"A Study on the Interaction Between the Rhizobacterium, *Pseudomonas putida* AKMP7, and, *Arabidopsis thaliana*, Under Drought Conditions: A Case of Conditional Pathogenesis"

THESIS

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by

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2024

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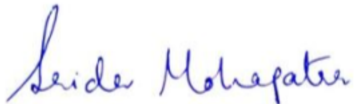
CERTIFICATE

This is to certify that the thesis titled **A Study on the Interaction Between the Rhizobacterium, *Pseudomonas putida* AKMP7, and, *Arabidopsis thaliana*, Under Drought Conditions: A Case of Conditional Pathogenesis** submitted by **Raja Gopalan N. S.** ID No **2019PHXF0004H** for award of Ph.D. of the Institute embodies original work done by him under my supervision.



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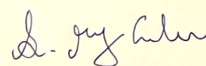
DECLARATION

This is to certify that the thesis titled **"A Study on the Interaction Between the Rhizobacterium, *Pseudomonas putida* AKMP7 and *Arabidopsis thaliana*, Under Drought Conditions: A Case of Conditional Pathogenesis"** is based on my own research work and has been carried out under the guidance and supervision of Dr. Sridev Mohapatra, Associate Professor, Dept. of Biological Sciences, BITS Pilani, Hyderabad Campus, Hyderabad, India.

The data and information which I have used from various sources have been duly acknowledged. I declare that this work has not been previously submitted by me to any other university/institute for the award of any other degree or diploma.



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Abstract:

Plant growth-promoting rhizobacteria (PGPR) are beneficial soil bacteria that reside in the rhizosphere and can colonize the roots of plants in their vicinity. They promote plant growth and can impart resistance/tolerance to various biotic and abiotic stress factors. Although, studies on the beneficial impact of PGPR have a long history, research on the behaviour of beneficial microbes that turn pathogenic to their host due to changes in environmental conditions remain non-existent. Previous research from our laboratory has uncovered a unique phenomenon that we have termed "conditional pathogenesis", in which, a particular strain of drought and thermo-tolerant PGPR, namely, *Pseudomonas putida* AKMP7, becomes pathogenic towards *Arabidopsis thaliana* under drought conditions, while being beneficial under well-watered conditions. In this study, with an extensive analysis of bacterial-secreted phytohormones, we unveiled that AKMP7 releases (a) free zeatin, (b) auxin derivatives such as indole-acetamide and (c) amino acid (alanine, phenylalanine, and aspartate) conjugates of auxin into the growth medium. Interestingly, the levels of most of these phytohormones were higher under osmotic/water-stressed conditions than under non-stressed conditions. Through studies involving the treatment of plants with the specific phytohormones/derivatives/conjugates mentioned above, we have identified that zeatin and the auxin derivative indole-acetamide can play a crucial role in aiding the drought-specific adverse effects of AKMP7. Apart from this, we have also studied the regulation of plant immune response during conditional parthenogenesis and identified that AKMP7 negatively regulates the salicylic acid-mediated plant immune response to cause early wilting of plants under water stress. The negative regulation of plant immune response also leads to enhanced susceptibility of *A. thaliana* seedlings to other pathogens. To understand the phenomenon of conditional pathogenesis from a genomics perspective, we have also performed functional genomic analysis of the AKMP7 genome and identified some important markers that can potentially contribute to conditional pathogenesis. Overall, our work lays a foundation for mechanistic understanding of the unique phenomenon of conditional pathogenesis.

Keywords: PGPR, *Pseudomonas putida* AKMP7, *Arabidopsis thaliana*, conditional pathogenesis, water stress, bacterial phytohormones, plant immunity, salicylic acid, functional genomics.

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List of Abbreviations:

1) PGPM- Plant Growth Promoting Microbes.
2) PGPR- Plant Growth Promoting Rhizobacteria
3) IAA- Indole-3-acetic acid
4) IA- Indole-3-acetamide
5) IA-Phe- Indole-3-acetyl-L-phenylalanine
6) IA-Ala- Indole-3-acetyl-L-alanine
7) IA-Asp- Indole-3-acetyl-L-aspartate
8) GA- Gibberellic acid
9) SA- Salicylic acid
10) ABA- Abscisic acid
11) tZ- trans-Zeatin
12) LC-MS/MS: Liquid chromatography-tandem mass spectrometry
13) ESI- Electron spray ionization
14) MRM- Multiple reaction monitoring
15) NPR- Non-expressor of pathogenesis related gene
16) PR- Pathogenesis related gene
17) ICS- Isochorismate synthase
18) EDS- Enhanced disease susceptibility gene
19) PBS- Polyhydroxy butyrate susceptible1
20) EPS- Enhanced *Pseudomonas* susceptibility
21) PAL- Phenylalanine-ammonia lyase
22) RAST- Rapid Annotation using Subsystem Technology
23) WGS- Whole genome sequencing

List of Symbols

1. µL-microlitre
2. µM- micromolar
4. nM- nanomolar
5. ng- nanogram
6. µg- microgram
8. ^{0}C- degree celsius
9. ng/ml- nanogram per mili litre
10. %- percentage
11. ml- millilitre
14. min- minutes
15. ± - plus/minus
17. < - lesser than
18. = - equal to
19. xg- times gravity
20. cm- centimetre
21. λ- Wave length
22. g- Grams

‘

Chapter 1

Introduction

1.1. Introduction:

Throughout their life cycle, plants interact with the microbiota present in their environment. Both, the aerial (leaf, flowers and fruits) as well as underground (roots) parts of the plants are exposed to various microbes. Roots interact with microbiota present in the rhizosphere, which is the soil adhering to the roots. Root-associated microbiota can be either free-living or endosymbiotic. Apart from this, the rhizosphere microbial community can be broadly classified as beneficial or pathogenic. Beneficial microbes are essential for improving plant growth, stress tolerance and disease resistance. In contrast, pathogenic microbes cause growth retardation, wilting and enhanced stress susceptibility. Beneficial soil microbes are commonly called plant growth-promoting microbes (PGPM), which can be further classified into plant growth promoting rhizobacteria (PGPR) or fungi (PGPF) (Kohler et al., 2008; Vejan et al., 2016).

Rapidly changing climatic landscapes across the globe pose a severe threat to global food security and sustainable agriculture (Ari et al., 2021; Singh et al., 2023). The usage of harmful chemical fertilisers to improve crop productivity can come at the cost of polluting the environment. Due to the adverse impact of chemical fertilisers on the environment, there is a pressing need to adopt eco-friendly, sustainable agricultural practices. Also, climatic changes across the globe require plants to adapt to various environmental stresses like heat, drought and cold stress. As mentioned earlier, PGPR possess various traits that make them suitable for agricultural applications. PGPR can promote crop yield and productivity, precluding the necessity for applying chemical fertilisers. They fix atmospheric nitrogen, enhancing nutrient uptake and producing plant growth regulators/phytohormones. Apart from plant growth promotion, PGPR can provide tolerance to various biotic and abiotic stress factors. PGPR can improve disease resistance by priming plant immune responses in their host plant and conferring broad-spectrum resistance to multiple pathogens.

While many studies highlight the beneficial aspects of PGPR, the adverse effects of PGPR due to changes in the host or climatic conditions remain non-existent.

A previous study from our laboratory has reported one such instance where a beneficial rhizobacterium *Pseudomonas putida* AKMP7 turns pathogenic to *Arabidopsis thaliana* seedlings under water stress conditions, while promoting plant growth under well water non-stressed conditions (Shah et al., 2017). This was a first report on such an unusual behaviour of rhizobacteria and we termed this phenomenon as "conditional pathogenesis". In this study, we elucidated the detailed mechanisms underlying this phenomenon of conditional pathogenesis. This research, therefore, uncovers the mechanisms by which otherwise beneficial rhizobacteria can turn harmful to plants, under adverse environmental conditions

Chapter 2

Review of Literature

2.1. Plants and their environment:

Unlike other living organisms, plants are sessile and cannot move from one place to another. Throughout their life cycle, plants interact with various environmental factors, both, biotic and abiotic, many of which can be adverse towards their growth and development. For example, the aerial parts of plants (leaves, stems, flowers and fruits) are directly exposed to factors like excessive heat, cold, rain, etc., which can affect their growth, development and survival (Liu et al., 2021). Similarly, the underground parts of the plants are directly exposed to drought, salinity and heavy metal contaminants. While plants have evolved to cope with these adverse abiotic factors, prolonged exposure or severity of stress can result in growth retardation or complete wilting of plants (Liu et al., 2021). This situation is exacerbated by the rapidly changing climatic landscape globally, which seriously threatens plant adaptability and survival (Ari et al., 2021; Singh et al., 2023). Apart from abiotic factors, plants also interact with various biotic factors in the environment. Biotic factors in the environment, like microbes, insects, herbivores, etc., can also influence plant growth, development and survival (Biere & Bennett, 2013). The biotic interactions can be beneficial or harmful to the plant, depending on the nature of these interactions. Harmful biotic interactions like pathogenic plant microbes, insects and herbivores can deteriorate plant health severely or, in the worst case, completely kill the plant (Saijo et al., 2022). On the contrary, beneficial biotic interactions (symbiotic/asymbiotic microbes, pollinators and insects that feed on plant pests, etc.) can promote plant growth and facilitate survival and reproduction. As mentioned earlier, in a rapidly changing climatic landscape across the globe, abiotic and biotic factors (lack of rainfall or emergence of a new disease) pose a severe threat to plant growth and development. Agronomically, these threats can lead to massive losses in plant productivity and lead to global food shortages (Ari et al., 2021; Singh et al., 2023). Hence, it is essential to understand the

mechanisms behind various abiotic and biotic factors affecting plant growth and develop innovations and solutions to promote sustainable agriculture.

2.2. Plant associated microbiota

As mentioned above, throughout their life cycle, plants interact with a variety of microbiota present in their environment (Berendsen et al., 2012; Binyamin et al., 2019; Han & Yoshikuni, 2022). Both, the aerial (leaf, flowers and fruits) as well as the underground parts of the plants are exposed to various microbes. The microbes residing in the leaves (and other aerial parts) are commonly referred to as phyllospheric microbes, which are mostly pathogenic in nature (Sohrabi et al., 2023). The soil microbial community residing near the roots, commonly referred to as rhizospheric microbes, can be either beneficial or pathogenic (Berendsen et al., 2012; Binyamin et al., 2019). These microbes can be free-living or endosymbiotic. Of the two categories, beneficial microbiota is essential for improving plant growth, stress tolerance and disease resistance (Backer et al., 2018; Hayat et al., 2010; Yu et al., 2019). They are important for various reasons, such as their role in nitrogen fixation, enhanced nutrient uptake, growth promotion and stress tolerance. In contrast, pathogenic microbes cause growth retardation, wilting and enhanced stress susceptibility (MacKenzie & Shane, 1984; Savary et al., 2019). Some beneficial soil microbes are known to possess bio-control properties that restrict pathogenic microbes' growth (Beneduzi et al., 2012a).

