yellowing of foliage and eventually plant death. Because the wilted leaves retain their green hue, the condition is often known as "green wilt disease" (Jiang *et al.*, 2017). Plant growth is also stunted, which is another indication of bacterial wilt. Infected vascular bundles in early tomato stems might appear as long, narrow, yellow to dark brown streaks. Cross-sections of stems may reveal a brown discoloration of the vascular system and other diseased tissues in well-established diseases. In most cases, vascular bundle infection is indicated by as large number of adventitious root buds on the stem near the root. If the cortex is extensively injured, water-soaked lesions on the exterior surface of the stem may occur. When a sick stem is cut crosswise, little drops of yellowish viscous, dirty white, or milky bacterial ooze appear, indicating infection by bacterial cells at the vascular bundles (Mamphogoro *et al.*,2020).

When the temperature is high (29-35°C), these symptoms are more likely to manifest. One of these signs is the wilting of young leaves at the branches' extremities during the hottest part of the day. Plants may appear to recover at this early stage when temperatures are cooler. Under hot, humid conditions, complete wilting occurs, resulting in the death of the diseased plant.

2.2.2 Diagnostic identification of bacterial wilt disease

The identification of symptoms is the first step in diagnosing bacterial wilt in tomatoes early. Early detection and identification of bacteria in vulnerable infected plants can be aided by screening assays. Stem streaming, immunofluorescence antibody staining, selective plating on South African selective medium (SMSA), nucleic-acid-based identification using *R. solanacerum* specific primers (DNA

probe hybridisation and PCR assays), and pathogenicity assessment using susceptible hosts are some of the screening tests available (e.g., tomato seedlings). For rapid and field identification of *R. solanacearum,* several rapid screening methods, such as ELISA testing employing immunostrips, are also commercially available (Mamphogoro *et al.*,2020).

Early detection and identification of bacteria in vulnerable infected plants can be aided by screening assays. Stem streaming, immunofluorescence antibody staining, selective plating on South African selective medium (SMSA), nucleic-acid-based identification using *R. solanacerum* specific primers (DNA probe hybridisation and PCR assays), and pathogenicity assessment using susceptible hosts are some of the screening tests available (e.g., tomato seedlings) (Narasimha Murthy *et al.,* 2012). For rapid and field identification of *R. solanacearum*, several rapid screening methods, such as ELISA testing employing immunostrips, are also commercially available (Patrice, 2008).

2.2.3 Ralstonia solanacearum *species complex (RSSC)*

R. solanacearum, originally Pseudomonas solanacearum, is a gram-negative phytopathogenic bacteria that causes wilt disease in tomatoes. It has a wide range of plant hosts, including a wide range of crop plants, ornamentals, and weeds from more than 54 botanical families around the world. It was first discovered in peanuts in the early 1930s (Pawaskar *et al.,* 2014).

2.2.4 Taxonomy and diversity of R. solanacearum

Kingdom	: Bacteria
Phylum	: Proteobacteria
Class	: β-proteobacteria
Order	: Burkholderiales
Family	: Burkholderiaceae
Genus	: *Ralstonia*

Species : *Ralstonia solanacearum*

Because of the great genetic variety within the species, *R. solanacearum* is classified as a "species complex" by Fegan and Prior (2005), who proposed a new hierarchical categorization method. *R. solanacearum* is divided into five races based on host range and six biovars based on ability to oxidise three disaccharides and three hexose alcohols, according to conventional classification (She *et al.*, 2017).

2.2.5 Isolation of Ralstonia solanacearum *from diseased plants*

After incubation at 28°C for 24 hours, the causal agent, *R. solanacearum*, can be isolated from the infected plant by streaking the bacterial slime streamed out into the water from the infected stem on Nutrient agar plates. *R. solanacearum* isolates can be purified after isolation by streaking a single colony of each isolate on Triphenyl Tetrazolium Chloride (TTC or TZC) agar medium, as Kelman describes (1954). Its cultural, biological, and molecular properties can all be used to evaluate it (Champoiseau *et al.*, 2009).

2.2.6 Cultural, phenotypic and biochemical characteristics of *R. solanacearum*

R. solanacearum is identified morphologically by the presence of irregular, fluidal white colonies with pink or red centres and whitish periphery on TZC agar medium. In Nutrient Agar medium, the bacteria form smooth, raised cream or off-white coloured colonies. The ideal temperature for growth is between 28°C and 32°C (Champoiseau *et al.*, 2009). Several selective mediums for isolating the pathogen from problematic substrates such as dirt, trash, or the surface are also available (Mamphogoro *et al.*,2020). *R. solanacearum* is a rod-shaped bacterium with dimensions of 1.5–3.12m X 0.25–2.5m and a diameter of 0.25–2.5m. Gram-negative bacteria have Gramnegative cell walls. The bacterium's metabolism is oxidative, and it is classified as a strict aerobe (Pawaskar *et al.*, 2014). They have one to four polar flagella that control their swimming motility (Tans-Kersten *et al.*, 2001).

In order to understand the characteristic features of the causal organism, biochemical tests such as Gram staining, potassium hydroxide solubility test, Kovac's oxidase test, Levan test, sugar fermentation test, starch hydrolysis test, hydrogen sulphide production test, and cellulose decomposition test can be performed (Sharma, 2018), (Pawaskar *et al.*, 2014), (Rahman *et al.*, 2010).

Table 1 Results of physiological and biochemical tests given by Ralstonia solanacearum

Sl No.	Physiological/ Biochemical tests	Result
1	Gram staining	Negative
2	Gram's Potassium hydroxide solubility test	Positive
3	Kovac's oxidase test	Positive
4	Levan test	Positive
5	Sugar fermentation test (Dextrose, sucrose, mannitol and lactose)	Positive
6	Starch hydrolysis test	Negative
7	Hydrogen sulphide production test	Negative
8	Cellulose decomposition test	Negative

2.2.7 Methods to control bacterial wilt disease

The disease's widespread dispersion and long-term survival in the environment, even in the absence of a vulnerable host, makes control more difficult, especially once it has established itself in the soil. Latent infections develop as a result of the prolonged survival. To combat the disease, a variety of physical, cultural, chemical, and biological control methods are used (Nion and Toyota, 2015). Various approaches to bacterial wilt disease management are summarised, along with their putative processes (Mamphogoro *et al.*, 2020), (Kurabachew and Ayana, 2016).

Solarization, hot water treatment and biological soil disinfection, biofumigation all can be counted as the physical methods for killing *R. solanacearum* with high or low temperatures. Cultural methods of using resistant cultivar, crop rotation and multi-cropping, soil amendment and grafting are utilised mainly for enabling limited pathogen movement from the primary xylem to other xylem tissues, induced uptake and distribution of nutrients, reduced disease inoculum and induced plant resistance. Use of chemicals like Algicide (3-3-Indolyl botanic acid), fumigants, acibenzolar-Smethyl, chitosan and sodium chloride bactericides, chloropicrin, silicon, thymol, weak acidic electrolyzed and phosphoric acid solution, methyl bromide coupled with 1,3-dichloropropene, carbendazim, flubendazole, propiconazole which uses the mechanism of inducing systemic resistance, increase the amount of soil micro-organisms or increase tolerance to *R. solanacearum*, antibacterial and bacteriostatic effect. Biocontrol agents like *Bacillus amyloliquefaciens, Bacillus cereus, Burkholderia nodosa, B. pyrrocinia, B. sacchari, B. tericola, Acinetobacter sp., Flavobacterium johnsoniae, Pseudomonas brassicacearum, Pseudomonas mallei, Ralstonia pickettii* etc are used. The specific mechanism that were involved like competition for nutrient and space, antibiosis, plant-mediated systemic resistance, parasitism, siderophore production, production of extracellular enzymes and decrease root colonization. Despite the fact that several tactics have been devised to control the bacterial wilt disease, none of them has been successful in entirely controlling the disease.

2.3 Acquired Resistance in Plants

The acquired resistance in plants can be broadly divided into Systemic Acquired Resitance and Induced systemic resistance. The non pathogenic microbes in soil such as rhizobacteria and fungi causes the induction of resistance called Induced Systemic Resistance (ISR) (Walters *et ai*., 2013). When the induction of resistance is naturally by a pathogen or with chemical inducers it is called Systemic Acquired Resistance (SAR) (Spoel and Dong, 2012).

2.3.1 Induced systemic Resistance in plants

There are certain microbes associated with plants that have beneficial roles in plant growth promotion as well as inducing defense against stress and pathogen. These beneficial microbes can be bacteria or fungi termed as plant growth promoting rhizobacteria (PGPR) and plant growth promoting fungi (PGPF) respectively. PGPRs are bacterial colonies rendering the rhizosphere hosting the root system of the plants. These microbes are responsible for the induction of enhanced defensive characteristics against a vast spectrum of pathogenic microbes as well as the stress tolerance caused due the infection of these microbes in plant. This type of resistance thus acquired is termed as Induces systemic resistance. Salicylic acid (SA) dependant as well as salicylic acid independent pathways are proceeded by PGPRs to induce resistance, salicylic acid independent pathway use jasmonic acid mediated as well as ethylene signalling for inducing resistance in plants.

Just as the immune system in primates works the concept of immune responds in plants also explains to be works in first and second line of defence system. The first line of defense use the initial morphological patterns of beneficial as well as the pathogenic organisms as the signal inducers, those were recognised by specific receptors namely pattern recognition receptor (PRR) (Zipfel, 2009). The specific patterns thus identified by the PRRs are categorized as pathogen- or microbe- associated molecular patterns (PAMPs or MAMPs) (Boller and Felix, 2009). Due to the attack of microbes there are certain molecular signals arises inside the cells of plants thus triggering further defence responds, these signal are called damage

associated molecular patterns (DAMPs)(Boller and Felix, 2009). The first line of defence thus triggered by PAMP are termed as PAMPtriggered immunity (PTI). The second line of defence is mediated by the signalling molecule called nucleotide binding leucine rich repeat receptor proteins(NB-LRR) which recognises specific effector molecules produced by attacking organisms in plants thus providing the effector triggered immunity(ETI)(Dodds and Rathjen, 2010).This signals will ultimately leads to apoptosis of the infected cells for preventing further infection to other plant parts as well as thus produces signals will often transmitted through out the plant system giving the systemic acquired resistance(SAR) which is usually induced due to the resistance by the infection of a pathogenic microbe or through the attack of herbivorous insects (Vlot *et al.,* 2009).

The SAR get well established due to the production of the plant hormone salicylic acid and its accumulation inside the plant cell. Pathogen related genes (PR gene) are involved in the production of specific PR proteins for the ultimate expression of SAR due the infection of specific pathogen. Both virulent and avirulent organisms are involved in the expression of SAR.

The main hormones involved in the production of ISR are jasmonates and ethylene (Pieters *et al.,* 2012). Jasmonate is the phytohormone which is basically a lipid derivative that involves in the expression of genes that deprive the cellular growth in plants and subsequently promote the evoking of specific defence mechanisms in plants. Ethylene is the gaseous phytohormone involved in the senescence of plant parts. The rhizosphere of the plants are rich source of C and N, for the growth of diverse microbes which further feeds up on the exudates and lysates released from the roots results in the formation of an excellent microflora. These provides both synergistic and antagonistic interaction to each other, and this antagonistic interaction of beneficial microbes eliminate pathogenic microbes from rhizosphere.

2.3.2 Salicylic acid receptor NPR1

NPR1 and NPR3/NPR4 are two types of receptors that respond to the plant defence hormone salicylic acid (SA). They regulate SA-induced defence gene expression through two different mechanisms (Ding *et al.*, 2018). *NPR1* and NPR4 must perceive SA in order to activate N-hydroxypipecolic acid production, which is required for generating systemic acquired resistance. In addition, in the *NPR1*-1 npr4-4D double mutant, both pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) are substantially affected. Through changes such as 5-hydroxylation and glycosylation, *NPR1* and NPR4 are engaged in positive feedback amplification of SA biosynthesis and regulation of SA homeostasis. As a result, the SA receptors *NPR1* and NPR4 have a wide range of functions in plant immunology (Liu *et al.*, 2020).

2.4 *Piriformospora indica* induced systemic resistance

Root endophytic fungi are found on nearly every plant in the world. They live asymptomatically in the tissue of the host plant. These microorganisms have been proven in studies to improve the ecological adaptations of plants living in severe settings. They have been discovered to boost plant stress tolerance (Yamaiji *et al.,* 2016). *Piriformospora indica,* a non-mycorrhizal mutualistic fungus belonging to the Sebacinales order, comes under the family of Sebacinaceae is regarded as a model root endophyte that benefits plant hosts significantly. It is a basidiomycete filamentous fungus associated with the roots of various plant species, including bryophytes, pteridophytes, gymnosperms and a large number of monocot and dicot plants (Thurich *et al.*, 2018), (Qiang *et al.,* 2012), (Varma *et al.,* 1999).

2.4.1 Taxonomic profile of P. indica

Kingdom : Fungi

Division : Basidiomycota

Class : Agaricomycetes

Order : Sebacinales

Family : Sebacinaceae

Genus : *Piriformospora*

Species : *Piriformospora indica*

Using a blast search against highly conserved core genes found in higher eukaryotes, the full 24.97 Mb genome of *P. indica* was sequenced and confirmed (Zuccaro *et al.,* 2011). It was discovered that the fungus has at least six chromosomes (Zuccaro *et al.,* 2009).

2.4.2 Phenotypic characteristics of **P. indica**

The mycelia are mostly flat and immersed in the substratum, and they are frequently intertwined and overlapping. As a result, the endophyte promotes rapid nutrient uptake thanks to its large hyphal network. The diameter of the hyphae varies from 0.7 to 3.5l m. Hyphae have uneven septations and frequently have anastomosis. As a result, a large number of cells have several nuclei. Chlamydospores are made up of thin-walled vesicles that can be found near the hyphae's terminals. The pear-shaped chlamydospores, which can be found alone or in clusters, distinguish them. The walls of very immature spores are thin and hyaline (Verma *et al.,* 2012).

2.4.3 Cultural characteristics of **P. indica**

P. indica may easily be propagated in axenic cultures, where it generates chlamydospores with 8 to 25 nuclei, unlike arbuscular mycorrhiza fungus (Verma *et al.,* 1998). The modified Hill–Kafer synthetic medium is ideal for cultivating *P. indica* . *P. indica 's* immature mycelia are white and practically hyaline, whereas older cultures contain subtle zonations. Under ideal culture circumstances (inoculum size: 5%; agitation speed: 200 rpm; working volume: 30%; starting pH: 6.5; temperature: 30 °C), maximum dry cell weight is attained after 5 days. After 6 days of growth in a 500-ml Erlenmeyer flask, sporulation commences, and after 8 days, the maximum spore yield is attained.

Due to more effective mixing and homogenised fungal suspension in the bioreactor, the fungus developed quicker than in the batch culture in shake flasks, resulting in early depletion of the carbon source and thus early sporulation.

The fungus colonises the roots and develops both inter- and intracellularly, generating pear-shaped chlamydospores in the cortex and rhizosphere zone (Mensah *et al.,* 2020) The biotrophic colonisation of local living cells is followed by a cell killing phase in which the fungus kills the cells in the roots in order for them to establish themselves in the host plant before reproducing. It does not, however, infiltrate the host plant's endodermis or aerial portions (Verma *et al.,* 2012).

A number of research have been carried out in attempt to better understand the relationship between the fungus and its host plants. It was hypothesised that the interactions can be separated into three stages based on the expression profiles of its colonisation with the host plants, which correspond to *P. indica* 's saprophytic and mutualistic properties. During the first encounter, the fungus suppresses plant defence mechanisms, followed by a phase in which the fungus suppresses the expression of genes linked to saprophism. Throughout the last phase, a long-term positive association is developed (Thurich *et al.,* 2018).

2.4.4 Stress tolerance imparted by P. indica

It is a near homologue of arbuscular mycorrhizal fungus (AMF) that uses numerous ways to stimulate host plant growth and development. Its potential to give (systemic) resistance to toxins, heavy metal ions, and pathogenic organisms has been demonstrated in studies, allowing plants to grow under harsh stress circumstances (Olemuller *et al.,* 2009), (Walter *et al.,* 2008). The cumulative effects of nutrients and phytohormones collected in combination with *P. indica* improve plant metabolism (Gill *et al.,* 2016), (Fakhro *yet al.,* 2010), (Xu *et al.,* 2017) (Lakshmipriya *et al.,* 2017) In micro propagated plants, it has also been demonstrated to increase early flowering, secondary metabolite or phytochemical production, and excessive biomass generation (Shermati *et al.,* 2008) (Abdelaziz *et al.,* 2019) (Ghorbani *et al.,* 2018), (Das *et al.,* 2012) (Dolatabati *et al.,* 2011)

(Satheesan *et al.*, 2012) (Aslam *et al.*, 2019). Treatment with *P. indica* on two varieties of tomato (Naveen- highly wilt susceptible hybrid and Vellayani Vijay (moderately wilt tolerant variety) results in 40% disease suppression of bacterial plant pathogen (Athira and Anith, 2020).

2.4.5 P. indica *and* NPR1 *signal expression*

P. indica colonisation is limited to the root cortex, implying that *P. indica* confers resistance to leaf diseases through systemic signalling. *P. indica* imparts resistance similar to induced systemic resistance (ISR) via *NPR1* cytoplasmic signalling, which is necessary but no nuclear localization signal of *NPR1* expression is observed (Stein *et al.*,2008). The priming of *NPR1*regulated genes in barley by *P. indica* suggests that the fungus activates common signaling pathways in monocotyledonous and dicotyledonous plants (Kogel and Langen.,2005).

2.5 Pathogenesis related (PR) proteins

PR proteins are a set of different molecules that are induced by phytopathogens and defense-related signalling molecules. They're important parts of systemic acquired resistance (SAR), which gives long-term protection against a wide range of infections (Gimenez *et al.*, 2018).

The PR proteins are thought to play a role in limiting pathogen development and dissemination in plants (Hamamouch *et al.*, 2011). Overexpression of PR genes, either separately or in combination, has been shown to improve plant defence response and resistance to a variety of diseases in studies. As a result, they're frequently used as a diagnostic marker for defensive signaling pathways (Jiang *et al.*, 2015).

Pathogenesis-related proteins were originally discovered in tobacco mosaic virus-infected Nicotiana tabacum in 1970. (TMV) (Van *et al.*,1970). After being found intracellularly and extracellularly in several plant species, PR proteins were described and grouped into 17 families based on their protein sequence similarities, enzymatic activity, and other biochemical characteristics. Glucanase (PR2), chitinases (PR3), thaumatin-like (PR5), peroxidases (PR9), plant defensins

(PR12), and thionins are only a few of the tasks they have (PR13) (Ali *et al.,* 2018).