Plants can release organic matter rich in sugar, amino acids, organic acids, etc., to recruit microbes to their roots (Bais et al., 2006). The root exudate can act as an energy source for the metabolism and reproduction of microbes. Depending on plants' various developmental or survival needs, such as growth or stress tolerance, roots can vary the composition of root exudates to attract different types of microbes. Thus, the contents of the root exudates can heavily influence the nature and population of the microbial community surrounding the roots. However, both beneficial and pathogenic microbes can be attracted to the roots via root

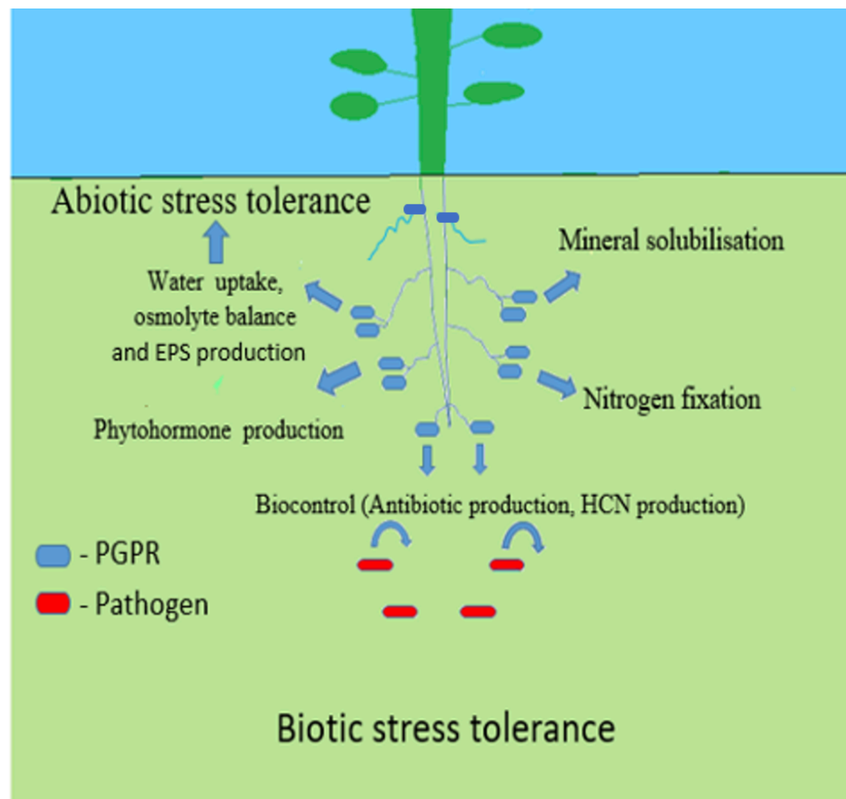
exudates, leading to contrasting outcomes. Similarly, microbes present in the environment can also influence plant growth by producing growth-regulating compounds such as phytohormones (i.e., auxin, gibberellins and cytokinin, etc.) and volatile organic compounds to modulate plant growth (Berg, 2009; Costacurta & Vanderleyden, 1995; Khan et al., 2020).

2.3. Beneficial soil microbes:

Recent advances in our knowledge of beneficial microbiota have helped identify natural microbial communities (bacterial/fungal) responsible for improving plant productivity, stress tolerance and disease resistance (Ghosh et al., 2019; Kohler et al., 2008; Sen & Mohapatra, 2021; Vejan et al., 2016). These microbial strains are commonly called plant growth-promoting microbes (PGPM), which can be further classified into PGPF (fungi) or PGPR (rhizobacteria). PGPR possess various traits that make them suitable for agricultural applications. PGPR can promote crop yield and productivity without applying chemical fertilizers. They fix atmospheric nitrogen, enhancing nutrient uptake and producing plant growth regulators/phytohormones. As mentioned earlier, apart from plant growth promotion, PGPR can also enhance tolerance of plants to various biotic and abiotic stress factors. PGPR can improve disease resistance by priming plant immune responses in their host plant and conferring broad-spectrum resistance to multiple pathogens (Beneduzi et al., 2012a). Rapidly changing climatic landscapes across the globe pose a severe threat to global food security and sustainable agriculture. The usage of harmful chemical fertilizers and biocontrol agents to improve crop productivity can come at the cost of polluting the environment. Due to the adverse impact of chemical fertilizers on the environment, there is a pressing need to adopt eco-friendly agricultural practices. Also, rapidly changing climatic conditions across the globe require plants to adapt to various environmental conditions like heat, drought and cold stress. Hence, application of PGPR can potentially solve various problems related to sustainable agriculture.

2.4. Molecular mechanisms involved in beneficial plant-microbe interactions

As mentioned earlier, plants interact with their surrounding microbes throughout their life. The outcome of these interactions can decide the fate of the plant. The rhizosphere consists of a complex network of microbes around the roots.



(Adapted from Ghosh, Gupta and Mohapatra, 2019a, 2019b)

Fig. 1: Mechanisms involved in beneficial plant-microbe interactions.

The beneficial rhizospheric microbes enhance nutrient uptake by solubilizing vital minerals in the soil (e.g., phosphorous, iron, etc.), which the roots cannot absorb directly (Pii et al., 2015). Microbe-mediated phosphate solubilization occurs via the release of phosphate solubilizing enzymes (Paries & Gutjahr, 2023). Similarly, microbe-mediated iron uptake occurs via the secretion of siderophores that reduce Fe^{3+} to Fe^{2+} for ease of uptake by plant roots (Pii et al., 2015). Nitrogen-fixing rhizobacteria can help plant growth by fixing atmospheric nitrogen in the soil (Han & Yoshikuni, 2022; Santi et al., 2013). Apart from this, the beneficial

microbes colonizing the roots can also enhance plant growth by suppressing the growth of harmful microbes in the rhizosphere by producing antibiotics and HCN (Beneduzi et al., 2012b). Besides promoting plant growth, beneficial rhizobacteria can help plants cope with various abiotic stress factors like drought, salinity and heavy metal stress by modulating metabolic and signalling pathways involved in abiotic stress tolerance in plants. Pathogenic microbes can colonize the plant roots and cause rot and wilting. They produce certain metabolites (phytohormones, secretory proteins/peptides, etc.) that suppress plant immunity and evade immune responses to cause disease (Bauters et al., 2021; Chen et al., 2017; Kunkel & Harper, 2018; Weiler & Spanier, 1981). Contrastingly, beneficial soil microbes produce certain metabolites such as phytohormones, volatile organic compounds and compatible osmolytes, etc., that can enhance plant growth (Ghosh et al., 2017; S.-M. Kang et al., 2014; Khan et al., 2020; Sen & Mohapatra, 2021). Among the metabolites released by soil microbes, phytohormones and phytohormone-mimicking substances play a multifaceted (beneficial and harmful) role in plant-microbe interactions (Djami-Tchatchou et al., 2020).

In plants, phytohormones regulate a wide range of physiological activities responsible for proper growth, development and stress tolerance (Waadt et al., 2022). Unlike phytohormones produced by plants, microbe-produced phytohormones play a dual role (beneficial and harmful) in plant-microbe interactions (Djami-Tchatchou et al., 2020) since they are released into the soil. As mentioned above, both beneficial and harmful microbes produce phytohormones to cause contrasting outcomes on plant growth. The beneficial microbes are known to produce major phytohormones such as auxin, gibberellin, cytokinin and salicylic acid (Akiyoshi et al., 1984; Bömke & Tudzynski, 2009; Conway et al., 2022; Mishra & Baek, 2021). Production of phytohormones by beneficial microbes can positively modulate plant growth. For example, auxin and cytokinin producing beneficial microbes can promote root growth and development to enhance overall plant growth (Finkel et al., 2020; Ghosh et

al., 2019). Similarly, gibberellin producing microbes can enhance plants' overall growth and biomass (S. M. Kang et al., 2014). The production of salicylic acid by certain microbes can prime plant defence and have biocontrol effects against pathogens (Mishra & Baek, 2021).

2.5. The plant-associated microbes for sustainable agriculture

In recent times, the potential of PGPR application as an alternative to the usage of harmful chemicals in agriculture has been extensively studied (Bhattacharyya & Jha, 2012; Grover et al., 2021; Mhatre et al., 2019). From the several studies that have been performed on PGPR application, it has been identified that PGPR can significantly reduce the usage of harmful chemicals in the field. Generally, the PGPR encompasses the microbial genera *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Enterobacter*, *Alcaligenes*, *Arthrobacter*, *Burkholderia*, *Bacillus*, *Serratia,* etc., which has been well-studied for their ability to promote plant growth and impart stress tolerance to heat, salinity and drought conditions (Beneduzi et al., 2012a; Khan et al., 2020). Several microbes belonging to the *Pseudomonas* genus are known to be beneficial to the plant by enhancing the growth or imparting stress tolerance towards adverse environmental factors. Among the diverse microbes encompassing this genus, species such as *P. putida*, *P. fluorescence,* and *P. aeruginosa* have been extensively studied for their plant growth-promoting traits and stress mitigation abilities.

Abiotic stress conditions like drought, salinity, cold, heat, etc., can severely affect plant (Liu et al., 2021) and lead to loss of productivity in many commercially important crops like wheat, rice, maize, etc. (Cramer et al., 2011). Many PGPR species alleviate abiotic stress conditions in plants by positively modulating their stress tolerance mechanisms at the molecular and physiological levels (Ghosh et al., 2017; Sen et al., 2018; Safdarian et al., 2019; Sen and Mohapatra, 2021). Among the abiotic stress factors affecting plant growth, drought has one of the most severe and widespread impacts on agriculture. Prolonged and sustained drought causes extreme osmotic shock in plants, leading to severe growth stunting and

accelerated cell death (Kapoor et al., 2020; Seleiman et al., 2021). The application of PGPR has been shown to be an effective strategy for mitigating drought stress in plants. While there are many reasons to be optimistic about the application of PGPR to improve plant growth and yield for sustainable agriculture, the several important molecular mechanisms underlying plant-PGPR interactions for ameliorating drought stress remain relatively unknown and need to be elucidated. Research from our laboratory has predominantly focussed on unearthing the molecular mechanisms involved in drought (water stress) alleviation in plants by PGPR (Ghosh et al., 2017; Shah et al., 2017; Sen et al., 2018; Ghosh, Gupta and Mohapatra, 2019b, 2019a; Sen and Mohapatra, 2021, Nikhil et al., 2023).

2.6. Conditional/opportunistic pathogenesis by otherwise beneficial soil bacteria:

In research pertaining to plant-PGPR interaction, there is one area that has not received its due attention. That is, host and environment-specific responses of PGPR. While PGPR are promising candidates for combating various environmental stress conditions affecting plant growth and yield, whether they have the same kind of beneficial impact with diverse hosts and different environmental conditions needs to be studied. PGPR isolated from the rhizosphere of one species of plants may not benefit other plant species due to lack of cross-compatibility between different species. What’s worse, sometimes, bacteria that are beneficial under certain environmental conditions may become pathogenic and, thus, deleterious towards plants under a different set of environmental conditions. Such an unusual and often overlooked aspect of plant-microbial interaction is a phenomenon that we have termed “conditional pathogenesis”. and have reported this in the interaction between *Arabidopsis thaliana* and an abiotic-stress tolerant rhizobacterium, *P. putida* AKMP7 (Shah et al., 2017, Raja Gopalan et al., 2022). *P. putida* AKMP7 was first isolated from the sorghum rhizosphere, and identified as a thermos-tolerant PGPR strain that imparts thermo-tolerance to wheat (Zulfikar Ali et al., 2011). Our

laboratory procured this strain from the National Bureau of Agriculturally Important Microbes (ICAR-NBAIM, Mau, Uttar Pradesh) and wanted to study its role in drought tolerance, using *A. thaliana* as the model plant. Preliminary studies in our laboratory on this bacterium and its interaction with *A. thaliana* revealed its conditionally pathogenic properties only under water-stress or drought (Shah et al., 2017). Probing further into this hitherto unreported phenomenon, we found that, AKMP7 promotes plant growth under watered conditions, while causing growth retardation under water-stress(Raja Gopalan et al., 2022),

We then proceeded towards unearthing the mechanisms involved in the differential behaviour of this bacterium under well-watered vs. water-stressed conditions, which became the premise of this study.