2.5.1 Structure of PR proteins

PR proteins are heat stable, protease resistant, and soluble at low pH proteins with a molecular weight of 6–43 kDa. In terms of biochemical properties, they are very different from one another. Acidic PR proteins are released into the extracellular space, while basic PR proteins are transported to the vacuole via a signal sequence at the C-terminal end (Ali *et al.,* 2018).

2.5.2 Function of PR protein as bacterial resistance entity

Pathogenesis-related proteins have been identified as potent antibacterial phytopathogen weapons. In vitro studies have shown that PR proteins such as PR10 (ribonuclease-like proteins), PR12 (defensins), PR13 (thionins), and PR14 (lipid-transfer protein) have antibacterial activities.

2.6 Descendants of primed plants exhibit resistance to stress

Priming can be defined as increased resistance and/or abiotic stress tolerance as a result of higher sensitivity and response to high levels of stress from prior experience and the induction of this specific priming is due to a signal that prepares a plant for enhanced responsiveness by causing subtle physiological, molecular, and epigenetic changes (Mauch *et al.,* 2017). Plants can develop a unique primed state of defence after being attacked by pathogens or being treated with particular resistance-inducing substances. When exposed to biotic or abiotic stress, primed plants exhibit heightened defence responses. This primed state of a plant is still functional in the next generation without additional treatment. After challenge inoculation with a virulent isolate of microbe, the offspring of primed plants demonstrated a faster and higher production of transcripts of defense-related genes in the salicylic acid signalling pathway, as well as improved disease resistance (Slaughter *et al.,* 2012). Transgenerational induction results due to DNA methylation, histone modifications, and small RNAs which explains a change in offspring phenotype that is triggered by an environmental signal in the parental generation and manifests itself irrespective of offspring genotype

changes. It can happen as a result of epigenetic in heritance or maternal influences (Holeski and Agarwal, 2012).

2.7 RNA isolation

Gene expression studies, reverse transcription quantitative PCR (RT-qPCR), transcriptome analysis employing next generation sequencing (NGS), Rapid Amplification of cDNA Ends (RACE), array analysis, northern blot analysis, and gene isolation all require high quality RNA. However, because RNA is an easy target for RNases, acquiring good quality RNA is a difficult task. As a result, the first and most important step is to prevent residues of ubiquitous RNases from contaminating the environment and consumables used on the job site. So, whatever isolation method is used, a few measures must be taken to avoid RNase contamination (Nadiya *et al.,* 2015). For RNA isolation across plant species, there is currently no globally approved standard procedure. The single-step method of RNA separation utilising acid guanidinium thiocyanate-phenol-chloroform extraction established by Sacchi and Chomcynzki (1987) is the most widely recognised of the protocols available so far.

2.7.1 cDNA synthesis

For PCR reactions, RNA cannot be used as a template. As a result, some steps must be completed before to PCR in order to convert RNA to complementary DNA (cDNA). Reverse Transcriptase, an RNA-dependent DNA polymerase enzyme, is used to do this. The most common template is total RNA or Poly A+ RNA, which is primed with either target specific primers or hexamers and oligo dT. Primers are chosen primarily for the function for which they are intended. By addressing reverse transcription specific factors, cDNA is employed for typical PCR reactions once it has been generated.

2.7.2 Primer designing

PCR is a ground-breaking scientific technique for amplification of DNA in vitro. Template DNA, primers, Taq polymerase, PCR buffer, dNTPs, and a thermocycler are all necessary components for performing a PCR reaction. A PCR reaction has a lot of complications, despite the fact that the processes and stages are quite straightforward. A good example is the specificity of a PCR reaction. Primers of good quality are required for a successful and specific PCR reaction. It is critical to rigorously follow the criteria for designing primers while designing primers. Etebu (2013) describes several essential design aspects targeted at obtaining precise amplification with high yield (Dieffebach *et al., 1993*)

The length of the primer is crucial, and it should be between 18 and 30 bp. Ensure that the Tm of both reverse and forward primers is the same and that it is always greater than 54 degrees Celsius. Primer dimers must be avoided (including self and cross dimers). At the 3' end, primers should not be complimentary, and secondary structures (hairpins) should be avoided. The number of GC residues in a primer sequence should not exceed four. One GC clamp is sufficient to assure specificity at the 3' end.

Manually designing primers is a time-consuming process, thus we prefer to employ user-friendly computational tools. Each platform has its own set of design rules. Primers can be made from either known or unknown sequences that have already been deposited in nucleotide sequence databases. When working with known sequences, constructing primers is simple since small oligos may be created from the flanking ends of our target. Things are different, though, when it comes to unknown sequences. Primers are designed using conserved sequences in this case.

2.7.3 Gene expression analysis

At the transcriptional or translational level, gene expression investigations are used to assess the presence and quantity of functioning gene product(s). It's a sensitive indicator of biological activity that may easily detect a change in gene

expression profile. A shift in biological process is reflected in this difference in signature pattern. Gene expression profiling is a dynamic functional perspective of an organism's biology that goes beyond the static information of the genomic sequence. For this objective, a number of procedures and protocols have been created. All of these, however, follow a similar technique that begins with RNA/protein isolation and continues with amplification, quantification, and detection methods. Some of them include Northern blotting, Microarray analysis, Serial analysis of gene expression (SAGE), qRT-PCR, In situ hybridization (ISH), and Next Generation Sequencing.

Gene expression analysis using next generation sequencing (NGS) technology-RNA-Seq-and reverse transcription polymerase chain reaction (RT-PCR) has gained popularity in past few decades because it enables for the measurement and comparison of millions of random location reads that are then mapped and aligned to each gene, providing an incredible level of detail.

2.7.4 Real -Time PCR

The advent of real-time PCR (qPCR) has drastically altered the landscape of gene expression research. Speed, simplicity, convenience, high sensitivity, specificity, robustness, and great performance are just a few of the characteristics that distinguish qPCR from traditional PCR. It gained popularity as a result of its ability to collect data in real time throughout the process, allowing researchers to skip the gel preparation phase at the end. For quantification, the equipment uses fluorescent dyes or probes, with the emitted fluorescence being detected to quantify the product concentration. A fluorescent dye called SYBR GREEN binds non-specifically to dsDNA and produces fluorescence that is proportional to the amount of DNA in the sample. Because the same dye can be used for multiple experiments, this is a very flexible method. Multiplexing, on the other hand, will result in false positives due to non-specificity. Melt curve analysis is used to assure reliable results.

Fluorescence emitting probes, such as Taqman probes, FRET probes, molecular beacons, and Scorpions probes, on the other hand, are extremely selective and bind to specific sequences.

2.7.5 Real-Time PCR dependant differential gene expression studies

Real-time PCR can be used to quantify genes in two ways: absolute quantification and relative quantification. Absolute quantification depicts initial number of copies of our target sequence, whereas relative quantification reflects a change in gene expression in a given sample relative to a reference sample. In contrast to absolute quantification, relative quantification does not necessitate the use of a standard curve for analysis.

Recent research has primarily focused on relative quantification using qPCR, which is an effective method for investigating tiny changes in gene expression. It's employed in research comparing normal and sick samples, mutant against wild-type samples, before and after treatment, time course during development or across treatment regimes, spatial variation within organs, tissues, and other sample types, and so on.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The study entitled "Salicylic acid and *Piriformospora indica* induced modulation of stress resistance mechanism in tomato (*Solanum lycopersicum* L.)." was conducted at the Department of Plant Physiology, Department of Plant Biotechnology and Department of Agriculture Microbiology, College of Agriculture Vellayani, Thiruvananthapuram, during 2020– 2021. Details on the experimental materials utilised and the methodology employed for the study are described in this chapter.

Bacterial wilt susceptible (Pusa Ruby, IARI) and resistant (Vellayani Vijay, KAU) varieties of tomato was used for the study.

Pusa ruby is the variety developed by IARI, New Delhi. It's an early-maturing cultivar with yellow stem ends, slightly wrinkled fruits, and even ripening. This particular variety is used as the bacterial wilt susceptible subject for the study.

The bacterial wilt resistant variety Vellayani Vijay was developed by KAU. The development of this particular variety was done along the duration of 2012-2013 through the study conducted for the development of tomato genotype possessing combined resistance to bacterial wilt disease and tomato leaf curl disease virus. The study showed the F2 generation selfed progeny segregant possess resistance to both the above mentioned diseases. A final development of this particular variety was obtained around 2014 with the phenotypic expression on total of 35F4 segregants (Dheemanth *et al.*, 2017).

3.1 Nursery production of tomato plants and application of bioagent *P.indica* and hormone salicylic acid

3.1.1 Preparation of potting mixture

The potting mix was prepared by combining vermiculite and perlite in the ratio 2:1. The potting mix was sterilised at 121°C by autoclaving for three consecutive days for one hour each. The sterilized potting mixture was filled into protrays (50 cells, each with a diameter of 5 cm).

3.1.2 Surface sterilisation of tomato seeds

Tomato seeds were surface sterilised in a laminar air flow chamber for 3 minutes in a 1% sodium hypochlorite (0.5%) aqueous solution. After that, the seeds were rinsed three times with sterile distilled water.

3.1.3 Experiment 1

3.1.3.1 Preparation of *Piriformospora indica* culture

The fungus was grown for 15 days in 250 ml conical flasks containing 100 ml of PDB (Potato Dextrose Broth) medium. Mycelium of the fungal endophyte was recovered by filtering the content from the flask using a muslin cloth. The mycelium was weighed and mixed with sterile potting mixture at a concentration of 1% (w/v) (Anith *et al.* 2015) and filled in the portray cavities (Plate 1).

Surface sterilised seeds were sown in the cavities. Later the seedlings were transferred to pots for further studies.

3.1.3.2 Staining of tomato roots for the confirmation of endophytic fungus *P. indica*

The roots of the *P. indica* treated tomato plants were collected and washed thoroughly to remove soil particles and other dirt. Then the roots were cut into small bits of length of about 1cm. They were boiled in 10% KOH solution for about 5 min and rinsed in water for three times. Acidification of the root bits was done by using 1N HCl by keeping the bits in acid for 3 min and then the acid drained out. Roots were then kept in lactophenol trypan blue stain (Appendix 1) for 10 min and further transferred in to lactophenol (Appendix II) for destaining.

The stained roots were viewed under a bright field microscope and images were captured. Those tomato plants confirmed with *P. indica* colonisation, were maintained in large pots and second generation progeny seedlings were obtained.

3.1.4 Experiment 2

Hormonal application of 100 ppm salicylic acid was done during the flowering time on another set of tomato plants. Salicylic acid was initially dissolved in 100% ethanol

and was making up to the volume for obtaining 100 ppm. This was then sprayed on to the tomato plants at the initial flowering stage. Those flowers were labelled and fruits developed from the labelled flowers were collected for generating seeds and progeny seedlings were obtained (Plate 2).

For both treatments (*P. indica* and salicylic acid) the second generation progeny seedlings of variety Vellayani Vijay were used for analysing transgenerational expression of *NPR1* defense gene and for evaluation of stress tolerance, against biotic and abiotic factors (Plate 3).

3.2 Biotic stress application

3.2.1 Isolation of *Ralstonia solanacearum*

Tomato plants which showing wilt symptom (drooping of leaves) were identified and collected from the field. Initially an ooze test was performed with stem portion of the suspected plant. Those stems showed positive results in ooze were surface sterilised and crushed in sterile water and streak plated in SMSA (SeMi Selective Agar) media with TTC (TriphenylTetrazolium Chloride), incubated in
28^0C. The virulent colonies were identified through proving Koch's postulate. The healthy plants were inoculated with isolated *Ralstonia solanacearum* by soil drenching and from those plants which gave wilt symptoms re-isolation was done. Low density isolates which gave an OD value of less than 0.5 were used for inducing biotic stress.

The progeny seedlings generated from the *P. indica* and salicylic acid treated plants were subjected to challenge inoculation with *Ralstonia solanacearum* (low density-10^6 cells/ml).

3.2 Abiotic stress application

A higher temperature was given to progeny Vellayani Vijay seedlings by keeping them in polyhouse (ambient + 4^0 C) for 7 days and these seedlings were used for physiological and biochemical observations.

The number of replication used was 4, for both biotic and abiotic stressors application.

3.3 Physiological observations

3.3.1 Relative water content

Leaf samples of progeny seedlings applied with both biotic and abiotic stressors were collected for analysing relative water content. The leaflet's fresh weight (FW) was measured right after it was cut. The leaflet was then immersed in double distilled water in a Petri plate and incubated at room temperature for 24 hours. The leaflet was removed after four hours, properly wiped to eliminate the water from the blade's surface, and weighed to acquire a turgid weight (TW). After that, the leaflet was dried in an oven at a temperature of 70 ^{0}C for 24 hours and weighed to determine its dry weight (DW).
The formulae used for measuring relative water content was, relative water content (RWC in %) = [(FW - DW)/(TW - DW)] ×100.

3.3.2 Membrane stability index

The procedure of Sullivan (1972) was used to determine the leaf membrane stability index (MSI). After removing the full-fledged highest leaves, spherical leaf discs of 0.75 cm in diameter were cut using a punch machine. Ten leaf discs were placed in two sets of 50 ml glass tubes and washed slowly with de-ionized distilled water three times to remove surface bound electrolytes. The washed leaf discs were then submerged 10 mL of distilled water in glass tubes. One set of test tubes was immersed in a water bath at 45°C for one hour. Then, for an overnight period, both sets were exposed to a temperature of 22°C in an air-conditioned environment. The electrical conductivity of each sample was measured the next day using the LF 538 EC metre after it had been well shaken. The leaf tissues were then killed by autoclaving at 121°C for 15 minutes at 15 lbs pressure, then allowing them to cool to 22°C overnight. The electrical conductivity was then measured for the second time. The amount of membrane integrity allows a measure of membrane stability to electrolyte leakage when the membrane was stressed. Using the first and second electrical conductivity data and the following method, the relative cell injury percentage (RCI %) was calculated as a measure of cell membrane thermostability.

CMT% = {1- (T1 / T2)} / {1- (C1 / C2)}] × 100

The subscripts 1 & 2 refer to the 1st and 2nd EC readings, respectively, while T & C denote electrical conductivity (EC) of heat treated and controlled sets of test tubes. Equations were used to calculate cell membrane thermostability and relative high temperature damage.

3.3.3 Chlorophyll stability index (CSI)

Koleyorea's (1958) approach was used to calculate CSI. The study employed 200mg of fresh leaf samples of uniform sizes. Two sets of leaves were placed in the test tubes: One pair was held at room temperature as a control, while the other was immersed in an 80ºC water bath for 30 minutes. The spectral absorbance of the water was then measured at 652 nm for each tube. Treated sample = 200 mg of leaf sample kept in a test tube containing

water at 55 °C for 1 h

Control sample = 200 mg of leaf sample kept in a test tube

containing water at room temperature

CSI = (OD at 652 nm of treated sample/OD at 652 nm of control) ×100

3.3.4 Photosynthetic rate (µmol CO_2 m^{-2} sec^{-1})

The photosynthetic rate was determined using a portable photosynthetic system (CIRAS-3 SW, PP System International, MA, USA) from leaf sample of tomato seedlings during the day between 9.00 and 10.30 a.m. and was expressed in µmole CO_2 m^{-2} s^{-1}.

3.4 Biochemical observations

3.4.1 Poly phenol oxidase activity

The method proposed by Mayer *et al.,* was followed for measuring polyphenol oxidase activity. The enzyme extract was prepared from grounded leaf samples by

centrifugation at 2500rpm for 20 minutes using 10mM phosphate buffer (pH 6.5). The supernatant was used as enzyme extract.1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5) and 200ml of enzyme extract were used in the procedure. The reaction was started by adding 200ml of 10mM catechol to the mixture. For 1 minute, the rate of rise in absorbance was measured at 420 nm. Change in absorbance min^{-1} mg^{-1} was used to measure activity. All of the trials were carried out in five replicates and three times each. The enzyme activity was expressed in unit per mg of sample.

3.4.2 Phenyl ammonia lyase activity

A revised version of Lisker *et al.,* (1983) procedure was used to measure PAL activity. The synthesis of trans-cinnamic acid from L-phenylalanine was measured spectrophotometrically to assess the enzyme activity. The enzyme extract was prepared from grounded leaf samples by centrifugation at 2500rpm for 20 minutes using 10mM phosphate buffer (pH 6.5). The supernatant was used as enzyme extract. 1 ml leaf enzyme extract, 0.5 ml 50 mM L-phenylalanine substrate, and 0.4 ml 25 mM Tris-HCl buffer were used in the process (pH 8.8). After a 2-hour incubation at 40°C, the activity was halted by adding 0.06 ml 5 N HCl; the absorbance was measured at 290 nm against a blank reaction mixture containing the same mixture of reaction except the volume of L-phenylalanine. The enzyme activity was measured as moles of trans-cinnamic acid mg^{-1} of protein $hour^{-1}$. The experiments were carried out in five replicates.

3.4.3 Super oxide dismutase

The activity of superoxide dismutase was determined using the technique given by Kono (1978). Grinding 0.5 g of fresh leaf samples in 50 mM potassium phosphate buffer (pH 6.5) and centrifuging at 10,000g for 12 mints yielded an enzyme extract solution. The supernatant was collected and used as an enzyme extract. A test tube was filled with 1.3 ml of 50 mM potassium phosphate buffer, 500ml nitroblue tetrazolium (NBT), and 100 ml Triton-X 100. The superoxide radical generation reaction was started by adding 100 l of hydroxylamine hydrochloride to the above

mixture. After 2 minutes, 70 l of enzyme extract was added, and absorbance at 540 nm was measured; an increase in absorbance was seen at 1 minute intervals. SOD activity was measured in units (the quantity of enzyme necessary to reduce NBT by 50%) min^{-1} mg^{-1} protein, which was subsequently converted to U mg^{-1} FW.

3.4.4 Malon di aldehyde activity

The procedure proposed by Hodges *et al.*,(1999) was used for analysing *Malon di aldehyde* content.. Frozen plant materials (0.5 g) were homogenised in a 5 mL trichloroacetic acid (TCA) solution at 5% (w/v). The supernatant was recovered after centrifuging the homogenate at 4 °C for 10 minutes at 4000 g. The aliquot supernatant was then treated with 2 mL TCA containing 0.67 percent TBA solution. After 30 minutes in a boiling water bath (100°C), the mixture was cooled in an ice bath. The absorbance of the supernatant was measured in a spectrophotometer at 450 nm, 532 nm, and 600 nm after centrifugation at 3000 g for 15 minutes. The MDA content was measured in nanomoles per kilogramme of fresh weight.

3.5 Molecular observation

The expression of *NPR1* was analysed in leaf samples on 5^{th} day and 7^{th} day after treatments with salicylic acid on both varieties (Vellayani Vijay and Pusa Ruby). *P. indica* treated leaf samples of Vellayani Vijay were collected and expression analysis was done in. The transgenerational expression of *NPR1* was analysed by obtaining the expression analysis of second generation progeny seedlings from both *P. indica* and salicylic acid treated Vellayani Vijay.

3.5.1 Isolation of total RNA by trizol method

RNase was removed from work benches and instruments with RNaseZap
(Ambion, USA). During RNA isolation, RNases were the most precarious threat to the procedure. To avoid infection, gloves were periodically cleansed with spirit. The RNases were removed from glass wares and solutions using diethylpyrocarbonate

(DEPC) (Sigma, USA), a potent RNase inhibitor. 1 ml DEPC was dissolved in 1000 ml autoclaved distilled water overnight in a Tarsons digital spinot (Tarsons Products Pvt. Ltd., India). By treating accessories including mortar and pestle, spatula, scissors, and forceps with 0.1 percent DEPC for 24 hours in the dark, they were rendered RNase-free. After wrapping in aluminium foil, these were sterilised at 180°C for 5 hours in a hot air oven (NSW India Limited, India). DEPC water was autoclaved twice to eliminate any traces of DEPC, which can limit the activity of RNA. All of the reagents for RNA isolation were made with DEPC-treated water that had been autoclaved. After soaking for 2 hours in a soaking buffer, the gel apparatus was rinsed thoroughly with DEPC treated water. TAE buffer for agarose gel preparation and tank buffer were produced in DEPC treated water. All pipettes and reagents used in RNA isolation were kept separate from the rest of the lab.

With a pre-cooled pestle and mortar, 1g of plant tissues were ground to a fine powder in liquid nitrogen. 10 ml TRIzol reagent extraction buffer was added to one gram sample and thoroughly mixed. At room temperature, the samples were incubated for 5 minutes. Following incubation, 0.2 ml chloroform was added to each one ml extraction buffer tube, and the tubes were vortex-shacked vigorously for 15 seconds. For 3 minutes, the tubes were incubated at room temperature. The samples were then centrifuged for 15 minutes at 10,000 g at 4°C. A clean screw-cap centrifuge tube was used to transfer the aqueous phase. Then, for each one ml of extraction buffer, add 0.5 ml isopropanol. Gentle inversion was used to cover and mix the tubes, which were then maintained at room temperature for 10 minutes. Tubes were centrifuged (10,000 g; 4°C; 10 min) after incubation, and the supernatant was discarded. The pellet was re-centrifuged (10,000 g; 4°C; 10 min) after being rinsed with 75 percent ice-cold ethanol. The supernatant was thrown away. The RNA pellet was dissolved in 50 µl RNase-free DEPC treated water. Total RNA concentration was determined by measuring absorbance at 260 and 280 nm. The isolated RNA samples were stored at -80°C.

3.5.2 Agarose gel electrophoresis

On a 1.2 percent Agarose gel, sample was loaded. 1 X TAE tank buffer electrophoresis was performed at 70 V. The gel was evaluated using the Gel Doc XR+ Gel Documentation system (Bio-Rad).

3.5.3 cDNA synthesis

cDNA synthesis was done by using the LunaScript® RT SuperMix Kit from New England Biolabs #E3010S/L (Version 2.0 1/20). The first strand of cDNA synthesis was carried out according to the kit manual's guidelines. Using the kit, the cDNA synthesis procedure was carried out at a temperature range of 45 °C to 65 °C. An optimal temperature of 55°C was selected for performing the procedure. The inclusion of genomic DNA or carry-over products might make precise target RNA quantification difficult, especially for low-copy targets. To account for these impacts, it's crucial to perform the required "No-RT" control reactions. To show that positive reactions are meaningful, no template control (NTC) reactions were also set up. All of the steps were carried out in an RNasefree environment by wearing gloves and using new sterile tips and tubes. The cDNA synthesis reactions were prepared as described below.

Table 2 Thermal profile used for cDNA synthesis

Cycle steps	Temperature	Time	Cycles
Primer Annealing	25°C	2 minutes	1
cDNA Synthesis	55°C	10 minutes	
Heat Inactivation	95°C	1 minute	

Table 3 Reaction mix used for cDNA synthesis

Componets	20µl reaction	Final concentration
LunaScript RT SuperMix(5X)	4 µl	1X
RNA Sample	1 µl	1 µg
Nuclease-free water	to 20µl	

3.5.4 Primer designing

The NCBI Primer-BLAST tool was used to create real-time primers. Primers that fulfilled the inclusion criteria were chosen. Primer lengths ranging from 17 to 30 bases were employed. The G:C proportion was found to be between 50% and 60%. The primer melting temperature (Tm) was found to be between 55 ^{0}C and 65^{0}C. G/C repeats of three base pairs were avoided. The Primer Probe Test Tool of Primer Express Software Version 3.0 was used to check the primers for selfdimer, cross dimer, and hairpin structures. The ubiquitin-conjugating enzyme E2 was employed as a reference gene in Real-Time PCR experiments.

3.5.4.1 Annealing temperature standardization

The temperature gradient of NEB Luna Universal Real Time PCR at 58 °C and 60 °C was used to estimate the annealing temperature of the *NPR1* gene. The optimum annealing temperature was chosen as the one with the lowest Ct value.

3.5.5 Real-Time PCR analysis qRT-PCR was used to examine the expression of the *NPR1* gene of cDNA was used as a template for the Real Time PCR study. The ubiquitin-conjugating enzyme E2 was used to normalise gene expression (reference gene). Each reaction was carried out in triplicate in a 20 µl volume. In addition, each primer's NTC was set by replacing the cDNA with 5 µl DNase-free water.

The NEB Luna Universal qPCR Master Mix was used for the real time PCR. The relative fold change in gene expression was evaluated using the Comparative Ct technique after amplification (Livak and Schmittgen, 2001). MS Excel 2010 was used to do the data analysis.

Table 4 Reaction mix used for Ta standardization

Components	20 µl reaction	Final concentration
Luna Universal qPCR Master Mix	10 µl	1X
Forward primer (10 µM)	0.5 µl	0.25 µM
Reverse primer (10 µM)	0.5 µl	0.25 µM
Template DNA	1 µl	2.5 ng
Nuclease-free Water	to 20 µl	

Table 5 Thermal profile used for Ta standardization

	Stage	**Repeat**	**Temperature (°C)**	**Time (s)**
Initial denaturation	1	1	95	300
Denaturation	2	35	95	45
Annealing			58/60/62	30
Primer extension			72	45
Final extension	3	1	72	300

Table 6 Reaction mix used for qPCR reaction

Components	20 µl reaction	Final concentration
Luna Universal qPCR Master Mix	10 µl	1X
Forward primer (10 µM)	0.5 µl	0.25 µM
Reverse primer (10 µM)	0.5 µl	0.25 µM
Template DNA	1 µl	2.5 ng
Nuclease-free Water	to 20 µl	

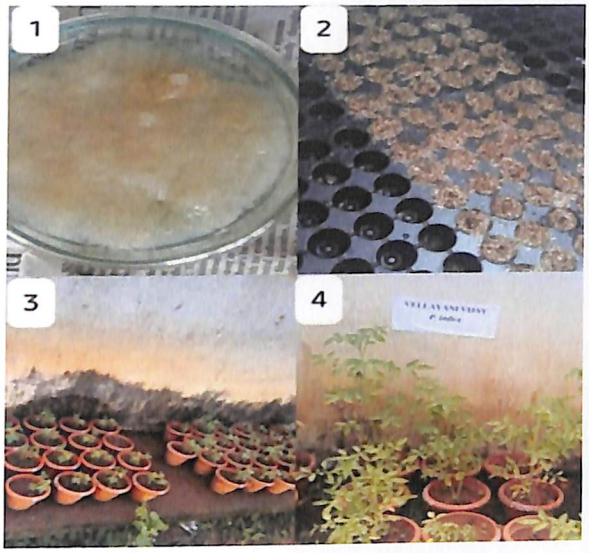

Plate 1- Inoculation of *P. indica* on tomato plants 1)*P. indica* culture, 2) Seeds sown on vermiculate mixture incorporated with 1% *P. indica*, 3) Pusa ruby treated with *P. indica* maintained with control, 4) Vellayani Vijay treated with 1% *P.indica.*

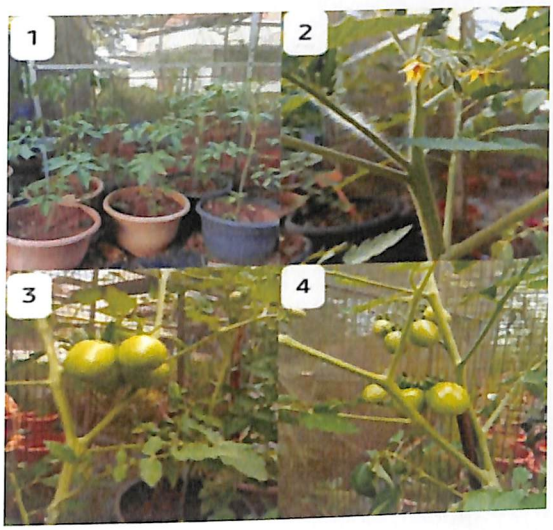

Plate 2- Application of salicylic acid 1) Tomato plants maintained for the application of salicylic acid, 2) Salicylic acid (100ppm) sprayed tomato flowers (labelled), 3&4) Fruits maintained for transgenerational study.

Plate 3- 1) Second generation progeny seedlings of Vellayani Vijay, 2,3&4) Maintenance of seedlings for evaluation of stress tolerance.

RESULTS

4. RESULTS

The title of the study was "Salicylic acid and *Piriformospora indica* induced modulation of the stress resistance mechanism in tomato (*Solanum lycopersicum* L.)" The objective of the study was to analyse salicylic acid induced *NPR1* gene expression as well as *Piriformospora indica* induced resistance modulation to stress and its persistence and transmission through seeds. The treatments such as *Piriformospora indica* (1%) and salicylic acid (100 ppm) were applied to first generation tomato plants, and the evaluation of stress tolerance was done on second generation seedlings. The evaluation of stress was done by subjecting the tomato plants to a higher temperature (abiotic stress) by keeping the treated plants in a polyhouse (ambient + 4^0 C) for 7 days and with *Ralstonia solanacearum* (low density-10^6 cells/ml), the causative agent of bacterial wilt disease (biotic stress). The transgenerational expression of the *NPR1* gene (a resistance related gene) was done by comparing the molecular expression in first generation leaves and second generation seedlings. The data collected throughout the study was statistically analysed, and the results are discussed in this chapter.

4.1 Experiment 1

4.1.1 Salicylic acid (hormone) and *P. indica* induced expression of plant resistance.

Microscopic observations on tomato roots infected with *P. indica* (Vellayani Vijay and Pusa Ruby) revealed the endophytic fungus' ability to colonise roots. On the outer surface and in the cortical sections of roots, dense development of *P. indica* hyphae was detected. Spherical to pear-shaped chlamydospores were observed within the root cortex cells (Plate 4).

4.2 Experiment 2

4.2.1 Evaluation for stress tolerance

Evaluation of stress tolerance was done on second generation tomato seedlings of variety Vellayani Vijay.

4.2.1.1 Isolation of *Ralstonia solanacearum*

Virulent strain of *Ralstonia solanacearum* was isolated from the infected tomato plants and reisolation was done to confirm the virulency by proving Koch's postulate (Plate 6).

The virulent *Ralstonia solanacearum* strain gave pink colonies with a whitish fluid texture (Figure 4.2). The re-isolated colonies were standardised for maintaining low density inoculum using a spectrophotometer. The inoculum used for the induction of biotic stress gave an O.D. value of less than 0.5

.4.2.2 Physiological observations

4.2.2.1 Relative water content

Based on the relative water content, the stress tolerance was evaluated on application of *P. indica* and salicylic acid. *P. indica* treated progeny seedlings induced tolerance against biotic stress and gave 56.646% relative water content, while salicylic acid treated plants had 45.1% relative water content. When seedlings were subjected to abiotic stress, *P. indica* treated progeny seedlings gave 45.1% and salicylic acid treated ones gave 46.880%. On the other hand, the control plants without any treatments gave 42.112% and 40.232% relative water content against biotic and abiotic stress, respectively. The treated plants without the application of any stress gave 87.652% and 84.526% (*P. indica* and salicylic acid, respectively). Absolute control without the application of any stress gave
72.686% relative water content (Table 7).

4.2.2.2 Membrane stability index

Progeny seedlings generated from *P. indica* treated tomato plants showed membrane stability index of 33.68% and 40.32% when subjected to biotic and abiotic stress, respectively. Salicylic acid treated progeny seedlings showed 34.7% and 39.496% membrane stability index. The control plants subjected to biotic and abiotic stress showed 30.6% and 34.48% membrane stability index, respectively. Without application of any stress, the progeny seedlings showed 81.26% and 80.42% membrane stability index when treated with *P. indica* and salicylic acid, respectively.

The absolute control progeny seedlings showed
75.12% membrane stability index in the absence of any stress (Table 8).

4.2.2.3 Chlorophyll stability index

P. indica treated second generation treated with to biotic stress gave chlorophyll stability index 48.31% and with abiotic stress showed 45.266%. In the absence of any stress under normal condition gave 68.218%. Salicylic acid treated progeny seedling resulted 49.362% and 43.258% when subjecting to biotic and abiotic stress respectively. In the absence of any stress chlorophyll stability index was
72.14%. Control plants showed 38.192% and 42.19% chlorophyll stability index when subjecting to biotic and abiotic stress respectively. Absolute control plants under normal condition gave 57.172% (Table 9).

4.2.2.4 Net photosynthetic rate

Subjecting the *P. indica* treated progeny seedlings to biotic and abiotic stress showed a photosynthetic rate of 16.484 and 16.574, respectively, and the photosynthetic rate was expressed in $\mu molesCO_2m^{-2}s^{-1}$. Salicylic acid treatment showed 15.54 and 15.588. Without application of any stress, the photosynthetic rate observed was 23.608 and 25.262, respectively, for *P. indica* and salicylic acid treatment. Control plants showed 14.252 and 13.452 when subjected to biotic and abiotic stress, respectively. Absolute control plants showed a 20.804 photosynthetic rate (Table 10).

4.2.3 Biochemical observations

4.2.3.1 Poly phenol oxidase activity

The poly phenol oxidase activity was found to increase in the presence of stress condition and the treated plants showed higher activity as compared to control plants. *P. indica* treated seedlings resulted 36.53 (min^{-1} mg^{-1}) and 29.092 (min^{-1} mg^{-1}) poly phenol oxidase activity when subjecting to biotic and abiotic stress respectively, whereas salicylic acid treated resulted 36.632 (min^{-1} mg^{-1}) and 26.8 (min^{-1} mg

^{1})activity. The control plants showed an activity of 23.536 (min^{-1} mg^{-1}) and 20.834 (min^{-1} mg^{-1}) against biotic and abiotic stress respectively. Without subjecting any stress the enzyme activity resulted as 19.792 (min^{-1} mg^{-1}) and 18.652 (min^{-1} mg^{-1}) respectively for *P. indica* and salicylic acid. The maximum activity was given by salicylic acid treated against biotic stress and thus enhanced the tolerance. For absolute control plants under normal condition poly phenol oxidase activity was 5.478 (min^{-1} mg^{-1}) (Table 11).

4.2.3.2 Phenyl ammonia lyase activity

The maximum activity of phenyl ammonia lyase was shown by progeny seedlings when parent tomato plants treated with *P. indica* were subjected to biotic stress (4458.6) followed by abiotic stress applied to progeny seedlings (4161.2). Salicylic acid treatment showed 3851.6 and 3945.6 activity against biotic and abiotic stress, respectively. Control plants showed phenyl ammonia lyase activity to a minimum range of 2265 and 2558 against biotic and abiotic stress, respectively. Without any stress application, the *P.indica* treated gave 1218 and the salicylic acid treated showed 1135.6 as the activity of this particular enzyme. Absolute control plants under normal conditions resulted in phenyl ammonia lyase activity of 835 only (Table 12).

4.2.3.3 Super oxide dismutase (SOD) activity

Tolerance to biotic and abiotic stress was evaluated based on SOD activity and the maximum activity was given by progeny seedlings of salicylic acid treated parent plants against abiotic stress (146.55) and against biotic stress, the activity observed was 88.584. Control plants against biotic stress gave an activity of 116.322 and against abiotic stress it was only 23.036. Without the application of any stress, *P. indica* treated gave 14.629 and salicylic acid treated gave 132.082 as SOD activity. *P. indica* treated progeny seedlings gave 23.794 and 26.55 as SOD activity against biotic and abiotic stress, respectively (Table 13).

4.2.3.4 Malondialdehyde content

The Malondialdehyde content determined the susceptible feature of a plant tissue when subjected to a stressful condition, i.e., the higher the Malondialdehyde content, the less the tolerance to stress, since Malondialdehyde is the by-product of lipid peroxidation of plant tissues during extreme conditions. Maximum content was given by the tissue sample of progeny seedlings from control plants when subjected to abiotic stress (32.384) and for biotic stress it was 29.486. Maximum tolerance based on Malondialdehyde content was observed against abiotic stress when treated with salicylic acid (22.41) followed by *P. indica* treated (23.254). With the application of biotic stress, the Malondialdehyde content observed was 28.236 and 27.398 for progeny seedlings from parent tomato plants treated with *P. indica* and salicylic acid, respectively (Table 14).

Table 7 Evaluation of stress tolerance on relative water content for Salicylic acid and P. indica treated second generation seedlings.

Treatment	Relative water content (%)	% increase or decrease over control
P. indica treated(T1)		
Biotic stress	46.400±1.093^{e}	(+)10.2
Abiotic stress	45.100±2.938^{e}	(+)12.09
Without stress	87.652±0.521^{a}	(+)19
Salicylic acid treated(T2)		
Biotic stress	56.646±1.903^{d}	(+)34.5
Abiotic stress	46.880±1.184^{e}	(+)16.5
Without stress	84.526±0.913^{b}	(+)16.8
Control		
Biotic stress	42.112±1.235^{f}	
Abiotic stress	40.232±0.486^{f}	
Absolute control	72.686±0.686^{c}	
SE(d)	0.899	
SE(m)	0.636	
CD(0.5%)	1.823	

The data represents mean value of 9 samples. Figures in a column followed by the same letter do not differ significantly ($P<0.05$)

Table 8 Evaluation of stress tolerance on membrane stability index for Salicylic acid and P. indica treated second generation seedlings.

Treatment	Membrane stability index	% increase or decrease over control
P. indica treated(T1)		
Biotic stress	33.680±0.719^{d}	(+)10
Abiotic stress	40.320±0.239^{c}	(+)16.9
*W*ithout stress	81.260±0.680^{a}	(+)8
Salicylic acid treated(T2)		
Biotic stress	34.700±0.464^{d}	(+)13
Abiotic stress	39.496±0.330^{c}	(+)14.5
*W*ithout stress	80.420±1.517^{a}	(+)7
Control		
Biotic stress	30.600±0.938^{d}	
Abiotic stress	34.480±1.477^{d}	
Absolute control	75.120±0.327^{b}	
SE(d)	0.551	
SE(m)	0.39	
CD(0.5%)	1.118	

The data represents mean value of 9 samples. Figures in a column followed by the same letter do not differ significantly ($P<0.05$)

Table 9 Evaluation of stress tolerance on chlorophyll stability index for Salicylic acid and P. indica treated second generation seedlings.