Knowledge Gaps and Objectives

2.7. Specific gaps in existing research:

As mentioned in the review of literature, in research pertaining to plant-microbe interactions, a lot of studies highlight the importance of beneficial plant-associated microbes on enhancing plant-growth, development and stress tolerance. There are also plenty of studies on mechanistic understanding of diseases caused by pathogenic rhizobacteria. However, till date, studies on the influence of host and environmental conditions on pathogenic potential of seemingly beneficial microbes remain almost non-existent. Through this study, we are trying to decode some of the fundamental molecular mechanisms involved in plant-microbe interactions, to understand why certain beneficial microbes turn conditionally pathogenic to the host-plant under certain adverse environmental conditions.

2.8. Broad objectives of the proposed study:

1) To understand the role of bacterial phytohormones in conditional pathogenesis by *P. putida* AKMP7 on *A. thaliana*.
2) Mechanistic insights into the modulation of *A. thaliana* immune-response by *P. putida* AKMP7.
3) Functional genome analysis of *P. putida* AKMP7 genome.

Chapter 3

The Role of Bacterial Phytohormones in Conditional Pathogenesis by *P. putida* AKMP7 on *A. thaliana*.

3.1. Introduction:

3.1.1. The conditionally pathogenic rhizobacterium *P. putida* AKMP7

As mentioned in the previous chapters, , a previous study from our laboratory has reported that, when AKMP7 is inoculated onto *A. thaliana* roots under water-stressed conditions, it adversely impacts plant growth and health, with no adverse effects seen under well-watered conditions (Shah et al., 2017). The same study also reported that AKMP7 released high amounts of auxin into the growth medium and that the levels of auxin produced were higher under water/osmotic-stressed conditions compared to non-stressed conditions (Shah et al., 2017). Since phytohormones at levels higher than optimal can become potentially toxic to plants, therefore, we hypothesized that, the higher levels of auxin may be responsible for turning the bacterium conditionally pathogenic, only under water-stressed conditions. However, that study was preliminary in nature did not involve any comprehensive analysis on the other bacterial-derived chemicals, especially phytohormones and their possible role in conditional pathogenesis in *A. thaliana* under water-stress. In the present study, we have probed deeper into this phenomenon of "conditional pathogenesis" by (a) performing a comprehensive analysis on the variety of phytohormones (including derivatives) released by this bacterium under water/osmotic stress (b) elucidated the rhizosphere colonization efficiency of AKMP7 and its impact on the physiology of *A. thaliana* under well-watered and water-stressed conditions (c) the specific roles of the bacterium-derived chemicals on conditional pathogenesis in *A. thaliana*. Data represented in this chapter have been published in the journals, Plant Physiology and Biochemistry (Raja Gopalan et al., 2022) and World Journal of Microbiology and Biotechnology (Sowmya et al., 2023).

3.2. Materials and methods

3.2.1. Extraction and quantification of bacteria-released phytohormones using LC-ESI-MS/MS

Extraction and quantification of phytohormones released by *P. putida* AKMP7 was carried out with slight modifications to methods described by Yasmin et al., 2017, Iqbal and Hasnain, 2013, Lee et al., 2012, and Ghosh et al. (2019a). The primary bacterial culture was prepared by inoculating a single colony of *P. putida* AKMP7 in 50 mL of Luria Bertani (LB) broth and growing it overnight to an optical density ($OD_{600\lambda}$) of 0.8 in a shaking incubator at 28 °C and shaken at 150 RPM. The extraction process was carried out by inoculating 2.5 mL of this overnight culture in 50 mL of fresh LB broth (with or without supplementation of 25% PEG to induce osmotic stress) and incubated for five days under the same conditions described above. Following growth for five days, bacterial cultures were centrifuged at 6700×*g* to separate the supernatant from the bacterial pellet. After centrifugation, the supernatant was transferred to a new 50 mL container and the bacterial pellet fresh weight was measured using a weighing a balance. Due to high viscous nature of 25% PEG supplemented broth, prior to fresh weight measurement, the pellet was gently rinsed twice with 2 mL of autoclaved water (without disrupting the pellet) to remove any residual PEG containing media from the pellet. The cell-free supernatant was acidified to pH 2.5 with 1M HCl and extracted twice by adding an equal volume of ethyl acetate followed by vigorous vortexing and centrifugation at 6700×*g* for 10 min. After centrifugation, the organic phase was collected and evaporated in a rotary evaporator, and the residue was resuspended in 2 mL of 80% MeOH. Then it was filtered using a 0.22 μm syringe filter and used for liquid chromatography-tandem mass spectrometry analysis. Prior to quantification, a screening was done to identify all major phytohormones and related metabolites in the bacterial supernatant using regular electron spray ionization mass

spectrometry by direct injection of 10 µL volume of samples (without column) into the mass analyzer. The mobile phase used for the analysis consisted of water (pump A) and acetonitrile (pump B) in a composition of 35%:65% (v/v) and the flow rate was set to 0.4 mL/min. The ionization was carried out in both negative (M-H) and positive (M + H) ionization modes under atmospheric ionization conditions (soft ionization). The detection mass range used for analysis was set to 50–800 m/z. Detection of precursor ions with mass to charge ratio (m/z) corresponding to the molecular weight of each metabolite indicates the presence of the compound in the samples (details provided in Table 2).

The Liquid Chromatography-Electrospray Ionization technique coupled with tandem mass spectrometry (LC-ESI-MS/MS) was used to quantify the identified phytohormones metabolites in bacterial extract. The analysis was carried out using a Kinetex core-shell 2.6µ polar C18 column (100 × 4.6 mm 100 Å). The mobile phase used consisted of 50 µL formic acid in 1L of Milli-Q water (A) and 100% acetonitrile (B). The mobile phase composition (A: B) used to analyze the various compounds is mentioned in Table 1. The flow rate used for analysis was 0.45 mL/min, and it was kept constant throughout the run (12 min) in an isocratic mode. The column oven temperature was maintained at 25 °C. The phytohormones were quantified using Multiple Reaction Monitoring (MRM) mode, and the intensity (m/z) of the most abundant ion was used for quantification. The concentration of each phytohormone was extrapolated from a standard curve of "area under the curve" vs concentration (of commercially procured phytohormone standards). A known quantity of an internal standard chloramphenicol was used to minimize variations. The mass to charge ratio (m/z) and optimized MRM conditions used for MS/MS quantification are given in Table 1 and *Appendix 1:* Supplementary Fig. 1 and 2. The method described here for phytohormone analysis from bacterial growth medium is a significant improvement over the existing methods for analyzing microbial secretions, with respect to efficiency, cost effectiveness and analysis time. In fact, it is the first

method reported for the simultaneous detection and precise quantification of multiple small molecules released by microbes. Hence, in order to test the generic efficacy and universality of this method, apart from AKMP7, we also used it to test phytohormones released by few other rhizospheric microbial strains, namely, *P. putida* PIK1, *P. otitidis* POPS1, *Sclerotium hydrophilum* PS 1, *Bacillus cabrialesii* BIK3 and *Trichoderma asperellum* TAIK 1. For experiments involving quantification of phytohormones from these additional strains, no PEG treatment was used. This work was carried out in collaboration with Dr. C. Kannan's laboratory (which was also source of these additional strains), principal scientist, Department of Plant Pathology, Indian Institute of Rice Research (ICAR-IIRR), Hyderabad and the data is published in Soumya et al. (2023).

Table 1. Operating conditions of LC-ESI-MS/MS for separation and quantification of bacterial secreted phytohormones.

Metabolite	**LC Pump % (A:B)**	**MRM (m/z of abundant ions)**
Indole acetamide (IA)	65:35	130.10 m/z (-ve mode)
Zeatin (tZ)	55:45	202.0 m/z (+ ve mode)
Indole acetic acid (IAA)	65:35	130.20 m/z (-ve mode)
Gibberellic acid (GA)	65:35	240.25 m/z (-ve mode)
Salicylic acid (SA)	65:35	93.00 m/z (-ve mode)
Abscisic acid (ABA)	65:35	153.0 m/z (-ve mode)
Indole-3-acetyl-L-alanine (IAA-Ala)	55:45	128.10 (-ve mode)
Indole-3-acetyl-L-phenyalanine (IAA-Phe)	55:45	164.20 m/z (-ve mode)
Indole-3-acetyl-L-aspartate (IAA-Asp)	65:35	132.10 m/z (-ve mode)

3.2.2. Plant growth, maintenance and treatments

Before germination, *A. thaliana* (Columbia-0) seeds were surface sterilized using 70% ethanol and vernalized (exposed to cold treatment) for 5 days at 4–8 °C under dark conditions. The vernalized seeds were germinated in an autoclaved soil mixture consisting of perlite, vermiculite, and peat (1:1:1 ratio) in a rectangular germination tray with a dimension of 15 cm × 10 cm x 8 cm (lxbxh). Before germination, the soil was drenched in autoclaved full strength Murashige and Skoog (MS) macro-nutrient solution. After two weeks of germination, the seedlings were transplanted to individual square pots with a dimension of 7 cm × 7 cm x 7 cm (lxbxh). Prior to transplantation, each pot was filled with approximately 75 ± 2 g of autoclaved dry soil mixture and drenched overnight in autoclaved full strength MS macronutrient solution. Post transplantation, each such pot contained one seedling, and there were 3 replicate pots per treatment (explained in detail below). After transplanting, the seedlings were grown in a controlled environment at a temperature range of 23–25 °C with a relative humidity of 55–60% and a light intensity of about 100 μmol m^{-2} s^{-1}. The soil continued to be watered intermittently (every 3 days) with water and full strength MS macronutrient solution (alternatively). The seedlings were allowed to grow for 4 weeks. Prior to treatment, seedlings with similar looking size were selected (*Appendix 1;* Supplementary Fig. 4 and 5), following which 50% of the plants/pots were inoculated with *P. putida* AKMP7.

For bacterial inoculation, a single colony of AKMP7 plated on cetrimide agar was revived in 50 mL of Luria Bertani (LB) broth and grown overnight in a shaking incubator at 28 °C and shaken at 150 RPM, as described above. A volume of 2 mL of this overnight culture was sub-cultured into a 50 mL LB broth media and grown to $OD_{600\lambda}$ 0.8 with the same growth conditions as described above. After that, the bacterial culture was centrifuged at 3720×*g* for 10 min at 4 °C, the supernatant was discarded, and the pellet was re-suspended in 5 mL of

autoclaved distilled water. About 400 µL (approximately 2.5×10^8 CFU) of the suspended culture was directly pipetted onto the root surface of 4 weeks old plants (400 µL/plant). Drought was induced by water withholding in 50% of the inoculated and non-inoculated plants. After inoculation and drought induction, only the well-watered treatments were watered following the same watering regime as mentioned above.