Treatment	Chlorophyll stability index	% increase or decrease over control
P. indica treated(T1)		
Biotic stress	48.310±0.166[e]	(+)26.4
Abiotic stress	45.266±0.175[f]	(+)7
*W*ithout stress	68.218±0.082[b]	(+)19
Salicylic acid treated(T2)		
Biotic stress	49.362±0.238[d]	(+)29
Abiotic stress	43.258±0.177[g]	(+)2
*W*ithout stress	72.140±0.016[a]	(+)26
Control		
Biotic stress	38.192±0.094[i]	
Abiotic stress	42.190±0.085[h]	
Absolute control	57.172±0.083[c]	
SE(d)	0.089	
SE(m)	0.063	
CD(0.5%)	0.18	

The data represents mean value of 9 samples. Figures in a column followed by the same letter do not differ significantly ($P<0.05$)

Table 10 Evaluation of stress tolerance on net photosynthetic rate for Salicylic acid and P. indica treated second generation seedlings.

Treatment	Net photosynthetic rate ($\mu molesCO_2m^{-2}s^{-1}$)	% increase or decrease over control
P. indica treated(T1)		
Biotic stress	16.484 ± 0.296^{c}	(+)15.6
Abiotic stress	16.574 ± 0.428^{c}	(+)23.2
*W*ithout stress	23.698 ± 0.467^{a}	(+)14
Salicylic acid treated(T2)		
Biotic stress	15.540 ± 0.489^{cd}	(+)9
Abiotic stress	15.588 ± 0.490^{cd}	(+)15.8
*W*ithout stress	25.262 ± 0.733^{a}	(+)21
Control		
Biotic stress	14.252 ± 0.317^{de}	
Abiotic stress	13.452 ± 0.373^{e}	
Absolute control	20.804 ± 3.793^{b}	
SE(d)	0.847	
SE(m)	0.599	
CD(0.5%)	1.717	

The data represents mean value of 9 samples. Figures in a column followed by the same letter do not differ significantly ($P<0.05$)

Table 11 Evaluation of stress tolerance on poly phenol oxidase activity for Salicylic acid and P. indica treated second generation seedlings.

Treatment	Poly phenol oxidase activity (min^{-1} mg^{-1})	% increase or decrease over control
P. indica treated(T1)		
Biotic stress	36.530±0.937[a]	(+)55.2
Abiotic stress	29.092±0.812[b]	(+)39.6
*W*ithout stress	19.792±0.501[e]	
Salicylic acid treated(T2)		
Biotic stress	36.632±0.697[a]	(+)55.6
Abiotic stress	26.800±0.875[c]	(+)28.6
*W*ithout stress	18.652±0.6[g]	
Control		
Biotic stress	23.536±0.548[d]	
Abiotic stress	20.834±0.936[e]	
Absolute control	5.478±0.518[h]	
SE(d)	0.464	
SE(m)	0.328	
CD(0.5%)	0.941	

The data represents mean value of 9 samples. Figures in a column followed by the same letter do not differ significantly ($P<0.05$)

Table 12 Evaluation of stress tolerance on phenyl ammonia lyase activity for salicylic acid and P. indica treated second generation seedlings.

Treatment	Phenyl ammonia lyase activity (µmtca/h/mg protein)	% increase or decrease over control
P. indica treated(T1)		
Biotic stress	4458.6±20.550[a]	(+)96
Abiotic stress	4161.2±22.917[b]	(+)62.7
*W*ithout stress	1218.0±16.852[g]	
Salicylic acid treated(T2)		
Biotic stress	3851.6±9.370[d]	(+)70
Abiotic stress	3945.6±19.437[c]	(+)54
*W*ithout stress	1135.6±30.501[h]	
Control		
Biotic stress	2265.4±29.022[f]	
Abiotic stress	2558.0±35.714[e]	
Absolute control	838.0±40.429[i]	
SE(d)	16.839	
SE(m)	11.907	
CD (0.5%)	34.15	

The data represents mean value of 9 samples. Figures in a column followed by the same letter do not differ significantly ($P<0.05$)

Table 13 Evaluation of stress tolerance on super oxide dismutase (SOD) activity for Salicylic acid and P. indica treated second generation seedlings.

Treatment	SOD activity (unit g^{-1} FW)	% increase or decrease over control
P. indica treated(T1)		
Biotic stress	23.794 ± 1.635^{f}	(+)3
Abiotic stress	26.550 ± 0.287^{e}	(+)5
*W*ithout stress	14.692 ± 0.294^{g}	
Salicylic acid treated(T2)		
Biotic stress	88.584 ± 0.294^{d}	(+)2.8
Abiotic stress	146.550 ± 0.312^{a}	(+)9
*W*ithout stress	132.082 ± 0.769^{b}	
Control		
Biotic stress	23.036 ± 0.707^{f}	
Abiotic stress	116.322 ± 0.456^{c}	
Absolute control	12.604 ± 0.475^{h}	
SE(d)	0.448	
SE(m)	0.317	
CD(0.5%)	0.909	

The data represents mean value of 9 samples. Figures in a column followed by the same letter do not differ significantly ($P<0.05$)

Table 14 Evaluation of stress tolerance on Malondialdehyde content for salicylic acid and P. indica treated second generation seedlings.

Treatment	Malondialdehyde activity g^{-1} (nmol FW)	% increase or decrease over control
P. indica treated(T1)		
Biotic stress	28.236 ± 0.155^{c}	(-)4
Abiotic stress	23.254 ± 0.167^{e}	(-)28
*W*ithout stress	12.502 ± 0.092^{h}	
Salicylic acid treated(T2)		
Biotic stress	27.398 ± 0.350^{d}	(-)7
Abiotic stress	22.410 ± 0.195^{f}	(-)30
*W*ithout stress	10.370 ± 0.190^{i}	
Control		
Biotic stress	29.486 ± 0.277^{b}	
Abiotic stress	32.384 ± 0.375^{a}	
Absolute control	17.404 ± 0.322^{g}	
SE(d)	0.16	
SE(m)	0.113	
CD(0.5%)	0.325	

The data represents mean value of 9 samples. Figures in a column followed by the same letter do not differ significantly ($P<0.05$)

4.2.4 Expression analysis of *NPR1* gene (Non expressor of PathogenesisRelated Genes 1) using Real-time PCR.

4.2.4.1 RNA isolation

Total RNA was isolated from fresh leaf tissues and seedlings of Vellayani Vijay and Pusa Ruby tomato plants using the TRIzol reagent method. The isolated RNA samples were kept at -80 °C.

4.2.4.2 Agarose gel electrophoresis

The RNA was observed as two bands (28 S and 18 S) in 1.2 % Agarose gel. (Plate 7 & 8).

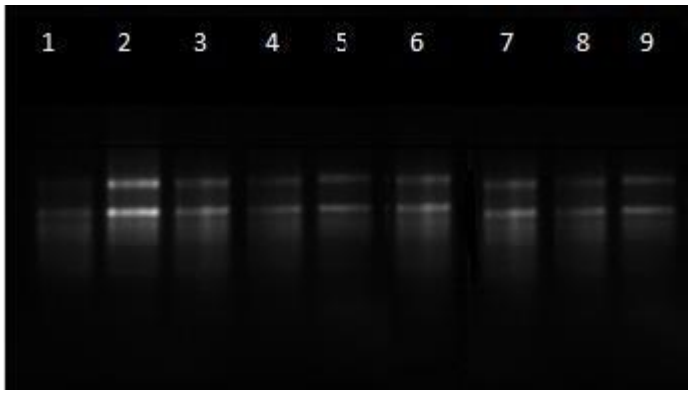

Plate 7 RNA isolated from first generation leaf samples of tomato plant, Lane 1- Control of *P. indica* treated Vellayani Vijay, Lane 2- *P. indica* treated Vellayani Vijay(T1), Lane 3- Vellayani Vijay treated with salicylic acid (5th day),Lane 4- Vellayani Vijay treated with salicylic acid (7th day), Lane 5& 6- Control for Vellayani Vijay treated with salicylic acid, Lane 7- Pusa ruby treated with salicylic acid (5th day), Lane 8- Pusa ruby treated with salicylic acid (7th day), Lane 9- Control for Pusa ruby treated with salicylic acid.

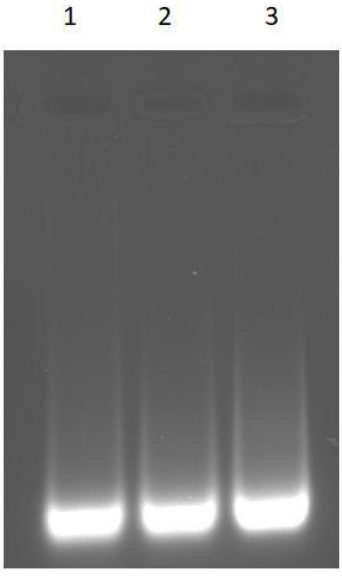

Plate 8 RNA isolated from second generation tomato seedlings, Lane 1- *P. indica* treated Vellayani vijay(T1), Lane 2- Vellayani vijay treated with salicylic acid, Lane 3- Control for second generation Vellayani vijay seedlings.

4.2.4.3 Quantification of RNA

Concentration as well as purity values of RNA isolated were represented in Table 15 & Table 16.

Table 15 Yield and purity of isolated RNA (first generation tomato leaves)

Treatment	Vellayani vijay		Pusa Ruby	
	Concentration (ng/µl)	A_{260}/A_{280}	Concentration (ng/µl)	A_{260}/A_{280}
P. indica treated (T1)	5.86	2.05	-	-
T1 control	44.1	2.01	-	-
5th day of S.A treated (T2)	13.9	2.12	5.69	2.06
7th day of S.A treated (T2)	2.98	2.15	17.6	2.12
Control of S.A treated	14.8	2.13	10.5	2.13

Table 16 Yield and purity of isolated RNA (Second generation tomato seedlings)

Treatment	Vellayani vijay	
	Concentration (ng/µl)	A_{260}/A_{280}
P. indica treated (T1)	100	2.12
T1 control	18.3	2.01
S.A treated (T2)	100	2.15
Control of S.A treated	18.3	2.05

4.2.4.4 cDNA synthesis

Both tomato varieties (Vellayani Vijay and Pusa Ruby) extracted RNA samples were diluted to a concentration of 50 ng/l. It was then converted to cDNA using the New England Biolabs #E3010S/L LunaScript® RT SuperMix Kit (Version 2.0 1/20). A 25ng RNA sample was produced for each 20µl reaction using suitable dilution from each sample in relation to the corresponding extracted RNA concentration. The generated cDNA was kept at -20°C.

4.2.4.5 Primer designing

The Primer-BLAST tool was used to design a total of four sets of primers, including a reference gene (Ubiquitin-conjugating enzyme E2). Primer Probe Test Tool of Primer Express Software Version 3.0 was used to check the primers for self-dimers, cross dimers, and hairpin structures. Following that, the gene's secondary structure was obtained using the RNA fold web server, and the primer's binding location was evaluated to ensure that the primer did not bind to the gene's secondary structures. The following was a list of the primers that were created as a result of this process.

T*able 17 List of primers designed for Real-Time PCR studies.*

Gene	Primers	Amplicon size
Ubiquitin-conjugating enzyme E2	**FW** GCTCTCTGTTGACAGACCCA **RW** GAGTCCAGCTACGAGCAGTG	108bp
NPR1	**FW** ATGTTGAATTACTAAGGATGTTGCT **RW** GAAGTACCGTGTGTCCTCTAGGA	112bp

4.2.4.6 Primer reconstitution and dilution

All of the primers were reconstituted according to the manufacturer's instructions. After dissolution, it was stored at -20 °C as a stock. Working standards were diluted stocks ten times. **4.2.4.7 Annealing temperature standardization**

After running thermal gradient qPCR at 58°C, 60°C, and 62°C, the best annealing temperature was determined to be 60°C. Since the reaction was carried out at 60°C, the lowest threshold cycle (Ct) values were obtained.

Table 18 Standardization of annealing temperature of Real-Time primers

Temperature	**Threshold cycle (Ct) values of *NPR1*gene**
58°C	23.56
60°C	22.21
62°C	23.09

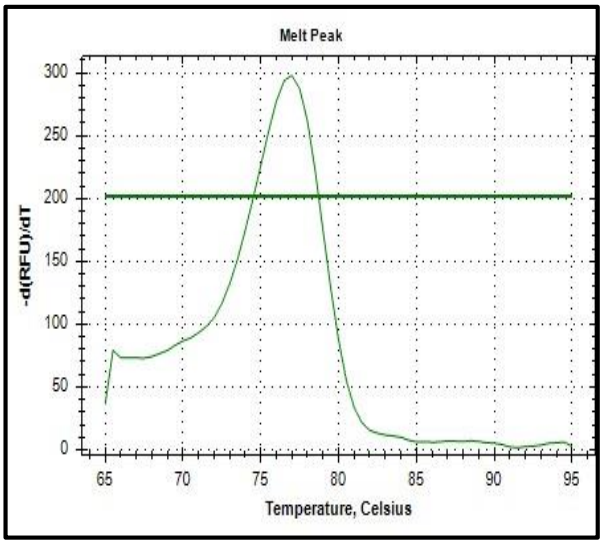

Plate 9 Melt curve analysis of *NPR1* gene

4.2.5 Expression analysis of *NPR1* gene.

4.2.5.1 Salicylic acid treated expression on susceptible variety (Pusa Ruby) and resistant variety (Vellayani Vijay)

Defence-related gene expression was analysed in parent tomato plants of both varieties (Vellayani Vijay and Pusa Ruby). Two treatments were used, salicylic acid and *P. indica* induced modulation, which were analysed based on the expression of the *NPR1* gene. A relative fold change in the expression of *NPR1* was given by salicylic acid treated leaf samples of Vellayani Vijay and Pusa Ruby. Vellayani Vijay treated with salicylic acid gave a 6.543 fold and a 3.58 fold expression after the 5^{th} day and 7^{th} day of treatment. Pusa Ruby treated with salicylic acid gave a 5.531 fold and a 1.635 fold expression. The resistant variety resulted in a higher expression of *NPR1* as compared to the susceptible variety.

Table 19 NPR1 expression for first generation

Treatments	Relative fold change (RFC)	
	Vellayani vijay	Pusa ruby
5^{th} day of S.A treated	6.543±0.21	5.531±0.1
Control	1.0±0.1	1.0±0.1
7^{th} day of S.A treated	3.58±0.01	1.635±0.04
Control	1.0±0.1	1.0±0.1
SE(d)	0.11	0.25
SE(m)	0.08	0.18
CD (0.5%)	0.48	1.07

4.2.5.2 Transgenerational expression of NPR1

Transgenerational expression of *NPR1* was analysed for both *P. indica* and the salicylic acid-treated tomato variety Vellayani Vijay. Induced Systemic Resistance (ISR) offered by *P. indica* and Systemic Acquired Resistance (SAR) offered by salicylic acid were analysed based on defense related gene expression *NPR1*, which was involved in the crosstalk of ISR and SAR. Salicylic acid and *P. indica* were applied to parent plants, and *NPR1* expression was determined to be 6.543 fold and 4.757 fold, respectively. This was then compared to the expression of *NPR1* in progeny seedlings and obtained a 5.31 fold and 1.83 fold expression. Induction of resistance in Vellayani Vijay was done in the first generation and the defense related gene expression was obtained in progeny seedlings.

Table 20 NPR1 trans generational expression

Treatments	Relative fold change (RFC)	
	P. indica	Salicylic acid
First generation	4.757±0.46	6.543±0.07
Control	1.0±0.1	1.0±0.1
Second generation	1.83±0.09	5.31±0.26
Control	1.0±0.1	1.0±0.1
SE(d)	0.14	0.35
SE(m)	0.07	0.13
CD (0.5%)	0.68	1.21

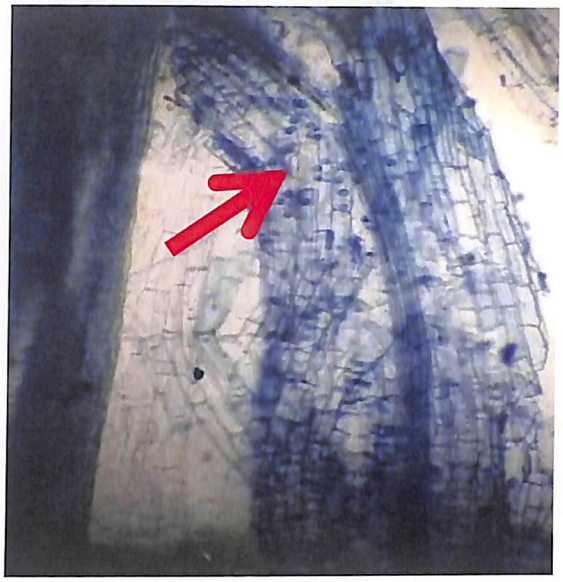

Plate 4 Root of tomato plant (Vellayani Vijay) stained with Lactophenol tryphan blue for confirming the colonisation of *P. indica*.

Plate 5- *Ralstonia solanacearum* isolated in SMSA media

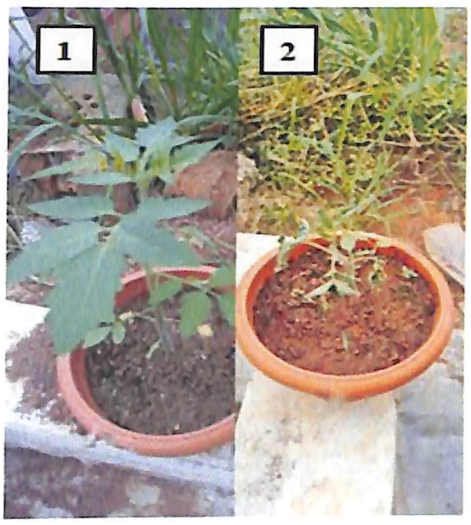

Plate 6- 1) Healthy tomato plant inoculated with *Ralstonia solanacearum* through soil drenching, 2) The plant showing symptom of bacterial wilt

DISCUSSION

5. DISCUSSION

Plants are vulnerable to a variety of environmental factors that diminish and limit their agricultural crop yield. Plants are exposed to two forms of environmental stress: (1) abiotic stress and (2) biotic stress. Radiation, salt, floods, droughts, temperature extremes, heavy metals, and other biotic stresses all contribute to the death of key crop plants around the world. Biological challenges, on the other hand, include attacks by pathogens such as fungi, bacteria, oomycetes, nematodes, and herbivores. Plants have evolved a variety of methods to deal with these biotic and abiotic challenges. They detect the environmental level of stress, become activated, and then produce the necessary cellular responses. They achieve this by receiving stimuli from sensors on the cell surface or in the cytoplasm and transmitting them to the nucleus' transcriptional machinery via several signal transduction channels. The plant's tolerance to stress was due to differential transcriptional modifications. The signalling pathways serve as a link between recognising the stress environment and generating a biochemical and physiological response that is suitable.

Tomatoes (*Solanum lycopersicum L.*) are one of the world's most popular vegetables. It is quite popular among consumers and is frequently served raw as a salad vegetable. Juices, ketchup, sauces, soups, and other products are made from the fruit. It is the vegetable that is canned the most. Tomatoes are a good source of vitamins A and C in most modern diets. About the consumption of this particular fruit in the state of Kerala: it is the state's most popular fruit, and a kilo or two of the scarlet fruit is a must-have on any housewife's grocery list. Tamil Nadu and Karnataka provide nearly all of the state's tomato supplies. One of the most common diseases of tomatoes and other solanaceous plants is bacterial wilt. The disease has been reported in the wet tropics, subtropics, and temperate parts of the globe. *Ralstonia solanacearum* is the causative agent of bacterial wilt, the most devastating tomato disease, especially infecting tomato cultivation in Kerala. Disease progression is aided by high soil moisture and temperatures. The quick and complete drooping of normal grown-up plants is a symptom of bacterial wilt. Lower leaves may fall off before they wilt. When infected plant portions are cut and immersed in clear water, a white stripe of bacterial slime appears. Through understanding the mechanism of resistivity of plants

aided by molecular techniques, the study provided a better development of cultivation practises for tomatoes as well as other food crops.

Understanding the methods by which plants protect themselves against pathogens, it could lead to new disease-resistant crop cultivation strategies. Both biotic stresses resulting from pathogens like *Ralstonia* and abiotic stresses like variation in atmospheric temperature can adversely affect plant growth and alter many physiological and molecular parameters.

Salicylic acid (SA), a common plant phenolic molecule, is known to be an endogenous plant metabolism regulator. SA has long been known to influence plant growth and development, allowing it to perform a variety of physiological and metabolic tasks. *P. indica* is an axenicically cultured fungus that uses signal transduction to develop local and systemic resistance to fungal and viral plant diseases. Stress tolerance can be conferred by *P. indica* - mediated activation of antioxidant defence system components and expression of stress-related genes. Exogenous application of salicylic acid and incorporation of *P. indica* inoculum on seeds or seedlings may eventually elicit the induced resistance mechanism in the plant systemic system (Gill *et al.,* 2016).Li *et al.,* 2019).

In rice and tomatoes, exogenous treatment with glutamate (Glu), an essential amino acid for all living creatures, causes resistance against fungal infections. According to transcriptome analysis, Glu substantially increases the expression of wound, defence, and stress-related genes in Arabidopsis seedlings. Glu, it turns out, promotes the expression of genes that are triggered by pathogens or damage-associated molecular patterns (PAMPs or DAMPs) (Goto *et al.,* 2020). In high-throughput reverse and forward genetic screens, virus-induced gene silencing (VIGS) has been used to investigate the functions of genes in various aspects of plant function (Burch-Smith *et al*. 2004). Chen *et al.,* 2009) discovered that ethylene, salicylic acid, and mitogen-activated protein kinase-related defence pathways are involved in tomato resistance to bacterial wilt through virusinduced gene silencing. Induced resistance (IR) is frequently linked to a greater ability to mobilise cellular defensive mechanisms in response to pathogen infection (Durrant and Dong, 2006). Systemic acquired resistance (SAR) is the most common type of IR, and it's governed by a signalling

pathway that relies on endogenous accumulation of SA, which is linked to the formation of defensive elements like PR proteins. Because tomatoes are a heat-susceptible crop, hormone-induced stress tolerance can be researched by utilising a variety of treatments (Stitcher *et al.*, 1997).

Slaughter *et al.,* 2012 suggested that treatment with the chemical BABA or inoculation with avirulent bacteria generates a primed condition in *Arabidopsis* plants, which is passed down to the progeny. Primed plant's offspring have a higher baseline level of disease resistance and a greater ability to respond to additional priming treatments. Greater levels of SA-dependent gene transcripts of PR1, PR2, and PR5 associated with increased disease resistance in the progeny of primed lines against virulent pathogen upon infection, showing widespread alterations in the regulatory mechanisms of defence gene expression. Specifically *NPR1* gene plays a major role in both systemic acquired resistance and induced systemic resistance in plant defence mechanism (Van loon *et al.,* 1998).

5.1 SALICYLIC ACID AND *Piriformospora indica (P. indica)* INDUCED EXPRESSION OF PLANT RESISTANCE IN TOMATO

In the current study two varieties of tomato, bacterial wilt susceptible Pusa Ruby (IARI) and resistant Vellayani Vijay (KAU), were used for the transgenerational expression of defence related gene *NPR1*, on elicitation with a chemical and biological agent,

5.1.1 EVALUATION OF TRANSFER OF INDUCED RESISTANCE IN PROGENY SEEDLINGS

5.1.1.1 Based on physiological parameters

Relative water content, which directly accounts for the osmotic adjustment and the powerful mechanism of conserving cellular hydration, was used as the first physiological parameter, and the results showed that the increase in percentage of relative water content for *P. indica* treated was about 10.2 and 12.09 for biotic and abiotic stress, respectively, and for salicylic acid treated it was observed as 34.5 and

16.5 respectively for biotic and abiotic stress application. Without application of any type of stress, the relative water content showed an increase in percentage of about 19 and 16.8 for *P.indica* and Salicylic acid treated second generation seedlings when compared with absolute control (Figure 1). Similar observations by Bagheri *et al.,* (2013) suggested that the application of endomycorhizal fungi *to* rice shows the accumulation of osmoprotetant proteins and showed an increase in relative water content in colonised plants as compared to the control. As suggested by Abdelaal *et al.,* (2020) when the study was conducted in barley plants, the exogenous application of salicylic acid resulted in an increase in relative water content when applied with abiotic stress.

Many conditions hazardous to plant growth and development target biological membranes as their initial site of action. It is widely acknowledged that maintaining membrane integrity and stability under water stress is a key component of drought tolerance in plants. According to Bajji et al. (2002), the membrane stability index (MSI) is another physiological parameter that has been widely used to evaluate drought and heat tolerance. Under stress, cell membrane rupturing occurs more often, resulting in decreased sustainability and osmotic potential. Drought also causes membrane breakage, reduced osmotic adjustments, and cytoplasm content deposition. Plants' ability to maintain MSI with water retention, on the other hand, suggests plant tolerance. Decreased membrane sustainability indicates the level of lipid peroxidation, which results in high numbers of oxidative bursts under water deficiency situations (Blackman *et al.,* 1995). The present study showed a relative increase in biotic and abiotic stress tolerance in terms of MSI of 10%, 16.9%, for *P. indica* treatment and 13% and 14.5% for salicylic acid treatment (Figure 2). George *et al*. (2015) studied tomatoes for water stress tolerance using polyethylene glycol and membrane stability index and obtained the same level of tolerance to applied stress when compared to control plants. Hosseini *et al*. (2017) studied the effect of *P. indica* colonisation on wheat against abiotic and mechanical stress and revealed an increase in the membrane stability index under stressed conditions on colonised plants as compared to non-colonised wheat plants. Samea *et al.* (2018) suggest that exogenous application of salicylic acid and cytokinin in faba beans showed a positive tolerance to saline stress

through the induction of physiological changes that result in an increased membrane stability index, antioxidant activity and sugar accumulation.

The chlorophyll stability index (CSI) is a measure of a plant's ability to withstand stress. A high CSI value indicates that the stress had little influence on plant chlorophyll concentration. Plants having a higher CSI have more chlorophyll available, which helps them tolerate stress. This results in a higher photosynthetic rate, dry matter production, and productivity. This shows how well chlorophyll can cope with stress. This study gave an increase in the percentage of CSI of 26.4% for *P. indica* treated against biotic stress and a 7% increase against biotic stress. Similarly, compared to the control used, an increase of 29% against biotic stress and 2% against abiotic stress was observed for salicylic acid treated tomato plants (Figure 3). Bhattarai *et al. (*2015) in an article on combining ability and gene action studies for heat-tolerance and physiobiochemical traits in tomato suggested the use of CSI for analysing the selection of tolerant varieties of tomato against stress. Farhengi *et al*. (2018) evaluated the effects of 1 mM salicylic acid (SA) under different levels of salt stress. Leaf cells were enriched with potassium and calcium ions, which increased glycine betaine, soluble sugars, proteins, antioxidant enzymes, chlorophyll content, and chlorophyll stability index. These advantages of SA treatment resulted in a significant increase in soybean plant biomass (10%) and seed yield (17%). Saddique *et al*. (2018) suggested in their study on three different varieties of rice that the *P. indica* symbiotic relationship enhanced seedling biomass, phosphorus uptake, and zinc uptake, and increased the fluorescence of chlorophyll, all of which are important for rice growth under drought stress.

The rate of photosynthesis is a broad indicator of how quickly a plant absorbs solar energy and converts it to organic carbon molecules. Plant photosynthetic efficiency is reduced by biotic and abiotic stress, which have detrimental impacts on photosynthetic pigment production, photosystem performance, electron transport mechanisms, gas exchange parameters, CO_2 fixation, carbohydrate metabolism, and many other processes (Dresselhaus *et al.,* 2018). The photosynthetic rate on the application of *P. indica* showed an increase in percentage of about 15.6 and 23.2 for biotic and abiotic stress, respectively, and with the exogenous application of salicylic acid, showed an

increase in percentage of 9 and 15.8 for biotic and abiotic stress. Without any stress application, an increase of 14% and 21% of photosynthetic rate as compared to absolute control for *P.indica* and salicylic acid application, respectively (Figure 4). Fariduddin *et al*. (2003) suggested that an improved photosynthetic rate was observed in *Brassica juncea* under the application of salicylic acid exogenously. Achalz *et al*. (2010) suggested an increase of 49% in net photosynthetic rate on barley under the colonisation of rood endophyte *P. indica* .

5.1.2 Based on biochemical parameters

Polyphenol oxidase (PPO) catalyses the conversion of phenols to quinines, which then self-oxidize and polymerize with cellular protein amino acids, resulting in brown and black coloration. Higher phenolic oxidation potential was linked to significantly higher levels of resistance to bacterial infection and other stressful situations. In a study on transgenic tomatoes, Li and Steffens (2002) suggested that the over-expressed PPO activity enhanced the resistivity in many varieties as compared to control. When the phytoremediation effect of cadmium in Medicago sativa was studied, Li *et al.,* (2020), discovered a higher PPO activity on plant samples having *P. indica* colonised roots. War *et al*. (2011) suggested that plants responded promptly to SA at 1.5 mM, with increased induction of PPO activity, as well as higher phenol and protein accumulation. The present study showed that an increase in percentage of about 55.2 and 28.6 was shown for *P. indica* treated second generation seedlings against biotic and abiotic stress, respectively. In the case of the application of salicylic acid, 55.6% and 28.6% were shown (Figure 5) to result in an increase in tolerance for seedlings generated from treated tomato plants.

The enzyme phenylalanine ammonia lyase (PAL) catalyses the deamination of phenylanine by forming a carbon-carbon double bond with the release of ammonia, resulting in trans-cinnamic acid. Using the technique of in vivo incubation of grape berries (Vitis vinifera L.), Wen *et al*. (2008) suggested the effects of exogenous salicylic acid on the gene expression of PAL, observed the synthesis of new PAL protein, and increased activity under high temperature stress. According to Ahanger *et al*. (2015), root colonisation of *P. indica* in wheat showed high expression levels of

PAL related defence genes and PAL activity against powdery mildew (biotic stress). The present study reveals an increase in the activity of PAL with the application of salicylic acid as well as *P. indica* . Salicylic acid increased PAL activity against biotic and abiotic stress by 70% and 54%, respectively, while *P. indica* increased it by 96% and 62.7% (Figure 6).

When plants are exposed to stress, reactive oxygen species (ROS) are produced, which can damage the cell membrane. As a result, they control scavenger enzymes (like SOD) expression in the cell membrane system against oxidative damage to protect themselves from oxidative stress and maintain their cellular activity. This can be considered a significant road to tolerance in unfavourable settings. Plants from oxidative damage are protected by the relationship between enzymatic and non-enzymatic antioxidants as well as cellular ROS stability. Super oxide dismutase (SOD) plays the basic function of converting reactive oxygen anions to hydrogen peroxide, which then by the action of catalase (CAT), produces water. Kumar *et al*. (2009) suggested that in maize colonised with *P. indica* there was an increase in antioxidant enzyme activity and, specifically, an eight fold increase was observed in the case of SOD. In tomato, the study showed an increase of 3% and 5% with the application of *P. indica* against biotic and abiotic stress, respectively. Similarly, with the application of salicylic acid, the increase observed was 2.8% and 9% (Figure 7). While studying the effect of salicylic acid on heat tolerance in Kentucky bluegrass, an increase in SOD activity resulted, this gave tolerance to stress (He *et al.,* 2005).

Various environmental stresses affect plant processes in different ways, resulting in a loss of cellular homeostasis and the generation of reactive oxygen species (ROS), which cause oxidative damage to membranes, lipids, proteins, and nucleic acids. Diverse environmental stresses differently affect the plant processes that lead to a loss of cellular homeostasis accompanied by the formation of ROS, which causes oxidative damage to membranes, lipids, proteins, and nucleic acids. Malondialdehyde (MDA) is a byproduct of lipid peroxidation, and the higher the content, the lower the tolerance. Savicka *et al. (*2010) suggested that the effect of a higher temperature on wheat led to the accumulation of MDA in wheat, and the concentration was observed to be different in different organs. Exogenous application of salicylic acid in plants could

affect their tolerance against abiotic stress like drought, salinity, and extreme temperatures, and thus the concentration of MDA is observed to be less as compared to those without treatment (Hasamuzzaman *et al.*, 2017). Ghorbani *et al*. (2018) suggested the application of *P. indica* in tomatoes to alleviate salinity and improve tolerance. *P. indica* inoculation also reduced H_2O_2, MDA, and superoxide anion. The present study on the application of *P. indica* reduces the MDA content by 4% and 28%, thus inducing tolerance against biotic and abiotic stress. With the application of salicylic acid, the reduction in content observed was between 7% and 30% (Figure 8). Thus, when observation on MDA content, the induction of resistance was evident in progeny seedlings with the application of *P. indica* and salicylic acid on parent plants.

5.1.3 Based on molecular parameters

Non-expressor of Pathogenesis-Related Genes 1 (*NPR1*) is a key player in a plant's response to pathogen infection. *NPR1* is involved in the development of both systemic acquired resistance (SAR) and induced systemic resistance (ISR). It mediates cross-talk between the salicylic acid (SA) and jasmonic acid/ethylene
(JA/ET) responses, acting as the master key to the plant defence signalling network. *NPR1* was considered to be the bonafied SA receptor. An essential component of the SA-defense response system was *NPR1,* the protein involved in fundamental responses to pathogenic challenges. The discovery of *NPR1*, a positive regulator of SAR, came as a result of the search for a SA responsive protein (Backer *et al.*, 2019). In *Arabidopsis*, the basidiomycete *Piriformospora indica* induced powdery mildew (*Golovinomyces orontii*) resistance, which required specialised defence pathways (Jahan *et al.*, 2019). In the wild-type, *Piriformospora indica* root colonisation reduced fungal conidia and *NPR1*-3 (nonexpressor of PR genes 1-3) expression. For *P. indica* -induced resistance, *NPR1* must be found in the cytoplasm, nuclear localization was not found (Jahan *et al.*, 2019). In the study done on tomatoes, the expression of *NPR1* was increased with the application of both *P. indica* and salicylic acid (Figure 9). When analysing the transgenerational expression, the *NPR1* expression was observed (Figure 10). The relative fold change in the *NPR1* gene was analysed in tomato and a 7 fold change in *NPR1* was observed when inoculated with *Xanthomonas* strains where salicylic acid

acts as a signalling molecule involved in the expression of defence related genes (Liu *et al.*, 2021). Ahangar *et al. (*2017) explained the expression of *NPR1* in wheat when treated with salicylic acid and *P. indica* against powdery mildew stress (biotic stress). At 12 hours after inoculation of the pathogen *Blumeria graminnis*, *NPR1* transcripts increased by 1.7 times in salicylic acid-treated plants compared to control plants, and at 24 hours after inoculation, *NPR1* expression reached its highest point. According to a comparison of transcript levels, the level of transcription in treated plants was around 2.1 times higher than in control plants. According to gene expression analysis, the expression of the *NPR1* gene was identical between *P. indica* colonised and non-colonized plants in the absence of a pathogen challenge. The expression of the *NPR1* gene increased in the early hours following infection. The levels of *NPR1* gene transcription in *P. indica* colonised plants were 1.7 times greater than in non-colonized plants.

SUMMARY

6. SUMMARY

The work entitled 'Salicylic acid and *Piriformospora indica* induced modulation of stress resistance mechanism in tomato (*Solanum lycopersicum* L.)' was conducted at the College of Agriculture Vellayani during the time frame of 2020– 2021. The objective of the study was to analyse salicylic acid induced *NPR1* gene expression as well as *Piriformospora indica* induced resistance modulation to stress and its persistence and transmission through seeds. The study emphasised the stress tolerance of tomato progeny seedlings against biotic (*Ralstonia solanacearum*) and abiotic (heat stress) stress when the parent plants were treated with salicylic acid and endophytic fungi (*P. indica).* Salicylic acid is usually involved in systemic acquired resistance (SAR), where SA acts as a key signalling molecule to impart resistance against infectious pathogens (biotic stress). *P. indica* is an endophytic, beneficial microbe whose colonisation in root devices an acquired resistance, namely induced systemic resistance (ISR). The results based on biochemical, physical, and molecular parameters showed a higher tolerance against stress was obtained for progeny seedlings generated from treated plants as compared to control plants.

Two varieties of tomato were involved in the study: Pusa Ruby (a bacterial wilt susceptible variety) and Vellayani Vijay (a bacterial wilt resistant variety). *Piriformospora indica* (1%) was applied along with the potting mixture and colonisation was confirmed by staining with lactophenol trypan blue stain. *P. indica* induced stress modulation was analysed by determining the expression of *NPR1* expression in leaves after confirming the colonisation. Salicylic acid (100 ppm) was applied during the flowering stage and expression of the *NPR1* gene was done by collecting the leaf samples on the 5^{th} and 7^{th} days after the treatment. The induced resistance transgenerational expression of the *NPR1* gene with the application of salicylic acid on both resistant (Vellayani Vijay) and susceptible (Pusa Ruby) varieties was analysed and compared to that of the leaf samples of the parent plant.