There were three replicate pots for each of the four different treatments-non-stressed (NS); non-stressed + inoculated (NS + I); water-stressed (WS); water-stressed + inoculated (WS + I). For inoculation studies using different volumes, 100, 200, and 400 µL of the AKMP7 aqueous suspension (as described above) were inoculated onto the roots of 4 weeks old *A. thaliana* seedlings. The phytohormone treatment study was carried out in square ice cube trays containing 15 compartments for plant growth with a dimension of 3 cm × 3 cm x 3 cm (lxbxh) per compartment. Prior to use, the soil was drenched in autoclaved full-strength MS macronutrient solution, following which, an equal amount of soil was added to each compartment. Seeds were then inoculated into the soil as described above in the same section. Following germination, the seedlings were watered every 3 days with an equal amount of water (i.e. 5 mL per compartment) and allowed to grow for 2 weeks (6-leaf stage). Thereafter, plants were subjected to the different treatments. In addition to the drought induction and AKMP7 inoculation (described above in the same section), phytohormone treatments were done under well-watered or water-stressed conditions, by adding a known concentration (i.e. 150 µM mL^{-1} in water) of commercially procured phytohormone or the related metabolite directly to the plant roots or to the *P. putida* AKMP7 cell suspension culture prior to inoculation. There were 3 replicate plants per treatment and each compartment consisted of one plant. After phytohormone treatment and drought induction, only the well-watered treatments (i.e., NS, NS + I) were supplemented with water with the same watering regime as mentioned above.

The phytohormone concentration chosen for the phytohormone treatment studies was in range with the concentration used in a similar study on *A. thaliana* by Ostrowski et al. (2016).

3.2.3. Plant growth parameters

The impact of the various treatments on the growth and development of *A. thaliana* was estimated under non-stressed and water-stressed conditions by measuring seedling fresh and dry weights after two weeks of treatments. To measure fresh weight, the entire seedling was uprooted and soil debris attaching to the roots was removed and measured using a weighing balance. For dry weight measurement, the seedlings were completely dried in a hot air oven at 50–60 °C, followed by weight measurement using a weighing balance. Also, pictures of the plants were taken to visually analyze the impact of various treatments on their morphology.

3.2.4. Root colonization studies

The colonization efficiency of *P. putida* AKMP7 was tested on cetrimide agar selective media by serial dilution plating of rhizosphere soil (root along with adhering soil) with slight modification to the method described by Somasegaran and Hoben (1994). The *A. thaliana* seedlings (3 replicate pots per treatment) were uprooted after 10 days of inoculation and drought induction without damaging the roots. After up-rooting, 1 g m of rhizosphere soil from all the treatments (NS, WS, NS + I and WS + I) was serially diluted (5 serial dilutions of 10-fold increments, i.e. up to 10^5-fold) and 200 µL of each dilution was spread in duplicates on Petri plates containing cetrimide agar selective media and incubated for 24 h at 28 °C. After 24 h of incubation, the plates were counted for the number of colony-forming-units (CFU) in each treatment. Colonies were also visualized under UV illumination since *P. putida* AKMP7 exhibits a characteristic round and bright green fluorescent colony morphology when observed under UV illumination.

3.2.5. Visualization of stress-induced cell damage

The SYTOX green nucleic acid staining method developed by Truernit and Haseloff, 2008), was used to visualize stress-induced cell damage (Truernit and Haseloff, 2008). Leaves were harvested from plants exposed to the different treatments (NS, NS + I, WS, and WS + I) after two weeks of inoculation and incubated with 1 mL of SYTOX green nucleic acid stain (dissolved in water to a final concentration of 250 nM mL^{-1}) for 25–30 min under dark conditions. After staining, the leaf tissue was placed over a clear glass slide, covered with a coverslip, and observed under confocal laser scanning microscopy. The SYTOX green dye can selectively stain only the nucleus of dead cells as it is impermeable to live cells. Therefore, green fluorescence indicates dead cells. The maximum excitation wavelength used was set at 504 nm, the emission wavelength was at 523 nm, the laser line used was at 488 nm and the emission collected was at 510–560 nm. The images consisted of superimposed pictures (of bright field and fluorescent microscopy) generated by the microscopy software at two different magnifications (10× and 40 ×).

3.3. Results

3.3.1. Detection and quantification of bacterial secreted phytohormones using LC-ESI-MS/MS

As shown in Table 2, a comprehensive analysis was performed to identify different types of phytohormone released by AKMP7 into the growth medium, both under normal and osmotic stressed (PEG treated) conditions. The LC-ESI-MS/MS technique used here helped us identify that, instead of free auxin, AKMP7 is releasing a known auxin precursor, indole-3-acetamide (IA) as well as auxin conjugated amino acids, i.e. indole-3-acetyl-L-alanine (IA-Ala), indole-3-acetyl-L-aspartate (IA-Asp), and indole-3-acetyl-L-phenylalanine (IA-Phe).

AKMP7 also releases the cytokinin, zeatin (tZ) into the medium. The presence of other phytohormones such as gibberellic acid and abscisic acid etc., could not be detected. As seen in Fig. 2 A, per mL of supernatant, the quantities of all auxin derivatives in PEG treated samples, were either similar to the control samples or higher. While the levels of IA-Ala, IA, and IA-Asp, were almost identical in both samples, a statistically significant increase was seen in the levels of IA-Phe in the PEG treated samples as opposed to the control samples. A similar increase was also seen in levels of tZ in the PEG treated bacterium viz-a-viz, the control bacterium (Fig. 2 C). As can be seen from Fig. 2B and D, when calculated per gram FW of bacterial pellet, all auxin derivatives and zeatin were quantified to be in higher quantities in PEG treated bacteria vs controls.

Table.2: Detection of phytohormones in AKMP7 cell free extracts using LCMS.

Phytohormones and their derivatives/conjugates screened in the cell-free bacterial extracts of *P. putida* AKMP7 using Mass spectrometry. A positive sign (+) indicates detection/presence of phytohormone in the sample and a negative sign (−) indicates no detection/absence in the sample.

Phytohormone/derivatives/ conjugates	Detectable (+)/Not-detectable (−) in cell free extracts of *P. putida* AKMP7
Indole-3-acetic-acid	(−)
Indole-3-pyruvic-acid	(−)
Indole-3-butyric acid	(−)
Indole-3-acetonitrile	(−)
Indole-3-acetamide	(+)
Gibberellic acid	(−)
Trans-Zeatin (tZ)	(+)
Abscisic acid	(−)
Indole-3-acetyl-L-alanine	(+)
Indole-3-acetyl-L-phenylalanine	(+)
Indole-3-acetyl-L-Aspartate	(+)

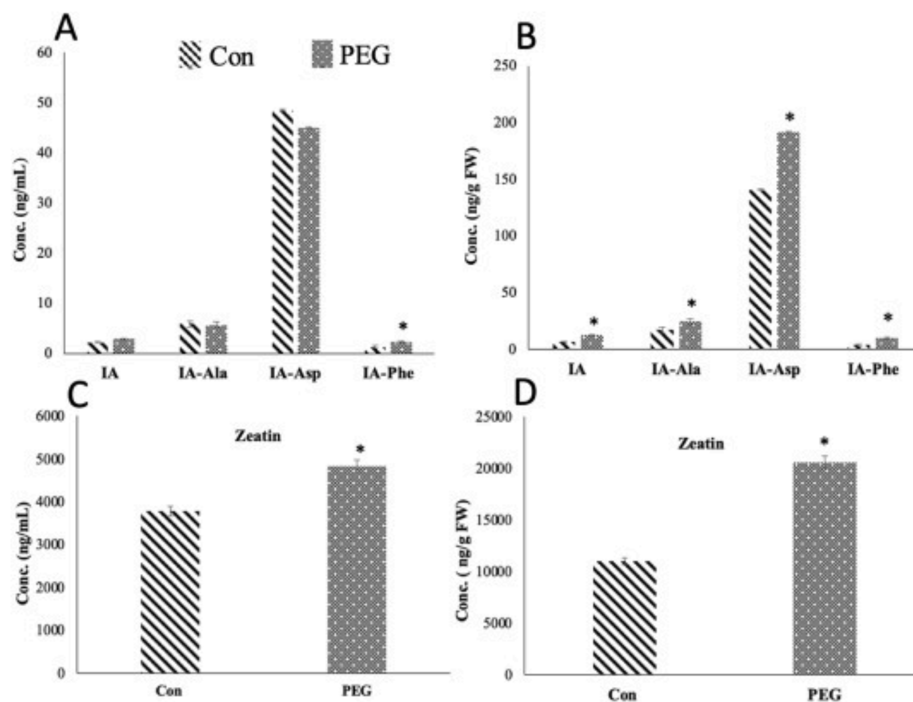


Fig. 2. Concentration of auxin derivatives, A -per mL of culture, B- per gram pellet fresh weight, the concentration of phytohormone zeatin, C -per mL of culture, D -per gram pellet fresh weight, released by *P. putida* AKMP7 into the broth medium under control and PEG treated conditions, quantified using LC-ESI-MS/MS. Each bar represents mean ± SE of 6 replicate flasks from 2 experiments. A * indicates a significant difference ($P \leq 0.05$) between PEG treated and control samples.

To reiterate from materials and methods, since the method used in this is an improved, highly efficient technique for the simultaneous detection and quantification of bacteria-derived phytohormones in the growth medium, we validated this method for the detection and quantification of phytohormones released by four other strains of rhizospheric microbes (both bacteria and fungi).

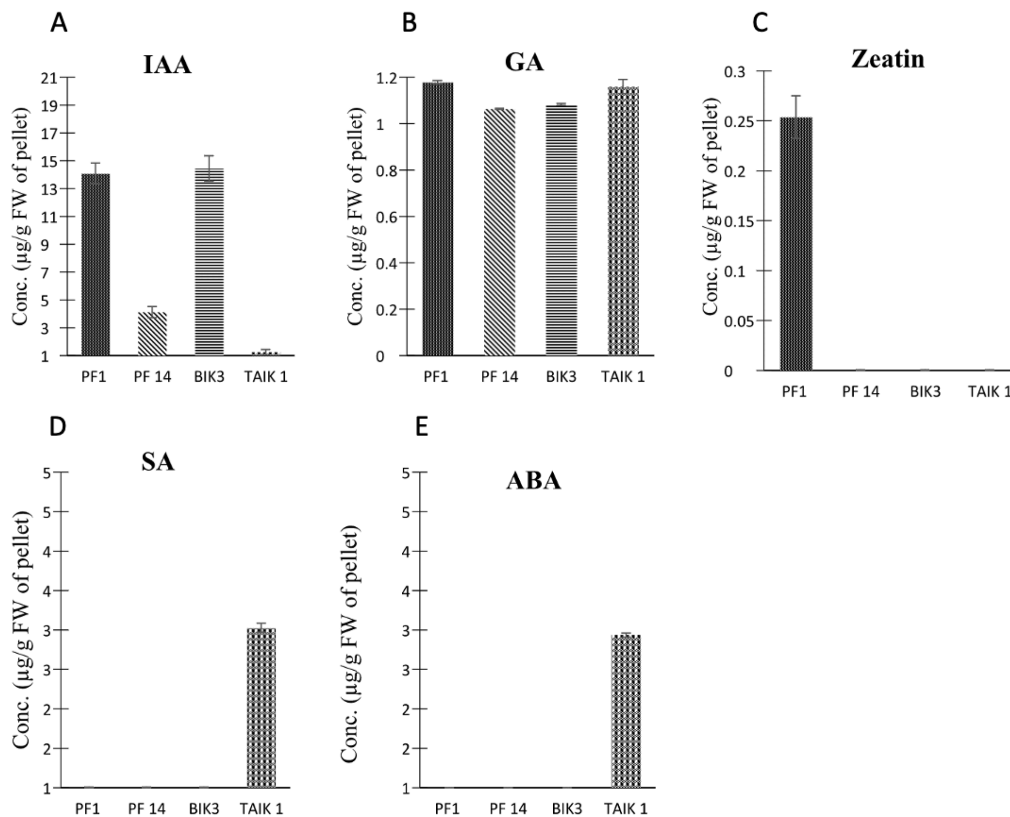


Fig. 3. A- indole acetic acid (IAA), B- gibberellic acid (GA), C-zeatin, D- salicylic acid (SA), E - abscisic acid (ABA). Each bar represents the mean ± SE of 6 biological replicate cultures from 2 experiments.