Induced resistance against biotic and abiotic stress was analysed by subjecting the progeny seedlings of salicylic acid and *P. indica* treated tomato plants with the bacterial wilt causative agent *Ralstonia solanacearum* (low density-10^6 cells/ml) and a higher temperature (ambient + 4^0 C) for 7 days. The experiment was conducted on

Vellayani Vijay progeny seedlings generated from parent plants treated with *P. indica* (1%) and salicylic acid (100 ppm) to analyse the transfer of induced resistance through seeds.

Observation based on relative water content (%) showed that salicylic acid treated progeny seedlings exhibit maximum tolerance compared to control plants under stressful conditions. For the treated plants, the tolerance against stress based on cell membrane stability index (%) was increased as progeny seedlings of *P. indica* treated were shown to show a higher value when subjected to abiotic stress. Chlorophyll stability index (%) based observation showed that salicylic acid treated progeny seedlings resuslted higher tolerance when subjected to biotic stress. When tolerance against stress was explained with photosynthetic rate ($\mu molesCO_2m^{-2}\ s^{-1}$), maximum tolerance was given by *P. indica* treated progeny seedlings when subjected to abiotic stress.

Biochemical observations were made on progeny seedlings generated from both *P. indica* and salicylic acid treated plants when subjected to both biotic and abiotic stress. The interplay between environmental stress and plants causes changes in cell metabolism, most notably in enzyme activity. Analysis of enzymes such as polyphenol oxidase (PPO), phenyl ammonia lyase (PAL), super oxide dismutase (SOD) and Malondialdehyde content (MDA) was done on progeny seedlings generated from *P. indica* and salicylic acid-treated tomato plants. Progeny seedlings generated from salicylic acid-treated tomato plants exhibit higher PPO activity when subjected to biotic stress. Phenylalanine ammonia lyase activity was expressed in µmtca/h/mg of total protein isolated and maximum activity was given by *P. indica* treated against biotic stress. Tolerance regarding super oxide dismutase activity was given by salicylic acid treated progeny seedlings against abiotic stress. The significant activity of SOD was shown by salicylic treated and *P. indica* treated plants showing comparable activity to that of control plants. Malondialdehyde content was analysed after being subjected to biotic and abiotic stress. Progeny tomato plants generated from both *P. indica* and salicylic acid-treated plants showed lower malondialdehyde content under stress when compared to that of control plants, thus imparting tolerance significantly. By analysing physiological and biochemical parameters of the progeny seedlings

generated after treatments, tolerance was imparted and significant resistance was shown as compared to control progenies generated from parent plants lacking any treatments.

Salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) were three essential signaling components of the induced defence system in plants. There was crosstalk between the ISR and SAR pathways because the ISR response was mediated by a mixture of JA and ET, but the SAR pathway can suppress JAdependent responses. Both the SAR and ISR pathways use *NPR1* as a common regulator. *NPR1* was shown to modulate the antagonistic effect of SA on JA signaling. The level of *NPR1* was sufficient for the expression of both the ISR and SAR pathways at the same time. Salicylic acid induced *NPR1* expression was analysed in a bacterial wilt susceptible variety (Pusa Ruby) and a resistant variety (Vellayani Vijay). The resistant variety showed significantly better *NPR1* expression when compared to the susceptible variety. Transgenerational expression of *NPR1* was analysed in Velayani Vijay for both *P. indica* and salicylic acid (SA) treated progenies. Significant expression was confirmed in both treatments for first generation leaves and second generation seedlings. Molecular observation regarding *NPR1* gene expression confirms the transfer of induced resistance to progeny seedlings when the treatment was applied to parent tomato plants.

The study concluded that an acquired resistance could be induced on parent tomato plants by using the hormone salicylic acid and endophytic fungi *P. indica* . This induced resistance was stored as molecular signals and could be expressed in progeny seedlings, confirming the transfer of resistance through the seeds. So the future line of study could be continued as the identification of signalling molecules (like transcription factors) associated with *P. indica* mediated induced resistance that allows the transgenerational expression of *NPR1* signalling. Dynamics of *P. indica* mediated defence signaling with respect to transgenerational expression analysis and use of the *NPR1* gene as a desired gene in genetic engineering for increasing the potential of plant resistance to pathogens could also be considered for further study.

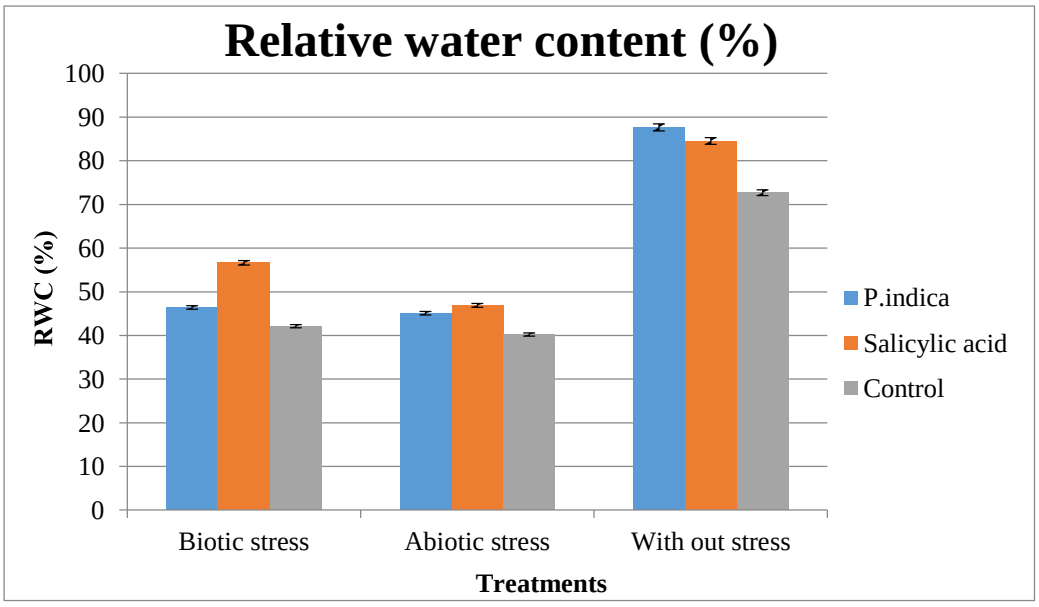

Figure 1 Graph showing relative water content for salicylic acid and P. indica treated second generation tomato seedlings.

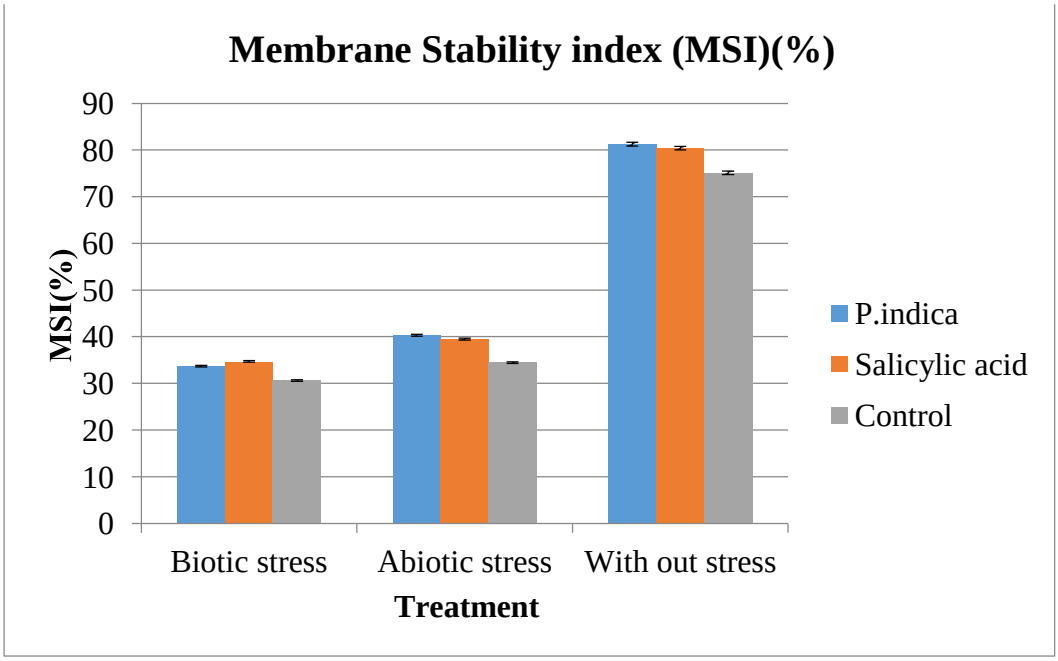

Figure 2 Graph showing membrane stability index for salicylic acid and P. indica treated second generation tomato seedlings.

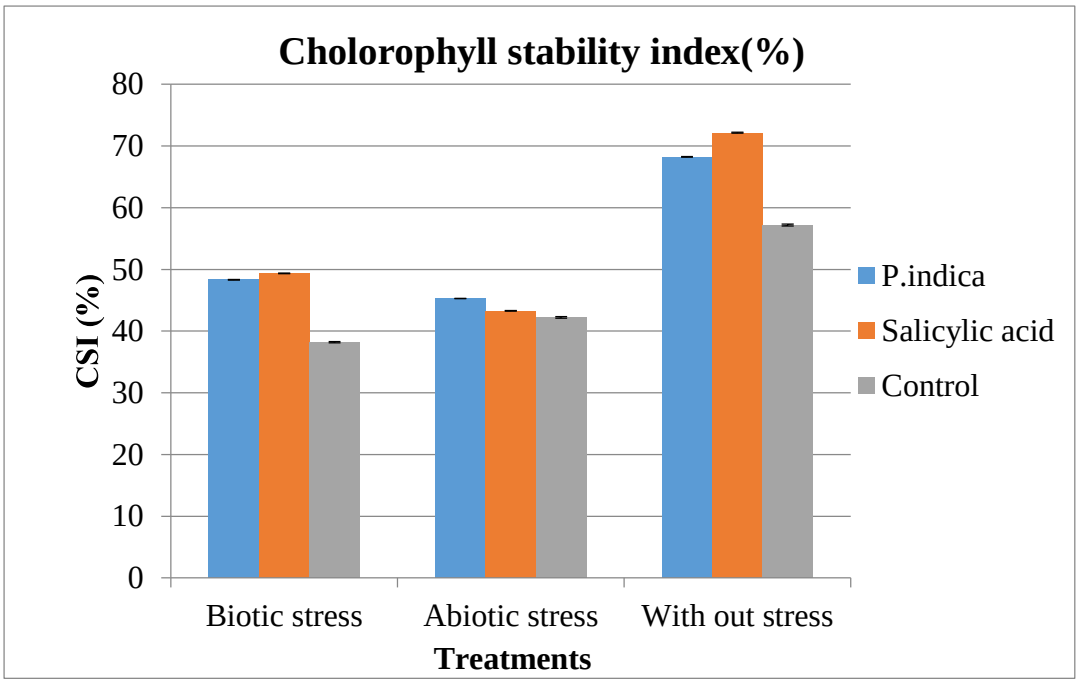

Figure 3 Graph showing chlorophyll stability index for salicylic acid and P. indica treated second generation tomato seedling.

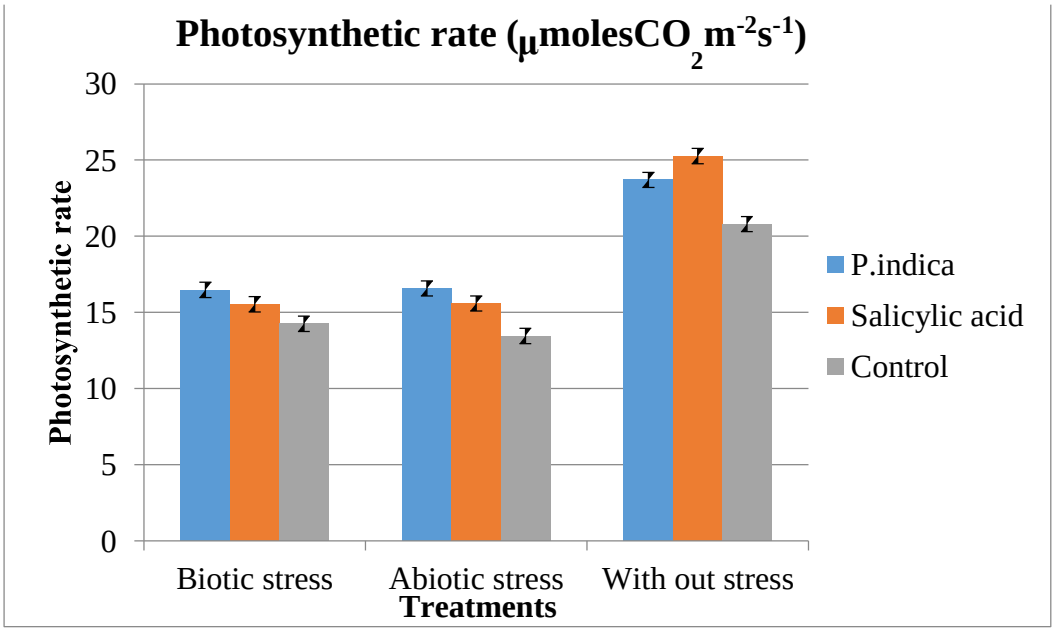

Figure 4 Graph showing photosynthetic rate for salicylic acid and P. indica treated second generation tomato seedlings expressed in μmoles$CO_2$$m^{-2}s^{-1}$.

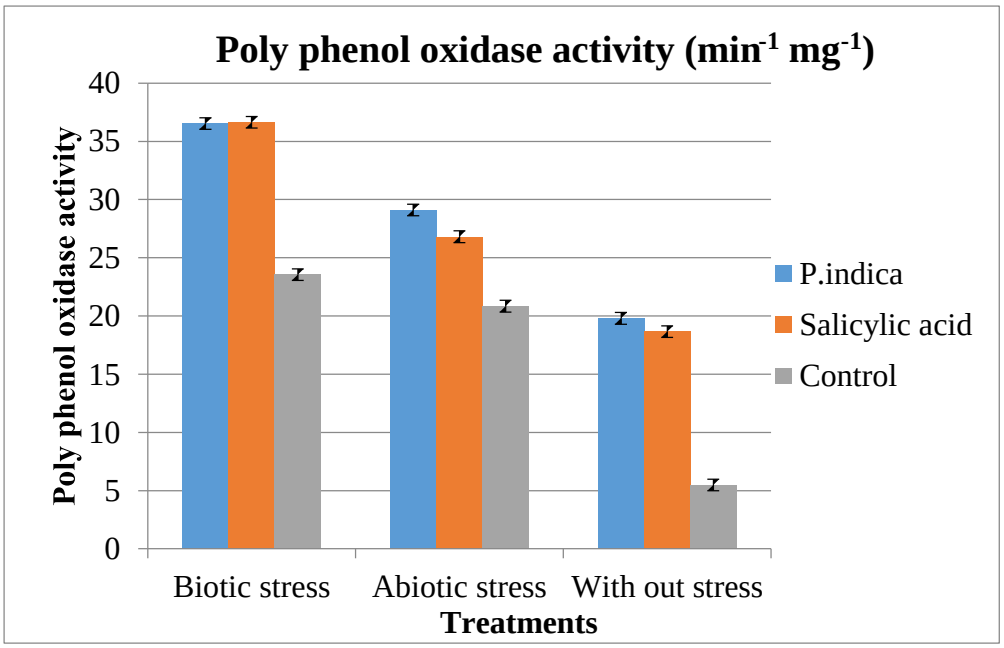

Figure 5 Graph showing poly phenol oxidase activity for salicylic acid and P. indica treated second generation tomato seedlings expressed in min^{-1} mg^{-}

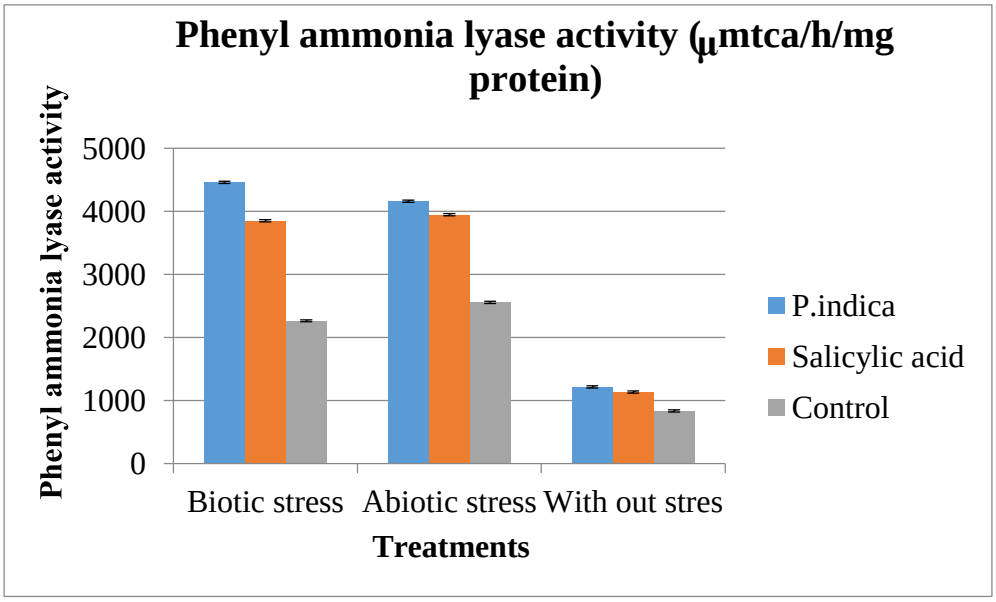

Figure 6 Graph showing phenyl ammonia lyase activity for salicylic acid and P. indica treated second generation tomato seedlings expressed in μmtca/h/mg protein.