As depicted in the Fig. 3, IAA and GA could be detected in all four strains (Fig. 3 A, B). While IAA levels varied greatly across strains, GA levels were somewhat higher in PF1 and AIK 1 than PF14 and BIK 3. Zeatin was detected only in PF1, and SA and ABA, only in

TAIK1. The fact that we were able to detect and quantify these other phytohormones in the four additional microbial strains proves that our method is applicable for the extraction and quantification of a wide range of phytohormones. This also validates that the lack of observable GA, SA and ABA in AKMP7 is not a limitation of the method.

3.3.3. Inoculation studies using different volumes of AKMP7 aqueous suspension

In order to understand the dose-dependent impact of AKMP7 on *A. thaliana*, we inoculated different volumes of this bacterium to the roots of the plants and studied their impact on the morpho-physiology of the plants under non-stressed (well-watered) and water-stressed conditions. As shown in Fig. 4 and 5, AKMP7 caused an enhancement of growth in *A. thaliana* seedlings under well-watered (non-stressed) conditions. There was increase in both the fresh and dry weights of *A. thaliana* with AKMP7 inoculation under well-watered conditions when (Fig. 5). Approximately, the respective percentage increase in fresh weight for each volume was, 6% for 100 µL, 10% for 200 µL and 36% for 400 µL. Similarly, the percentage increase in dry weight was 6% for 100 µL, 10% for 200 µL and 22% for 400 µL. Among these volumes, 400 µL showed a statistically significant difference in terms of growth enhancement (Fig. 4, 5 and *Appendix 1:* Supplementary Fig. 6). Contrarily, inoculation with AKMP7 resulted in retardation of growth under water-stressed conditions for the same inoculum volumes (Fig. 4, 5). Approximately, the respective percentage decrease in fresh weight for each volume was 23% for 100 µL, 26% for 200 µL and 47% for 400 µL. Similarly, the percentage decrease in dry weight is, 10% for 100 µL, 19% for 200 µL and 33% for 400 µL volume. This impact is statistically significant only in the highest volume, i.e. 400 µL (Fig. 5, 5 and *Appendix 1:* Supplementary Fig. 6).

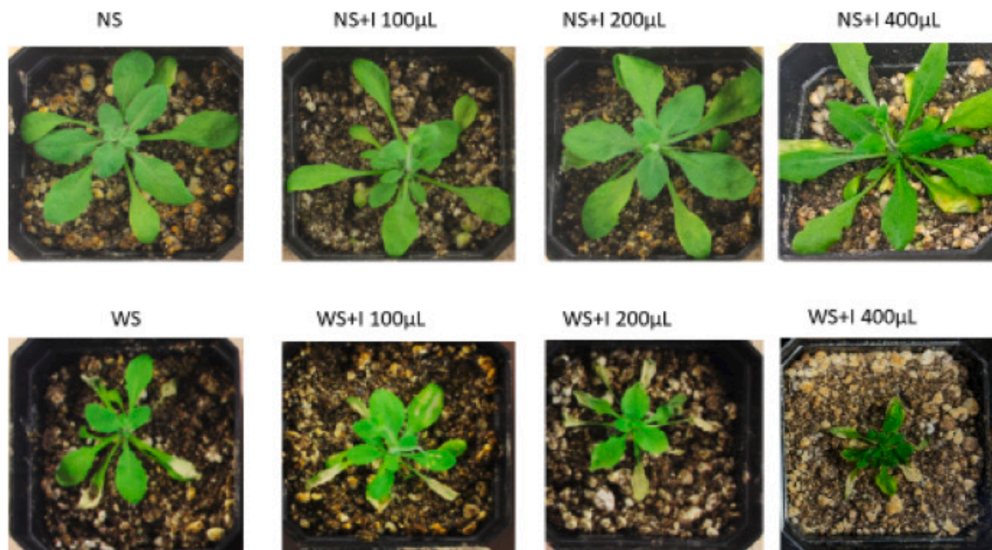


Fig. 4. Observation of growth and development of *Arabidopsis* seedlings inoculated with different volumes (100, 200, and 400 µL) of *P. putida* AKMP7 under non-stressed and water-stressed conditions. The non-stressed plants are represented as NS, non-stressed + inoculated as NS + I, water-stressed plants are represented as WS, and water-stressed + inoculated plants are represented as WS + I.

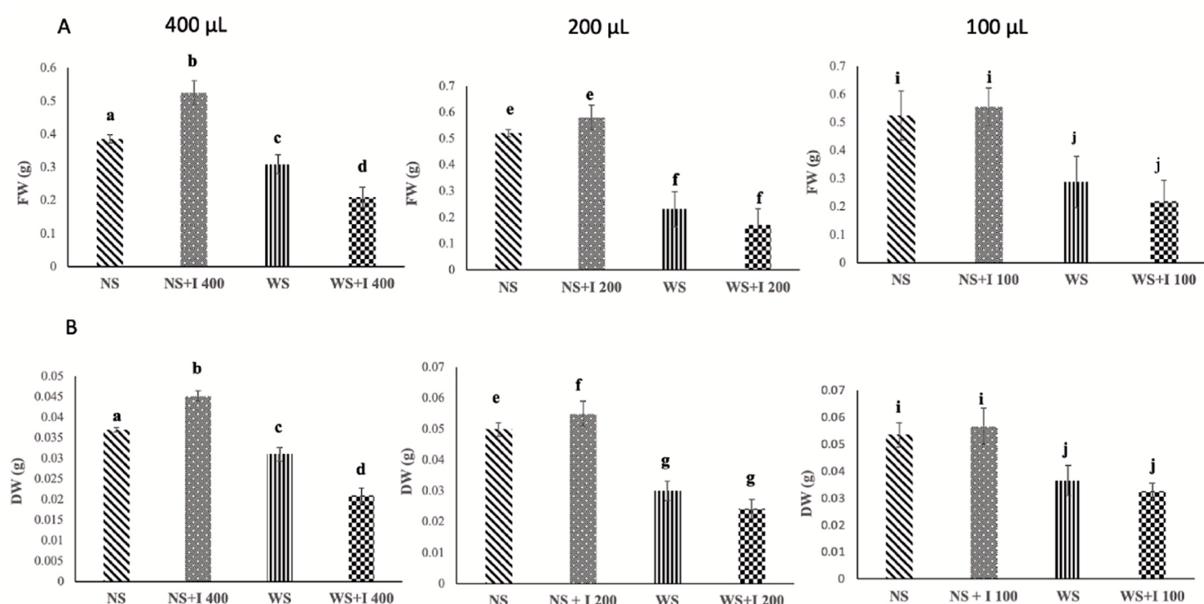


Fig. 5. Representation of fresh weight (A) and dry weight (B) data of *Arabidopsis* seedlings after two weeks post-inoculation with different volumes- 100, 200, 400 µL of *P. putida* AKMP7 culture. The experiment consisted of four different treatments (NS, NS + I, WS and WS + I). Each bar represents mean ± SE of 6 replicate pots from 2 experiments. Each inoculum volume has been assigned with different lettering scheme (i.e. 400 µL: letters a, b, c and d; 200 µL:

letters e, f, g and h; 100 μL: I, j, k and l) and difference in letter within the same treatment indicate a statistically significant difference between the four treatments, within the same volume.

3.3.4. Root colonization assay

Root colonization assay was performed in order to estimate the colonization efficiency of AKMP7 on the surface of roots and adhering soil of *A. thaliana* under non-stressed and water-stressed conditions. As can be seen from Fig. 6, the rhizosphere (root and adhering soil) suspension corresponding to inoculated seedlings (NS + I- Non stressed inoculated and WS + I-Water stressed inoculated) shows abundant growth and a characteristic green fluorescence (when subjected to UV-illumination) similar to the pure culture plate of AKMP7 (AKMP7). Plates corresponding to non-inoculated plants (NS- non stressed non-inoculated, WS- water-stressed non-inoculated)- show minimal growth of microbes. They have a faint glow and morphology that does not resemble AKMP7 colonies on cetrimide agar selective plates. The data represented in Table 3 is a measure of bacterial colony-forming unit (CFU) per gram of rhizosphere soil.

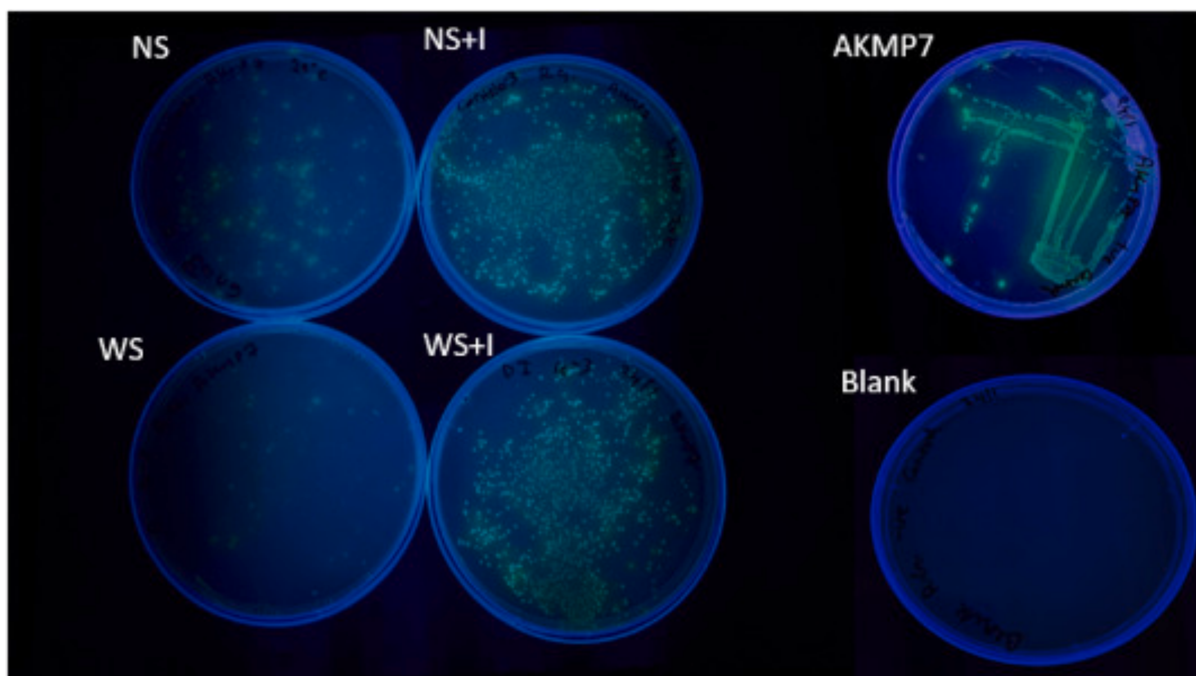


Fig. 6. Representative images of root colonization assay of *P. putida* AKMP7 inoculated seedlings after two weeks of inoculation (NS; NS + I; WS; WS + I; AKMP7 -pure culture and Blank-control).

Table 3. Microbial CFU. data of *A. thaliana* roots inoculated with *P. putida* AKMP7 and plated on cetrimide agar selective medium after two weeks of inoculation and drought induction.

Treatments	**CFU/g of rhizosphere soil**
NS.	Not detected
NS + I	$31 \times 10^4 \pm 4$
WS	Not detected
WS + I	$28 \times 10^4 \pm 4$

3.3.5. Visualization of stress-induced cell damage by confocal microscopy

We hypothesized that under water-stress, AKMP7 could be potentially causing cell damage in the plants, and hence, performed a confocal microscopy-based experiment to

confirm the same. As can be seen from Fig. 7, leaves of plants inoculated with AKMP7 under drought conditions (A4, B4) show cells with several green fluorescent spots stained with SYTOX Green nucleic acid stain when observed under confocal microscopy, indicating stress-induced cell damage. The leaves of non-inoculated drought treated plants (A3, B3) showed significantly fewer green fluorescent spots than water-stressed inoculated plants but showed more green fluorescent spots than non-stressed plants indicating early signs of drought-induced cell damage. Whether inoculated or not, leaves from the non-stressed plants showed very minimal or no green fluorescent spots, indicating no stress-induced cell damage (A1, B1, A2, B2).

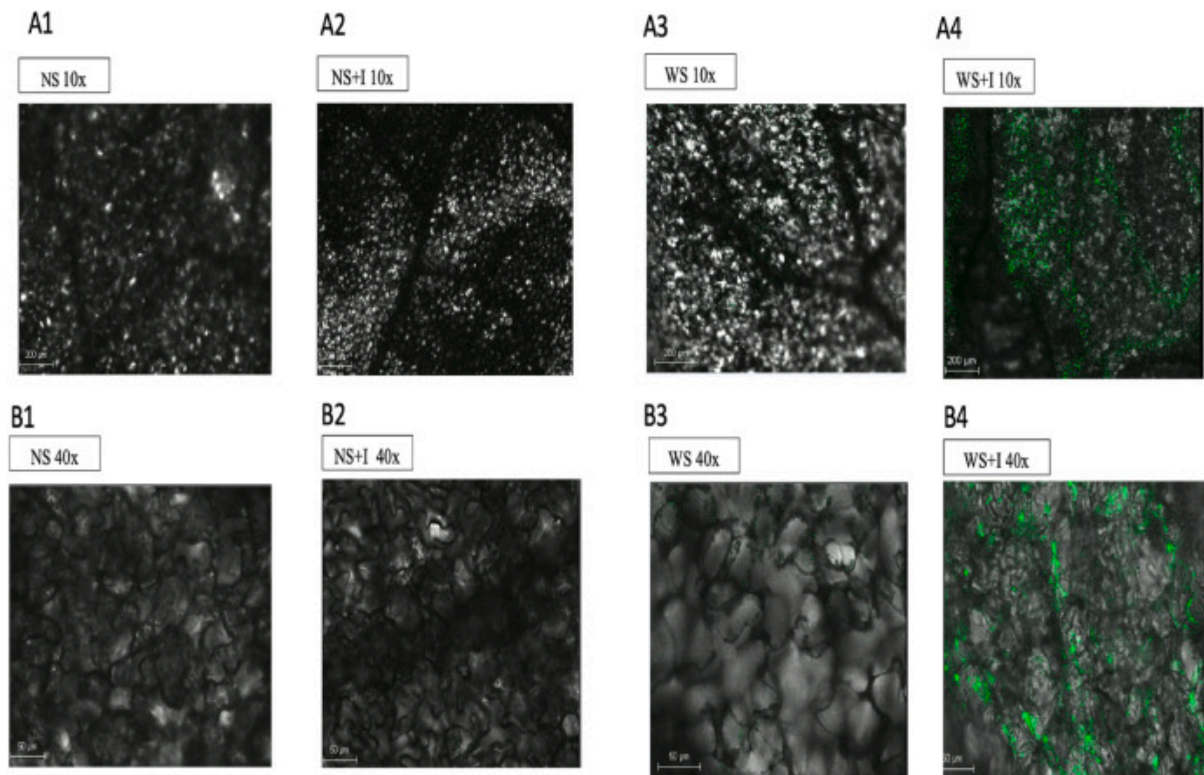


Fig. 7. Confocal microscopy images of *A. thaliana* leaf stained with SYTOX Green nucleic acid stain after two weeks of inoculation. Two different magnifications- 10× (A1-4) and 40× (B1-4) were used for observation of stained samples (NS- A1, B1; NS + I- A2, B2; WS- A3,

B3 and WS + I- A4, B4). Green fluorescent spots in the image indicate the presence of dead cells in the tissue.

3.3.6. Phytohormone treatment studies

In order to understand the impact of the bacteria-released phytohormones or their derivatives/conjugates on *A. thaliana* under non-stressed and water-stressed conditions, we treated the plants with commercially available forms of these chemicals. As can be seen from Fig. 8, under non-stressed non-inoculated (NS) conditions when plants were treated with commercially procured zeatin and auxin derivatives alone, none of the plants showed any signs of growth retardation and wilting. They looked similar to the NS plants after 10 days of treatment (Fig.8A). Similarly, under non-stressed conditions when plants were subjected to AKMP7+phytohormone treatment, no signs of growth improvement nor retardation was observed when compared to NS after 10 days of inoculation (Fig.8B). On the contrary, under the water-stressed, non-inoculated conditions (WS), when plants were treated with the same set of phytohormones/derivatives, there was severe retardation of growth and enhanced wilting observed in the case of plants phytohormone treated with tZ (WS + ZEA) and indole acetamide (WS + IA) after around 10 days of phytohormone treatment and drought induction (Fig.8C). While the auxin conjugates (IA-Ala, IA-Asp, and IA-Phe) did not induce any growth retardation or wilting when compared to water-stressed, non-inoculated plants. Subjecting water-stressed, inoculated (WS + I) plants to tZ and IA resulted in severe growth retardation and wilting, while moderate growth retardation was observed in the case of IA-Asp and IA-Ala. Plants exposed to IA-Phe did not look much different from water-stressed + inoculated controls (Fig.8D).

Fig. 8. Representative image of *Arabidopsis* seedling ten days after inoculation with *P. putida* AKMP7 and phytohormone co-inoculation. The denotations are, NS- non-stressed non-inoculated; WS- water-stressed non-inoculated; NS + I- non-stressed inoculated; WS + I- water-stressed inoculated; IA-Ala-indole-3-acetyl-L-alanine; IA-Asp-indole-3-acetyl-L-aspartate; IA-Phe-indole-3-acetyl-L-phenylalanine; IA-indole acetamide and zeatin-tZ.

3.4. Discussion

3.4.1. *P. putida* AKMP7 releases high levels of tZ, IA auxin conjugates under osmotic stress conditions

It has been known that many plant-associated rhizobacteria (beneficial as well as pathogenic) produce phytohormones such as auxin, cytokinin, gibberellin etc., and also modulate the host phytohormone levels (Dobbelaere et al., 2003;Beneduzi et al., 2012; Patten and Glick, 1996). As stated earlier, our laboratory has previously reported that AKMP7 releases

copious amounts of auxin under osmotic stress conditions and exhibits "conditional pathogenesis" on *A. thaliana* under water stress (Shah et al., 2017).

In this study, we wanted to (a) perform a comprehensive analysis on all phytohormones released by this PGPR strain and (b) understand if one or more of these phytohormones could be a causative factor for the conditional pathogenesis exhibited by this bacterial strain. As described here (Fig. 2, 3 and Table 2), using mass spectrometry analysis (direct injection without column) we primarily screened for many important phytohormones in the cell-free extracts (supernatant) of *P. putida* AKMP7 and have identified and quantified the cytokinin tZ, an auxin precursor, IA and auxin-amino acid conjugates IA-Asp, IA-Ala and IA-Phe.

Interestingly, under osmotic stress, while bacterial growth is relatively low (Shah et al., 2017), the production of the aforementioned compounds is almost equal or significantly higher. In fact, when the concentration of phytohormones is normalized to per gram of bacterial pellet fresh weight, we found that the concentration of all of these compounds was significantly higher under osmotic stressed conditions compared to non-stressed conditions. This suggests that, when stressed, the rate of production/secretion of these compounds increases in this bacterium. The overproduction of phytohormones under osmotic-stressed conditions can be a key factor contributing to the drought specific adverse impact of *P. putida* AKMP7 (discussed in detail in the following section).

The biosynthesis and external release of major phytohormones such as auxin, cytokinin, gibberellin and ABA are well reported in plant-associated bacteria (Koga et al., 1991; Tully et al., 1998; Takei et al., 2001; Patten and Glick, 2002; Xie et al., 2005; Sakakibara, 2006). Thus, there are studies that corroborate our observation on the release of tZ into the growth medium (Barry et al., 1984; MacDonald et al., 1986; Akiyoshi et al., 1989). However, there is very limited information on the auxin derivatives, conjugates etc. that we observed in AKMP7.

While the metabolism of these compounds has been relatively well studied in plants, the same has been extremely under-explored in microbes. In plants, these compounds are used as precursors or storage forms of auxin and help in maintaining auxin homeostasis (Woodward and Bartel, 2005; Ludwig-Müller, 2011), while their precise function in microbes is not clearly understood. There are reports though, that, plant-associated bacteria such as *P. fluorescens, Erwinia herbicola, Erwinia crysanthemi* etc. can biosynthesize auxin via an IA mediated pathway (Spaepen and Vanderleyden, 2011; Kunkel and Harper, 2018). Also, the plant pathogen *P. savastanoi* produces the conjugated form of auxin, IA-lysine which aids in pathogenesis (Glass and Kosuge, 1986; Yang et al., 2007; Kochar et al., 2011). However, as far as we know, there are no reports so far on the release of such phytohormone conjugates/derivatives/precursors into the growth medium by plant-associated bacteria. Hence, ours is the first report on the presence of (a) three different amino acid conjugated forms of auxin (IA-Asp, IA-Phe and IA-Ala) and (b) the auxin biosynthesis precursor, I.A., in the bacterial secretions of a PGPR strain.

3.4.2. *P. putida* AKMP7 enhances the growth of *A. thaliana* under well-watered conditions while curtailing the same under water stress

There is an exhaustive history on the positive impacts of PGPR on plant growth, development and stress alleviation. However, it must be kept in mind that all/most microbes can be opportunistic pathogens and even beneficial microbes can turn pathogenic under challenging adversities such as environmental stress or resource starvation. While there are some studies on such instances of "conditional pathogenesis" in animal/human-associated microbes, such studies are lacking in plant-associated microbes. For example, there are reports on the possibility of generally harmless gut microbe *Escherichia coli* turning pathogenic in chicken, which can possibly further evolve (to overcome species barriers) to be able to infect

the human population (Mageiros et al., 2021). Thus, it can be hypothesized that a potential PGPR can become a negative regulator of growth and development under certain environmental conditions either due to (a) the challenges of environmental adversity or (b) cross-compatibility issues between the PGPR strain and its "host" plant or (c) both.