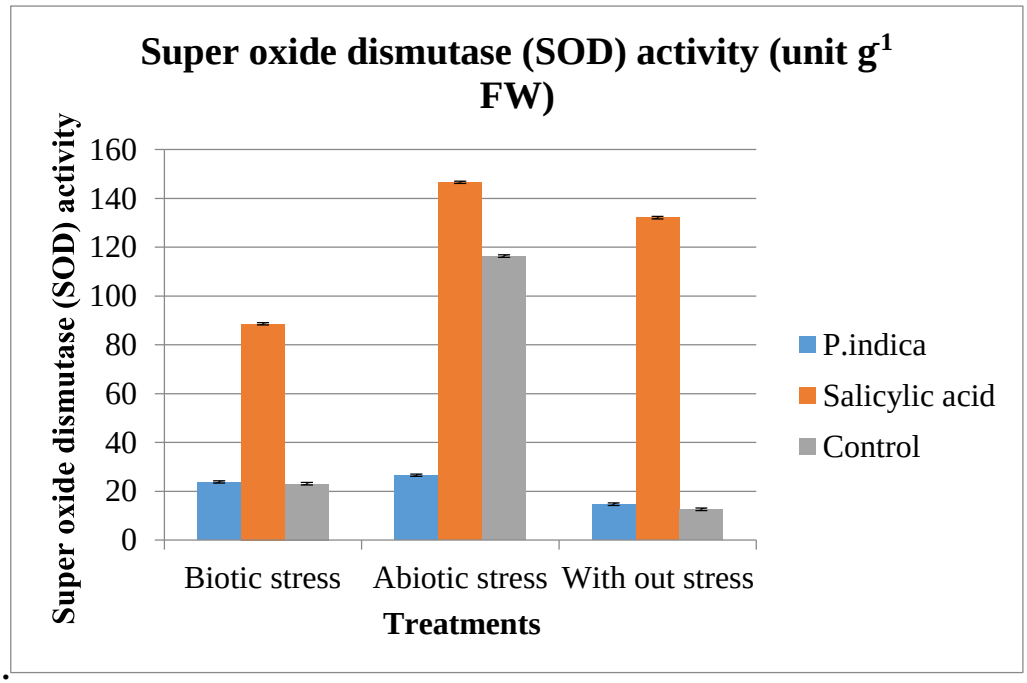

Figure 7 Graph showing super oxide dismutase (SOD) activity for salicylic acid and P. indica treated second generation tomato seedlings.

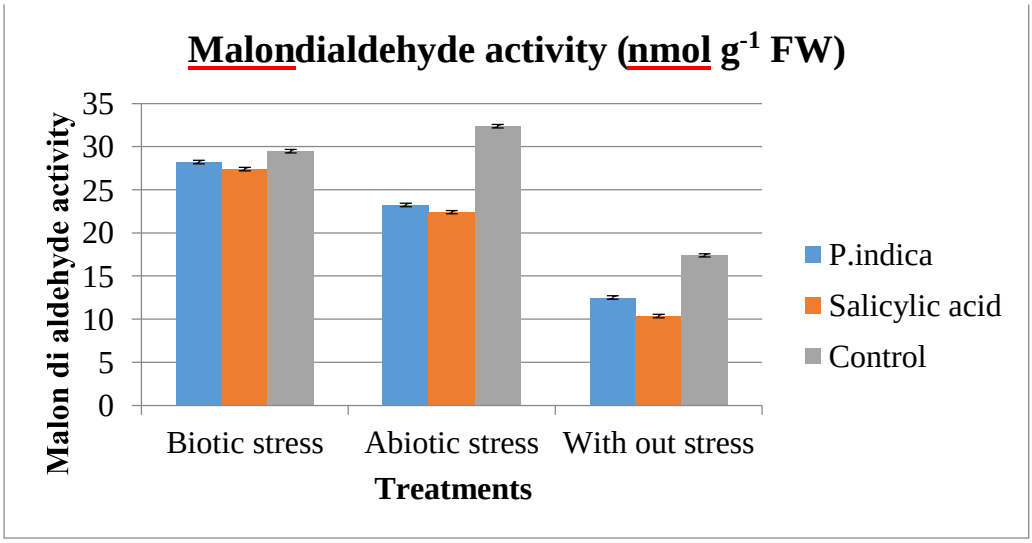

Figure 8 Graph showing Malondialdehyde content for Salicylic acid and P. indica treated second generation tomato seedlings expressed in nmol g^{-1} FW.

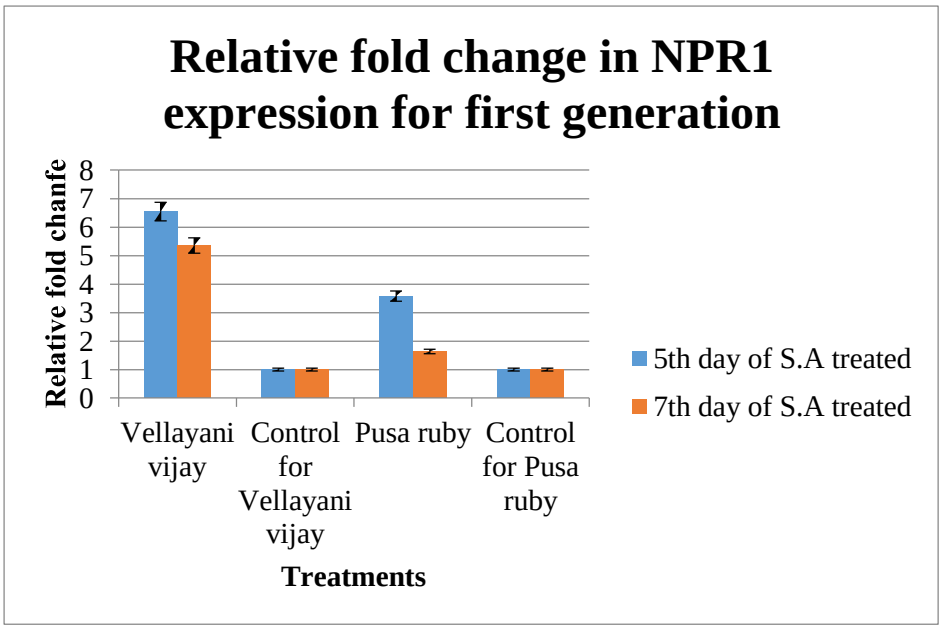

Figure 9 Graph showing relative fold change in NPR1 expression for first generation tomato plants.

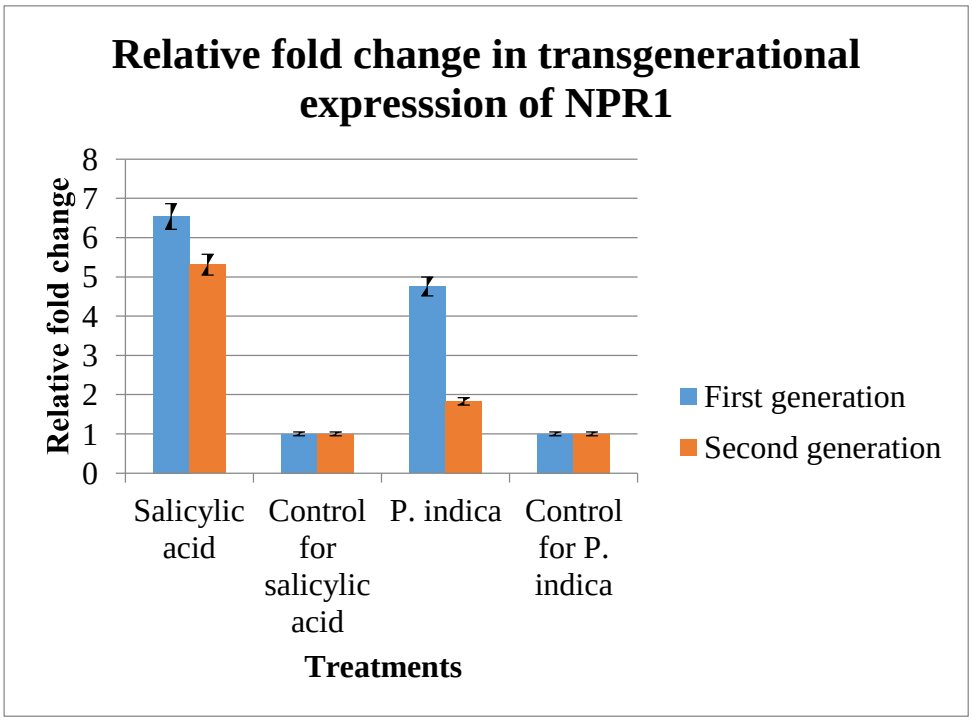

Figure 10 Graph showing relative fold change in NPR1 expression for second generation tomato plants.

REFERENCES

7. REFERENCES

Abo-Elyousr, K.A.M. and Asran, M.R. 2009. Antibacterial activity of certain plant extracts against bacterial wilt of tomato. *Archives of Phytopathology and Plant Protection*. *42*(6):573-578.

Ahangar, L., Ranjbar, G.A., Babaeizad, V., Najafi H. and Biabani, A.2015. Study on expression of phenylalanine ammonia lyase and pathogenesis related genes in wheat symbiont with endomycorrhyzal fungus *Piriformospora indica* after infection with powdery. *Iranian Journal of Plant Pathology*, *50*(4):369-384.

Ahangar, L., Ranjbar, G.A., Babaeizad, V., Najafi Zarrini, H. and Biabani, A. 2017. Assay of *NPR1* gene expression in wheat under powdery mildew stress. *Journal of Crop Protection*, *6*(1):157-166.

Alexandersson, E., Mulugeta, T., Lankinen, Å., Liljeroth, E. and Andreasson, E.2016. Plant resistance inducers against pathogens in Solanaceae species—From molecular mechanisms to field application. *International journal of molecular sciences*, *17*(10):1673.

Ali, S., Ganai, B.A., Kamili, A.N., Bhat, A.A., Mir, Z.A., Bhat, J.A., Tyagi, A., Islam, S.T., Mushtaq, M., Yadav, P. and Rawat, S. 2018. Pathogenesisrelated proteins and peptides as promising tools for engineering plants with multiple stress tolerance. *Microbiological Research*, *212*:29-37.

Anith, K.N., Sreekumar, A. and Sreekumar, J. 2015. The growth of tomato seedlings inoculated with co-cultivated *Piriformospora indica* and Bacillus pumilus. *Symbiosis*, *65*(1):9-16.

Aslam, M.M., Karanja, J. and Bello, S.K. 2019. *Piriformospora indica* colonization reprograms plants to improved P-uptake, enhanced crop performance, and biotic/abiotic stress tolerance. *Physiological and Molecular Plant Pathology*, *106*:232-237.

Athira, S. and Anith, K.N. 2020. Plant growth promotion and suppression of bacterial

wilt incidence in tomato by rhizobacteria, bacterial endophytes and the root endophytic fungus *Piriformospora indica*. *Indian Phytopathology*, *73*(4):629-642.

Backer, R., Naidoo, S. and Van den Berg, N. 2019. The NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (*NPR1*) and related family: mechanistic insights in plant disease resistance. *Frontiers in plant science*, *10*:102.

Bajji, M., Kinet, J.M. and Lutts, S., 2002. The use of the electrolyte leakage method for assessing cell membrane stability as a water stress tolerance test in durum wheat. *Plant growth regulation*, *36*(1):61-70.

Bhattarai, U., Talukdar, P., Sharma, A. and Das, R. 2015. Combining ability and gene action studies for heat-tolerance physio-biochemical traits in tomato. *Asian Journal of Agricultural Research*, *10*(2), pp.99-106.

Blackman, S.A., Obendorf, R.L. and Leopold, A.C. 1995. Desiccation tolerance in developing soybean seeds: the role of stress proteins. *Physiologia Plantarum*, *93*(4):630-638.

Boller, T. and Felix, G. 2009. A renaissance of elicitors: perception of microbeassociated molecular patterns and danger signals by pattern-recognition receptors. *Annual review of plant biology*, *60*:379-406.

Burch-Smith, T.M., Anderson, J.C., Martin, G.B. and Dinesh-Kumar, S.P. 2004. Applications and advantages of virus-induced gene silencing for gene function studies in plants. *The Plant Journal*, *39*(5):734-746.

Champoiseau, P.G., Jones, J.B. and Allen, C.2009. Ralstonia solanacearum race 3 biovar 2 causes tropical losses and temperate anxieties. *Plant Health Progress*, *10*(1):35.

Chen, Y.Y., Lin, Y.M., Chao, T.C., Wang, J.F., Liu, A.C., Ho, F.I. and Cheng, C.P.2009. Virus-induced gene silencing reveals the involvement of ethylene-, salicylic acid- and mitogen-activated protein kinase-related defense pathways in the resistance of tomato to bacterial wilt. *Physiologia*

Plantarum, *136*(3):324-335.

Das, A., Kamal, S., Shakil, N.A., Sherameti, I., Oelmüller, R., Dua, M., Tuteja, N., Johri, A.K. and Varma, A. 2012. The root endophyte fungus *Piriformospora indica* leads to early flowering, higher biomass and altered secondary metabolites of the medicinal plant, Coleus forskohlii. *Plant signaling & behavior*, *7*(1):103-112.

Dheemanth, T.L., Kumar, P.G., Nazeem, P.A., Mathew, S.K. and Reddy, M.A. 2017. Field screening of F3 and F4 generations of tomato for combined resistance to bacterial wilt and tomato leaf curl virus (ToLCV) diseases. *Vegetable Science*, *44*(1):81-85.

Dieffenbach, C.W., Lowe, T.M. and Dveksler, G.S. 1993. General concepts for PCR primer design. *PCR methods appl*, *3*(3):S30-S37.

Ding, Y., Sun, T., Ao, K., Peng, Y., Zhang, Y., Li, X. and Zhang, Y. 2018. Opposite roles of salicylic acid receptors *NPR1* and NPR3/NPR4 in transcriptional regulation of plant immunity. *Cell*, *173*(6):1454-1467.

Dodds, P.N. and Rathjen, J.P. 2010. Plant immunity: towards an integrated view of plant–pathogen interactions. *Nature Reviews Genetics*, *11*(8):539-548.

Dolatabadi, H.K., Goltapeh, E.M., Moieni, A., Jaimand, K., Sardrood, B.P. and Varma, A.2011. Effect of *Piriformospora indica* and Sebacina vermifera on plant growth and essential oil yield in Thymus vulgaris in vitro and in vivo experiments. *Symbiosis*, *53*(1):29-35.

Durrant, W.E. and Dong, X. 2004. Systemic acquired resistance. *Annu. Rev. Phytopathol.*, *42*:185-209.

Fakhro, A., Andrade-Linares, D.R., von Bargen, S., Bandte, M., Büttner, C., Grosch, R., Schwarz, D. and Franken, P. 2010. Impact of *Piriformospora indica* on tomato growth and on interaction with fungal and viral pathogens. *Mycorrhiza*, *20*(3):191-200.

George, S., Minhas, N.M., Jatoi, S.A., Siddiqui, S.U. and Ghafoor, A.2015. Impact of

polyethylene glycol on proline and membrane stability index for water stress regime in tomato (Solanum lycopersicum). *Pakistan Journal of Botany*, *47*(3):835-844.

Ghorbani, A., Razavi, S.M., Ghasemi Omran, V.O. and Pirdashti, H.2018. *Piriformospora indica* inoculation alleviates the adverse effect of NaCl stress on growth, gas exchange and chlorophyll fluorescence in tomato (Solanum lycopersicum L.). *Plant Biology*, *20*(4):729-736.

Gill, S.S., Gill, R., Trivedi, D.K., Anjum, N.A., Sharma, K.K., Ansari, M.W., Ansari, A.A., Johri, A.K., Prasad, R., Pereira, E. and Varma, A. 2016. *Piriformospora indica*: potential and significance in plant stress tolerance. *Frontiers in Microbiology*, *7*:332.

Gill, S.S., Gill, R., Trivedi, D.K., Anjum, N.A., Sharma, K.K., Ansari, M.W., Ansari, A.A., Johri, A.K., Prasad, R., Pereira, E. and Varma, A.2016. *Piriformospora indica*: potential and significance in plant stress tolerance. *Frontiers in Microbiology*, *7*:332.

Gimenez, E., Salinas, M. and Manzano-Agugliaro, F.2018. Worldwide research on plant defense against biotic stresses as improvement for sustainable agriculture. *Sustainability*, *10*(2):391.

Goto, Y., Maki, N., Ichihashi, Y., Kitazawa, D., Igarashi, D., Kadota, Y. and Shirasu, K. 2020. Exogenous treatment with glutamate induces immune responses in Arabidopsis. *Molecular Plant-Microbe Interactions*, *33*(3):474-487.

Hamamouch, N., Li, C., Seo, P.J., PARK, C.M. and Davis, E.L.2011. Expression of Arabidopsis pathogenesis-related genes during nematode infection. *Molecular Plant Pathology*, *12*(4):355-364.

Hasanuzzaman, M., Nahar, K., Bhuiyan, T.F., Anee, T.I., Inafuku, M., Oku, H. and Fujita, M.2017. Salicylic acid: an all-rounder in regulating abiotic stress responses in plants. *Phytohormones-Signaling Mechanisms and crosstalk in plant development and stress responses*:31-75.

Hodges, D.M., DeLong, J.M., Forney, C.F. and Prange, R.K. 1999. Improving the

thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. *Planta*, *207*(4):604-611.

Holeski, L.M., Jander, G. and Agrawal, A.A., 2012. Transgenerational defense induction and epigenetic inheritance in plants. *Trends in ecology & evolution*, *27*(11), pp.618-626.

Jahan, M.S., Wang, Y., Shu, S., Zhong, M., Chen, Z., Wu, J., Sun, J. and Guo, S., 2019. Exogenous salicylic acid increases the heat tolerance in Tomato (Solanum lycopersicum L) by enhancing photosynthesis efficiency and improving antioxidant defense system through scavenging of reactive oxygen species. *Scientia Horticulturae*, *247*:421-429.

Jakab, G., Cottier, V., Toquin, V., Rigoli, G., Zimmerli, L., Métraux, J.P. and Mauch-Mani, B. 2001. β-Aminobutyric acid-induced resistance in plants. *European Journal of plant pathology*, *107*(1), pp.29-37.

Jiang, G., Wei, Z., Xu, J., Chen, H., Zhang, Y., She, X., Macho, A.P., Ding, W. and Liao, B. 2017. Bacterial wilt in China: history, current status, and future perspectives. *Frontiers in Plant Science*, *8*:1549.

Jiang, L., Wu, J., Fan, S., Li, W., Dong, L., Cheng, Q., Xu, P. and Zhang, S. 2015. Isolation and characterization of a novel pathogenesis-related protein gene (GmPRP) with induced expression in soybean (Glycine max) during infection with Phytophthora sojae. *PLoS one*, *10*(6):e0129932.

Kashyap, S.P., Prasanna, H.C., Kumari, N., Mishra, P. and Singh, B. 2020. Understanding salt tolerance mechanism using transcriptome profiling and de novo assembly of wild tomato Solanum chilense. *Scientific reports*, *10*(1):1-20.

Kelman, A. 1954. The relationship of pathogenicity of Pseudomonas solanacearum to colony appearance in a tetrazolium medium. *Phytopathology*, *44*(12).

Kogel, K.H. and Langen, G. 2005. Induced disease resistance and gene expression in cereals. *Cellular Microbiology*, *7*(11):1555-1564.

Kono, Y. 1978. Generation of superoxide radical during autoxidation of hydroxylamine and an assay for superoxide dismutase. *Archives of biochemistry and biophysics*, *186*(1):189-195.

Kumar, M., Yadav, V., Tuteja, N. and Johri, A.K. 2009. Antioxidant enzyme activities in maize plants colonized with *Piriformospora indica*. *Microbiology*, *155*(3):780-790.