There are a few reports that high quantities of some phytohormones, especially auxin and cytokinin can negatively impact root growth in plants. According to a review by Kudoyarova et al. (2019), the impact of PGPR on root development is two-fold; a positive impact by some strains and a negative impact by others. The negative impact has been correlated with the inhibitory roles of higher concentrations of auxin and cytokinin on root growth (Taiz et al., 2015). Other than our previous report on the AKMP7 (Shah et al., 2017), some cytokinin producing microbes, such as *Bacillus amyloliquefaciens* UCMB5113 have been reported to inhibit primary root growth in *A. thaliana* (Asari et al., 2017). Moreover, it is known that auxin is responsible for activating virulence in pathogenic bacteria (Jameson, 2000). Moreover, the roles of auxin and cytokinin in root gall formation and disease progression in *Agrobacterium tumefaciens* infection is well known (Weiler and Spanier, 1981; Nilsson and Olsson, 1997; Jameson, 2000). Thus, the role of phytohormones in plant rhizobacterial interaction can purely be contextual with respect to whether the interaction is beneficial or not.

One of the most unique observations in our study has been the fact that this PGPR promotes plant growth significantly under well-watered conditions and deters plant growth under water-stressed conditions in *A. thaliana* seedlings (Fig. 4, 5). Therefore, it can be hypothesized that the adversity of water deprivation challenges this bacterium to behave like an opportunistic pathogen. This possibility of beneficial soil microbes turning into opportunistic/conditional pathogens needs further exploration and can open the doors to an

entirely novel area of research in plant-associated microbes. Presently we can only hypothesize that the difference in bacterial released phytohormone concentration in normal vs stressed conditions that we observe in the growth medium, maybe mimicking in the soil, somehow contribute to the phenomenon of conditional pathogenesis. Further studies need to be done to understand this differential behaviour of the bacterium.

3.4.3. Role of tZ, IA and auxin conjugates in conditional pathogenesis

As discussed earlier, *P. putida* AKMP7 produces high amounts of tZ, IA and the auxin-amino acid conjugates under water-stressed conditions and becomes conditionally pathogenic towards *A. thaliana*. Considering the fact that some of these bacteria-derived chemicals are potentially hazardous towards plant growth in high concentrations, it becomes imperative to test the impact of these chemicals individually on plant growth under water-stressed conditions. Hence, to understand whether the phytohormone tZ and auxin derivatives produced by *P. putida* AKMP7 play a role in conditional pathogenesis under water-stressed conditions, we performed phytohormone treatment studies using commercially procured tZ and the auxin derivatives/conjugates. This study was done by exposing plants to known concentrations of these commercially available chemicals either singly or in combination with AKMP7. This study not only helped us understand whether the phytohormone/derivative alone can have a negative impact on the plants but also established the role of individual phytohormone/derivatives on enhancing pathogenesis in the bacterium. As evident from the visual observation made in the phytohormone co-treatment studies (Fig. 8), we have identified that phytohormone tZ and the auxin precursor IA, when co-treated with the bacterium, contribute to the drought-specific adverse impact of *P. putida* AKMP7 on *A. thaliana* seedlings. The negative effects were more severe than those of regular water-stressed inoculated plants without co-treatment (exogenously supplied phytohormone). We have also

found that plants treated with tZ alone were more susceptible to water stress, highlighting that tZ can play a crucial role in dehydration-mediated conditional pathogenesis. While IA treatment alone showed some adverse effects on plant growth under water stress, it was not as severe as in the case of co-treated plants. The other auxin derivatives showed no negative impact upon treatment alone (without AKMP7) under well-watered and water-stressed conditions but showed minimal/slight adverse effects upon co-treatment with AKMP7 under water-stressed conditions. Further studies need to be done to identify the precise role of these metabolites and to know how exactly they are contributing to the adverse effects seen under drought.

There are reports on the accumulation of IA leading to curtailing of growth in *A. thaliana* seedlings (Sánchez-parra et al., 2021). There are also reports that IA is involved in the cross-talk between phytohormones indole-3-acetic acid and abscisic acid, which leads to the biosynthesis of abscisic acid and consequently leads to enhanced stress response in plants (Pérez-Alonso et al., 2021). What is unknown is whether the accumulation of higher than optimal levels of endogenous IA can lead to abscisic acid (ABA) overproduction in plants and result in growth inhibition. IA is also prevalent in many pathogenic *Pseudomonas* Spp., which manipulates host auxin levels at the advent of infection (Kunkel and Harper, 2018; Djami-Tchatchou et al., 2020). Some studies indicate that genetic transformation of pathogenic fungi with IA biosynthesis genes iaaH and iaaM leads to enhanced IAA production and hypervirulence (Cohen et al., 2002). There are also reports on auto-oxidation of indole acetamide and related compounds to IAA (Ernsten et al., 1986). It is possible that the bacterial derived phytohormone can be employed to manipulate host phytohormone homeostasis by producing free phytohormone (i.e. tZ) or the precursors (i.e. IA) as well as storage forms (indole-3-acetyl-L-alanine) to disrupt the host hormonal homeostasis under stress conditions. Disrupting the phytohormone homeostasis might also disrupt the underlying signalling

mechanisms necessary for the host's survival. While the auxin derivative IA is known to be present in the culture medium extract of some bacterial species, to our knowledge, this is the first report on the presence of an auxin precursor IA along with three different auxin amino acid conjugates (storage/degradative forms of auxin in plants) such as IA-Asp, IA-Ala and IA-Phe in the bacterial extract of a PGPR. While the plant hormone auxin plays a role in mediating plant defence responses, it also promotes the susceptibility of the host towards pathogens in certain instances by regulating/activating the expression of virulence genes, thereby, enhancing the pathogenicity (Mutka et al., 2013; Djami-Tchatchou et al., 2020). Hence based on the evidence from previous research and the current study, we suspect the involvement of bacterial produced phytohormone and phytohormone-derivatives on the drought specific deterioration in the health of *A. thaliana* seedlings. Further studies need to be done to understand how these two bacterial produced phytohormone metabolites contribute to the negative impact seen under drought and the key pathways that they regulate, leading to conditional pathogenesis.

3.5. Conclusions from chapter 3:

Based on our study we can conclude that:

1. *P. putida* AKMP7 releases higher amounts of tZ, IAA. and auxin conjugates into the growth medium under osmotic stress than under non-stressed conditions.

2. *P. putida* AKMP7 enhances plant growth significantly under well-watered conditions, while contrarily exhibits significant deterioration of plant growth under water stressed conditions.

3. Among all the phytohormones analyzed, tZ and IA seem to contribute maximally towards conditional pathogenesis.

4. Apart from detection/quantification of phytohormones in AKMP7, our improvised method can also be used for detection of major phytohormones such as IAA, ABA, GA, SA and tZ in various microbial extracts of bacterial and fungal origin.

<u>Chapter 4</u>

Mechanistic Insights into the Modulation of *A. thaliana* Immune Response by *P. putida* AKMP7.

4.1. Introduction

Having established that AKMP7 releases the phytohormones/phytohormone-derivatives, some of which become detrimental towards plant growth and physiology under water-stress, we proceeded to understand the regulation of plant immune response during AKMP7-mediated conditional pathogenesis in *A. thaliana* under water-stress. Since (a) plant immune response is upregulated during pathogen invasion, as a defense mechanism to prevent disease and (b) since pathogens manipulate plant immune response to cause disease as a virulence mechanism, therefore, studying the regulation of plant immune response in *A. thaliana* by AKMP7 is crucial to understanding the molecular mechanisms behind the conditional pathogenesis caused by the bacterium. The importance of the defense-related phytohormone salicylic acid (SA) in plant defense response is well known (Corina Vlot et al., 2009; Ding and Ding, 2020). Through several decades of breakthrough research, many important genes, enzymes, and metabolites involved in SA biosynthesis and signaling have been deciphered. Accumulation of SA upon pathogen infection in plants is required for the activation of plant defense related signaling genes and to restrict the progression of the disease. To understand the impact of AKMP7 on plant immune response in *A. thaliana* during the onset of conditional pathogenesis we have study the regulation of major defense hormone SA and it's signaling genes involved in plant defense. Our data, thus, throws light on the modulation of plant immune response by a conditionally pathogenic rhizobacterium under water deprivation.

4.1.1. Biosynthesis of SA in plants:

SA is mainly biosynthesized via two different biosynthetic pathways, using chorismate as a common precursor (Fig. 9). In *A. thaliana*, SA is predominantly made via the isochorismate synthase (ICS) route, in which chorismate is converted into isochorismate and which, in turn, is subsequently converted to SA (Torrens-Spence *et al.*, 2019). The alternative mode of SA biosynthesis uses the phenylalanine (Phe) ammonia lyase (PAL) route, in which Phe generated

from chorismate is converted into trans-cinnamic acid and, which, in turn is subsequently converted to SA. The PAL pathway functions as an alternative/complementary biosynthetic pathway in *A. thaliana.* As can be seen from Fig. 8, there are three key genes (*ICS1*, *PBS1* and *EPS1*), involved in SA biosynthesis via the ICS pathway. Mutation in any of these genes can cause drastic abnormalities in SA biosynthesis (Huang *et al.*, 2010; Torrens-Spence *et al.*, 2019; Peng *et al.*, 2021). Similarly, the PAL pathway relies on the PAL family of genes (*PAL1*, *PAL2*, *PAL3* and *PAL4*), which encodes phenylalanine ammonia-lyase, an enzyme that converts phenylalanine into trans-cinnamic acid (Huang *et al.*, 2010; Zhang *et al.*, 2021). Subsequently, a key gene called *AIM1* plays a crucial role in converting trans-cinnamic acid into benzoic acid, which is subsequently converted into SA by a proposed/hypothesized gene, benzoic-acid hydroxylase (*BA2H*). While the PALs have been involved in SA biosynthesis in many plant species, there are some contrasting reports on their function in *A. thaliana* (Wu et al., 2022). However, previous studies have shown that *A. thaliana* mutants lacking functional *ICS* and *PAL* genes (disrupted/silenced genes) are compromised on SA biosynthesis and immune response, highlighting the importance of both pathways in plant immunity (Huang *et al.*, 2010; Torrens-Spence *et al.*, 2019; Zhang *et al.*, 2021).