Kurabachew, H. and Ayana, G. 2016. Bacterial Wilt caused by Ralstonia solanacearum in Ethiopia: StatusaAnd Management Approaches: A Review. *International journal of Phytopathology*, *5*(3):107-119.

Lakshmipriya, P., Nath, V.S., Veena, S.S., Anith, K.N., Sreekumar, J. and Jeeva, M.L. 2017. *Piriformospora indica*, a Cultivable Endophyte for Growth Promotion and Disease Management in Taro (Colocasia esculenta (L.). *Journal of Root Crops*, *42*(2):107-114.

Li, L. and Steffens, J.C. 2002. Overexpression of polyphenol oxidase in transgenic tomato plants results in enhanced bacterial disease resistance. *Planta*, *215*(2):239-247.

Li, L., Zhu, P., Wang, X. and Zhang, Z. 2020. Phytoremediation effect of Medicago sativa colonized by *Piriformospora indica* in the phenanthrene and cadmium co-contaminated soil. *BMC biotechnology*, *20*(1):1-14.

Li, T., Huang, Y., Xu, Z.S., Wang, F. and Xiong, A.S. 2019. Salicylic acidinduced differential resistance to the Tomato yellow leaf curl virus among resistant and susceptible tomato cultivars. *BMC plant biology*, *19*(1):1-14.

Liao, T., Zhou, L., Liu, J., Zou, L., Dai, T. and Liu, W. 2021. Inhibitory mechanism of salicylic acid on polyphenol oxidase: A cooperation between acidification and binding effects. *Food Chemistry*, *348*:129100.

Lisker, N., Cohen, L., Chalutz, E. and Fuchs, Y. 1983. Fungal infections suppress ethylene-induced phenylalanine ammonia-lyase activity in grapefruits. *Physiological Plant Pathology*, *22*(3):331-338.

Liu, X., Meng, G., Wang, M., Qian, Z., Zhang, Y. and Yang, W. 2021. Tomato SlPUB24 enhances resistance to *Xanthomonas euvesicatoria* pv. perforans race T3. *Horticulture research*, *8*(1):1-18.

Liu, Y., Sun, T., Sun, Y., Zhang, Y., Radojičić, A., Ding, Y., Tian, H., Huang, X., Lan, J., Chen, S. and Orduna, A.R. 2020. Diverse roles of the salicylic acid receptors *NPR1* and NPR3/NPR4 in plant immunity. *The Plant Cell*, *32*(12):4002-4016.

Livak, K.J. and Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *methods*, *25*(4):402-408.

Luna, E., Bruce, T.J., Roberts, M.R., Flors, V. and Ton, J. 2012. Next-generation systemic acquired resistance. *Plant physiology*, *158*(2):844-853.

Mamphogoro, T.P., Babalola, O.O. and Aiyegoro, O.A. 2020. Sustainable management strategies for bacterial wilt of sweet peppers (Capsicum annuum) and other Solanaceous crops. *Journal of applied microbiology*, *129*(3):496-508.

Martineau, J.R., Specht, J.E., Williams, J.H. and Sullivan, C.Y. 1979. Temperature tolerance in soybeans. I. Evaluation of a technique for assessing cellular membrane thermostability 1. *Crop Science*, *19*(1):75-78.

Mauch-Mani, B., Baccelli, I., Luna, E. and Flors, V. 2017. Defense priming: an adaptive part of induced resistance. *Annual review of plant biology*, *68*:485-512.

Mayer, A.M., Harel, E. and Shaul, R.B. 1965. Assay of catechol oxidase a critical comparison of methods. vol. 5. *Phytochemistry*:83660-2.

Mensah, R.A., Li, D., Liu, F., Tian, N., Sun, X., Hao, X., Lai, Z. and Cheng, C. 2020. Versatile *Piriformospora indica* and its potential applications in horticultural crops. *Horticultural Plant Journal*, *6*(2):111-121.

Mohammed, A.F., Oloyede, A.R. and Odeseye, A.O. 2020. Biological control of

bacterial wilt of tomato caused by Ralstonia solanacearum using Pseudomonas species isolated from the rhizosphere of tomato plants. *Archives of Phytopathology and Plant Protection, 53*(1-2):1-16.

Mohan, M.M., Narayanan, S.L. and Ibrahim, S.M. 2000. Chlorophyll stability index (CSI): its impact on salt tolerance in rice. *International Rice Research Notes, 25*(2):38-39.

Molitor, A. and Kogel, K.H. 2009. Induced resistance triggered by *Piriformospora indica*. *Plant signaling & behavior*, *4*(3):215-216.

Nadiya, F., Anjali, N., Gangaprasad, A. and Sabu, K.K. 2015. High-quality RNA extraction from small cardamom tissues rich in polysaccharides and polyphenols. *Analytical biochemistry*, *485*:25-27.

Nahakpam, S. 2017. Chlorophyll Stability: A Better Trait for Grain Yield in Rice under Drought. *Indian Journal of Ecology*, *44*(4):77.

Narasimha Murthy, K. and Srinivas, C. 2012. Invitro screening of bioantagonistic agents and plant extracts to control bacterial wilt (Ralstonia solanacearum) of tomato (Lycopersicon esculentum). *Journal of Agricultural Technology*, *8*(3):999-1015.

Naveed, Z.A. and Ali, G.S. 2018. Comparative transcriptome analysis between a resistant and a susceptible wild tomato accession in response to Phytophthora parasitica. *International journal of molecular sciences*, *19*(12):3735.

Nion, Y.A. and Toyota, K. 2015. Recent trends in control methods for bacterial wilt diseases caused by Ralstonia solanacearum. *Microbes and environments*:ME14144.

Noutoshi, Y., Okazaki, M., Kida, T., Nishina, Y., Morishita, Y., Ogawa, T., Suzuki, H., Shibata, D., Jikumaru, Y., Hanada, A. and Kamiya, Y. 2012. Novel plant immune-priming compounds identified via high-throughput chemical screening target salicylic acid glucosyltransferases in Arabidopsis. *The Plant Cell*, *24*(9):3795-3804.

Oelmüller, R., Sherameti, I., Tripathi, S. and Varma, A. 2009. *Piriformospora indica*, a cultivable root endophyte with multiple biotechnological applications. *Symbiosis*, *49*(1):1-17.

Patrice, G. 2008. R. solanacearum race 3 biovar 2: detection, exclusion and analysis of a Select Agent Educational modules. The United States Dept. *Agric.-Nat. Res. Initiative Program*:1-4.

Pawaskar J, Joshi MS, Navathe S, Agale RC. Physiological and biochemical characters of ralstonia solanacearum. International Journal of Research in Agricultural Sciences. 2014;1(6):2348-3997.

Pieterse, C.M., Van der Does, D., Zamioudis, C., Leon-Reyes, A. and Van Wees, S.C. 2012. Hormonal modulation of plant immunity. *Annual review of cell and developmental biology*, *28*:489-521.

Qiang, X., Weiss, M., KOGEL, K.H. and Schäfer, P. 2012. *Piriformospora indica*—a mutualistic basidiomycete with an exceptionally large plant host range. *Molecular plant pathology*, *13*(5):508-518.

Rahman, M.F., Islam, M.R., Rahman, T. and Meah, M.B. 2010. Biochemical characterization of Ralstonia solanacerum causing bacterial wilt of brinjal in Bangladesh. *Progressive Agriculture*, *21*(1-2):9-19.

Ryals, J.A., Neuenschwander, U.H., Willits, M.G., Molina, A., Steiner, H.Y. and Hunt, M.D. 1996. Systemic acquired resistance. *The plant cell*, *8*(10):1809.

Satheesan, J., Narayanan, A.K. and Sakunthala, M. 2012. Induction of root colonization by *Piriformospora indica* leads to enhanced asiaticoside production in Centella asiatica. *Mycorrhiza*, *22*(3):195-202.

Seleim, M.A., Abo-Elyousr, K.A., Abd-El-Moneem, K.M. and Saead, F.A. 2014. First report of bacterial wilt caused by Ralstonia solanacearum biovar 2 race 1 on tomato in Egypt. *The plant pathology journal*, *30*(3):299.

Sharma, D.K. 2018. Morphological and biochemical characterization of Ralstonia solanacearum (Smith) in brinjal (Solanum melongena L.) in Rajasthan

(India). *Advances in Plants & Agriculture Research*, *8*(3):284288.

She, X., Yu, L., Lan, G., Tang, Y. and He, Z. 2017. Identification and genetic characterization of Ralstonia solanacearum species complex isolates from Cucurbita maxima in China. *Frontiers in plant science*, *8*:1794.

Sherameti, I., Tripathi, S., Varma, A. and Oelmüller, R. 2008. The rootcolonizing endophyte Pirifomospora indica confers drought tolerance in Arabidopsis by stimulating the expression of drought stress–related genes in leaves. *Molecular Plant-Microbe Interactions*, *21*(6):799-807.

Slaughter, A., Daniel, X., Flors, V., Luna, E., Hohn, B. and Mauch-Mani, B. 2012. Descendants of primed Arabidopsis plants exhibit resistance to biotic stress. *Plant physiology*, *158*(2):835-843.

Spoel, S.H. and Dong, X. 2012. How do plants achieve immunity? Defence without specialized immune cells. *Nature reviews immunology*, *12*(2):89-100.

Stein, E., Molitor, A., Kogel, K.H. and Waller, F. 2008. Systemic resistance in Arabidopsis conferred by the mycorrhizal fungus *Piriformospora indica* requires jasmonic acid signaling and the cytoplasmic function of *NPR1*. *Plant and cell physiology*, *49*(11):1747-1751.

Sticher, L., Mauch-Mani, B. and Métraux, A.J. 1997. Systemic acquired resistance. *Annual review of phytopathology*, *35*(1):235-270.

Tans-Kersten, J., Huang, H. and Allen, C. 2001. Ralstonia solanacearum needs motility for invasive virulence on tomato. *Journal of bacteriology*, *183*(12):3597-3605.

Thürich, J., Meichsner, D., Furch, A.C., Pfalz, J., Krüger, T., Kniemeyer, O., Brakhage,

A. and Oelmüller, R. 2018. Arabidopsis thaliana responds to colonisation of *Piriformospora indica* by secretion of symbiosis-specific proteins. *PLoS One*, *13*(12):e0209658.

Tomato Genome Consortium, X. 2012. The tomato genome sequence provides insights into fleshy fruit evolution. *Nature*, *485*(7400):635.

Van Loon, L.C. and Van Kammen, A. 1970. Polyacrylamide disc electrophoresis of the soluble leaf proteins from Nicotiana tabacum var.'Samsun'and 'Samsun NN': II. Changes in protein constitution after infection with tobacco mosaic virus. *Virology*, *40*(2):199-211.

Van Loon, L.C. 1997. Induced resistance in plants and the role of pathogenesisrelated proteins. *European journal of plant pathology*, *103*(9):753-765.

Van Loon, L.C., Bakker, P.A.H.M. and Pieterse, C.M.J. 1998. Systemic resistance induced by rhizosphere bacteria. *Annual review of phytopathology*, *36*(1):453-483.

Varma, A., Bakshi, M., Lou, B., Hartmann, A. and Oelmueller, R. 2012. *Piriformospora indica*: a novel plant growth-promoting mycorrhizal fungus. *Agricultural research*, *1*(2):117-131.

Varma, A., Verma, S., Sudha, Sahay, N., Bütehorn, B. and Franken, P. 1999. *Piriformospora indica*, a cultivable plant-growth-promoting root endophyte. *Applied and environmental Microbiology*, *65*(6):2741-2744.

Verma, S., Varma, A., Rexer, K.H., Hassel, A., Kost, G., Sarbhoy, A., Bisen, P., Bütehorn, B. and Franken, P. 1998. *Piriformospora indica*, gen. et sp. nov., a new root-colonizing fungus. *Mycologia*, *90*(5):896-903.

Vlot, A.C., Dempsey, D.M.A. and Klessig, D.F. 2009. Salicylic acid, a multifaceted hormone to combat disease. *Annual review of phytopathology*, *47*:177-206.

Waller, F., Mukherjee, K., Deshmukh, S.D., Achatz, B., Sharma, M., Schäfer, P. and Kogel, K.H. 2008. Systemic and local modulation of plant responses by *Piriformospora indica* and related Sebacinales species. *Journal of plant physiology*, *165*(1):60-70.

Walters, D.R., Ratsep, J. and Havis, N.D. 2013. Controlling crop diseases using induced resistance: challenges for the future. *Journal of experimental botany*, *64*(5):1263-1280.

Wang, Y., Tao, X., Tang, X.M., Xiao, L., Sun, J.L., Yan, X.F., Li, D., Deng, H.Y. and

Ma, X.R. 2013. Comparative transcriptome analysis of tomato (Solanum lycopersicum) in response to exogenous abscisic acid. *BMC genomics*, *14*(1):1-14.

War, A.R., Paulraj, M.G., War, M.Y. and Ignacimuthu, S. 2011. Role of salicylic acid in induction of plant defense system in chickpea (Cicer arietinum L.). *Plant signaling & behavior*, *6*(11):1787-1792.

Ward, E.R., Uknes, S.J., Williams, S.C., Dincher, S.S., Wiederhold, D.L., Alexander, D.C., Ahl-Goy, P., Metraux, J.P. and Ryals, J.A. 1991. Coordinate gene activity in response to agents that induce systemic acquired resistance. *The Plant Cell*, *3*(10):1085-1094.ss

Wen, P.F., Chen, J.Y., Wan, S.B., Kong, W.F., Zhang, P., Wang, W., Zhan, J.C., Pan, Q.H. and Huang, W.D. 2008. Salicylic acid activates phenylalanine ammonia-lyase in grape berry in response to high temperature stress. *Plant Growth Regulation*, *55*(1):1-10.

Xu, L., Wang, A., Wang, J., Wei, Q. and Zhang, W. 2017. *Piriformospora indica* confers drought tolerance on Zea mays L. through enhancement of antioxidant activity and expression of drought-related genes. *The Crop Journal*, *5*(3):251-258.

Yamaji, K., Watanabe, Y., Masuya, H., Shigeto, A., Yui, H. and Haruma, T. 2016. Root fungal endophytes enhance heavy-metal stress tolerance of Clethra barbinervis growing naturally at mining sites via growth enhancement, promotion of nutrient uptake and decrease of heavy-metal concentration. *PloS one*, *11*(12):e0169089.

Zhan, Y., Qu, Y., Zhu, L., Shen, C., Feng, X. and Yu, C. 2018. Transcriptome analysis of tomato (Solanum lycopersicum L.) shoots reveals a crosstalk between auxin and strigolactone. *Plos one*, *13*(7):e0201124.

Zhou, R., Wu, Z., Cao, X. and Jiang, F.L. 2015. Genetic diversity of cultivated and wild tomatoes revealed by morphological traits and SSR markers. *Genet. Mol. Res*, *14*(4):13868-13879.

Zipfel, C. 2009. Early molecular events in PAMP-triggered immunity. *Current opinion in plant biology*, *12*(4):414-420.

Zuccaro, A., Lahrmann, U., Güldener, U., Langen, G., Pfiffi, S., Biedenkopf, D., Wong, P., Samans, B., Grimm, C., Basiewicz, M. and Murat, C. 2011. Endophytic life strategies decoded by genome and transcriptome analyses of the mutualistic root symbiont *Piriformospora indica*. *PLoS pathogens*, *7*(10):e1002290.

8. APPENDICES

APPENDIX I

Reagents required for staining of P. indica

Composition of Staining solution (lactophenol trypan blue stain)(for 60ml)

Lactic acid-10ml

Phenol-10ml

Trypan blue- 0.012mg (0.02%)

Glycerol-20ml

APPENDIX II

Composition of Destaining solution (Lactophenol) (for 60ml)

Lactic acid - 10ml

Phenol -10ml

Glycerol -20ml

Water -20ml

ABSTRACT

SALICYLIC ACID and *Piriformospora indica* INDUCED MODULATION OF STRESS RESISTANCE MECHANISM IN TOMATO (*Solanum lycopersicum* L.).

by

DEEPTHI MOHAN

(2016-09-021)

ABSTRACT OF THESIS

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ABSTRACT

Deepthi Mohan **Date – 28/12/2021**

2016-09-021

The study entitled "Salicylic acid and *Piriformospora indica* induced modulation of stress resistance mechanism in tomato (*Solanum lycopersicum* L.)." was conducted at College of Agriculture, Vellayani, Thiruvananthapuram, during 2020 – 2021. The primary objective of this study was to analyse the salicylic acid induced *NPR1* gene expression as well as *Piriformospora indica* induced resistance modulation to stress and its persistence and transmission through seeds.

The induced resistance in plants can be classified as Systemic Acquired Resistance (SAR), mediated by the signalling molecule salicylic acid and Induced Systemic Resistance (ISR) which was induced by the presence of non-pathogenic plant growth promoting fungi or bacteria. In the present study two treatments, plant growth promoting endophytic basidiomycete fungus *Piriformospora indica* (biological inducer) and salicylic acid (chemical inducer) were applied on bacterial wilt resistant (Vellayani Vijay, KAU) and susceptible (Pusa Ruby, IARI) varieties of tomato and the expression rate of *Non-expresser of pathogenesisrelated genes1* (*NPR1*) gene was evaluated using q-RTPCR.

In first generation, one set of seed were inoculated with *P. indica* (1%) by incorporating it into the potting mixture before filling the pot-tray cavities. After confirming endomycorhizal root colonization, leaf samples were collected for analyzing *NPR1* expression. Seeds from these plants were collected and progeny seedlings were maintained for analysing transgenerational expression of *NPR1* gene. Second set of seeds were sown in the nursery and two weeks old seedlings were

transplanted in to pots. Salicylic acid(100ppm) was sprayed at flowering stage and *NPR1* gene expression was analysed on 5^{th} day and 7^{th} day after treatment in leaves and compared to next generation (in two weeks old seedlings) for understanding transgenerational expression. The evaluation of stress tolerance of the treated (*P. indica* and S.A) plants was done on progeny seedlings of Vellayani Vijay by treating it with a low density inoculum of virulent *Ralstonia solanacearum* (10^6 cells/ ml). Similarly abiotic stress tolerance evaluation was carried out by subjecting the plants to a higher temperature in polyhouse.

Physiological and biochemical observations showed that application of *P. indica* and salicylic acid can induce resistance by regulating the enzyme activities and other physiological parameters. The resistant variety (Vellayani Vijay) gave a relative change of 7 fold and 4 fold for *NPR1* expression (on 5^{th} day and 7^{th} day respectively) on the application of salicylic acid. The susceptible variety (Pusa ruby) showed a relative change of 6 fold and 2 fold for *NPR1* expression on the application of salicylic acid. A relative change of 7 fold and 5 fold was shown respectively for first generation and second generation on treatment with salicylic acid, which confirms a transgenerational expression of the defence related gene.