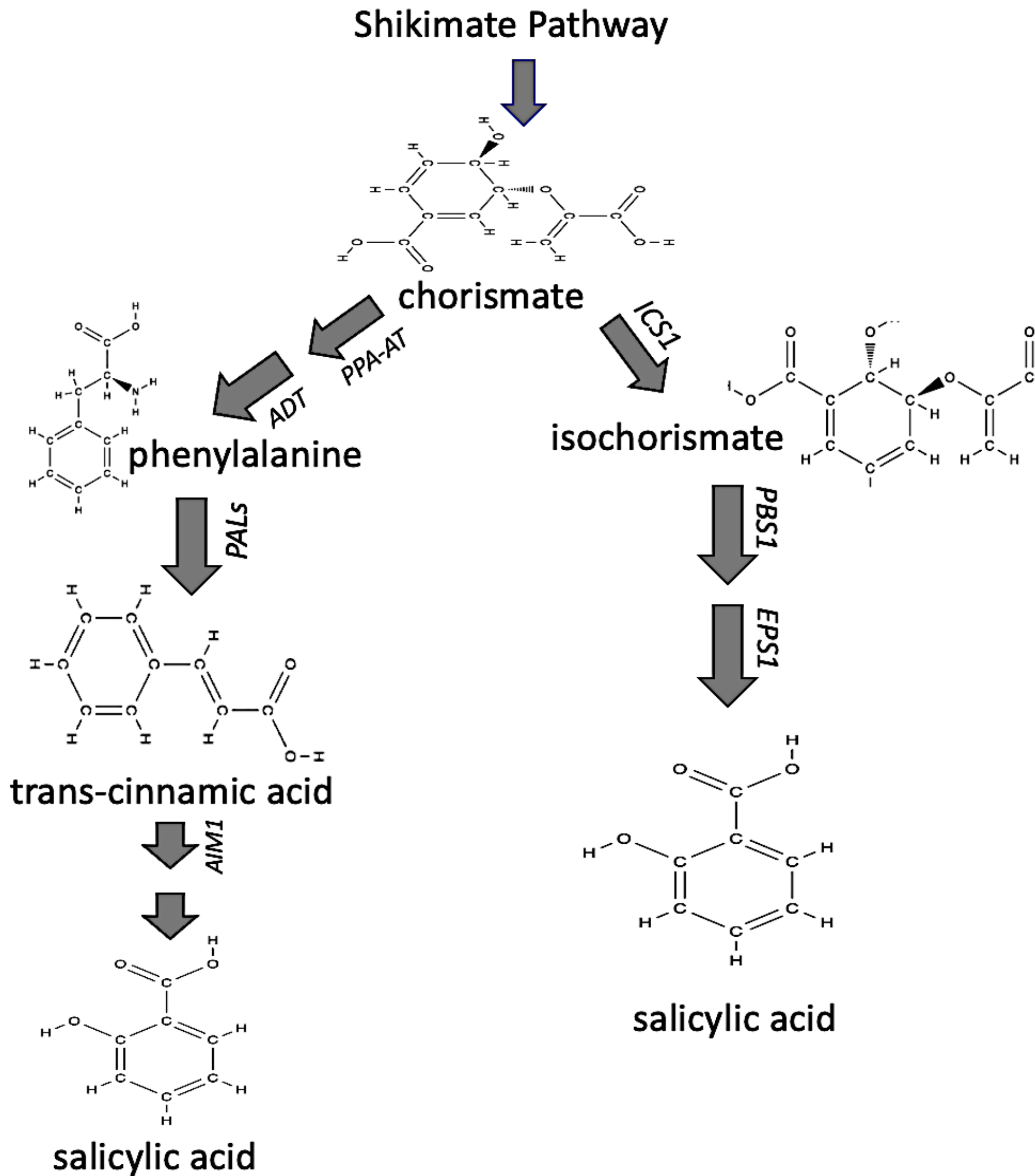


Fig. 9. Schematic representation of SA biosynthesis pathway in plants. The model was adopted from the information based on the works of Torrens-Spence et al., 2019; Ding and Ding, 2020.

4.1.2. Activation of plant immune response by the signaling trio of SA, *NPR1* and *PR1*:

As discussed above, SA is a major defense-related phytohormone and master regulator of plant immunity against different types of pathogens. The accumulation of SA in the infected plant tissues contributes to disease resistance in local and distal infection sites by activating

various defense-responsive genes, leading to systemic acquired resistance (SAR) (Fu and Dong, 2013). Previous studies have established, the role of Non-expresser of Pathogenesis Related (*NPR*) family of genes in SA-mediated defense response (Fu *et al.*, 2012; Ding *et al.*, 2018; Liu *et al.*, 2020). It has been reported that *A. thaliana* has 4 different isoforms of *NPR* (*NPR1-4*). While *NPR1* and *NPR2* play positive roles in regulating SA-induced immunity and activating SAR response, *NPR3* and *NPR4* negatively regulate the same (Ding *et al.*, 2018). It's known that both, NPR3 and NPR4 proteins bind SA and mark the degradation of NPR1 proteins (Fu *et al.*, 2012).

Previous studies with *A. thaliana* have identified that *NPR1* mutants exhibit enhanced susceptibility to bacterial pathogens and fail to mount SAR response (Fu *et al.*, 2012; Liu *et al.*, 2020). *NPR1* signaling activates most SA-induced genes and functions as a master regulator of SA-mediated plant defense (Pajerowska-Mukhtar et al., 2013). As seen from Fig. 10, under normal conditions, NPR1 protein is inactivated by low cytosolic SA concentration and remains relatively at very low levels in the cytosol as a nonfunctional oligomer. Also, when the cytosolic SA levels are low, NPR3 and NPR4 prevent NPR1-dependent activation of SA signalling by triggering proteasome-mediated degradation of NPR1. NPR1 is activated when the cytosolic SA levels are high, leading to co-localization of SA-bound NPR1 into the nucleus, which is essential to suppress negative regulators of plant defence and activate the SA-responsive genes involved in plant defence. Also, under high cytosolic SA, the *NPR3/4* are suppressed/inactivated, leading to NPR1-mediated induction of the Pathogenesis related gene 1 (*PR1*), another essential component of SAR (Pajerowska-Mukhtar et al., 2013; Ding *et al.*, 2018; Liu *et al.*, 2020; Wang *et al.*, 2020).

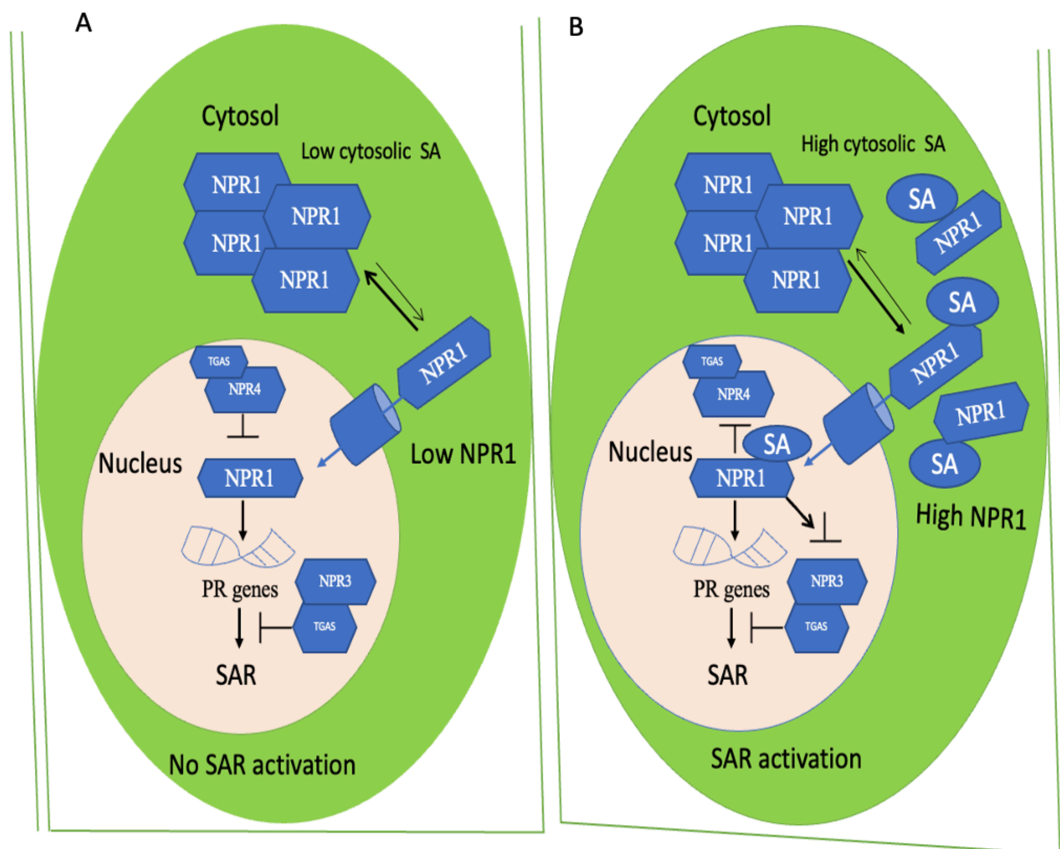


Fig. 10. Graphical representation of SA-mediated activation of immune response and SAR in plants during A: normal conditions vs B: interaction with pathogen. Adapted from Ding et al. (2018); Torrens-Spence et al. (2019); Ding and Ding (2020).

4.1.3. Modulation of SA perception during plant-pathogen interaction:

Plant-associated pathogens employ different strategies to manipulate phytohormone crosstalk/signaling inside the host to suppress immune responses and promote virulence. Some pathogenic microbes are known to disrupt SA homeostasis inside the plant to promote disease (Venturuzzi et al., 2021; Uppalapati et al., 2007; Schell, 2000; Nomura et al., 2005; Chen et al., 2017). While a lot of information has been uncovered concerning plant-pathogen interactions, due to the astonishing diversity of microbes interacting with plants, the exact molecular mechanisms orchestrating an event of successful pathogenic invasion or disease resistance (i.e.,

signaling pathways involved in disease/resistance) in many cases remain vastly unknown. They need to be further investigated and studied. There are many reports on the modulation of SA biosynthesis by both beneficial and harmful microbes leading to better disease resistance (in beneficial microbe interaction) or enhanced susceptibility (in harmful microbe interaction). However, to our knowledge, there are no studies on the differential regulation of SA signaling by a plant-associated microbe, which becomes preferentially beneficial or detrimental to the host plant, depending upon changes in the environment.

In this study, we analyzed the following parameters to understand how SA biosynthesis/immune response is regulated upon inoculation of *P. putida* AKMP7 with *A. thaliana* during drought-induced conditional pathogenesis.

(a) SA biosynthesis: SA accumulation and expression pattern of both the ICS and PAL pathway related genes
(b) SA signaling: Expression of *NPR1* and *PR1* genes involved in plant immunity and SAR
(c) We also studied the impact of AKMP7 inoculation on the pathogenesis of *Pseudomonas syringae* DC3000 (a known phyllospheric pathogen of tomato and Arabidopsis) on *A. thaliana*

4.2. Materials and Methods:

4.2.1. Plant growth and treatments:

The conditions used for growing *A. thaliana* seedlings and for subsequent treatments have already been described in Chapter 4 (Raja Gopalan et al., 2022).

4.2.2. Extraction of SA from plant tissue and its quantification using LC-ESI-MS/MS:

The phytohormone, SA, was extracted from *A. thaliana* leaves at two different time points, i.e., 1 week, and 2 weeks post treatments. The extraction was performed with slight modifications to the method described by (Ghosh et al., 2019). Before extraction, the leaf

samples were ground to a fine powder using liquid nitrogen with a mortar and pestle. Before extraction, 2µg of internal standard chloramphenicol was added to the ground tissue to minimize variations. A volume of 2 mL 80% methanol (HPLC grade) was added to 200 mg ground tissue, the samples were extracted overnight at -20° C, followed by centrifugation at 6700 xg for 10 mins at 4° C to remove plant debris. After centrifugation, the supernatant (extract) was collected in a fresh tube, and the samples were purified/cleaned-up using an anion exchange C18 solid phase extraction (SPE) cartridge (Phenomenex- Strata Screen-A), using a slightly modified method from Cao et al., 2016. The extract (2 mL) was loaded into the SPE cartridge, and the eluent was collected in a separate tube. Following washes with methanol and water, the cartridges were eluted with 2 mL of methanol containing 0.5% formic acid. The eluent containing internal standard and SA were then pooled together and concentrated using a centrifugal vacuum concentrator. For quantification, 500µL of final concentrate was taken and filtered using 0.22µM syringe filters and subjected to liquid chromatography coupled with electron spray ionization tandem mass spectrometry (LC-ESI-MS/MS) analysis. The LC-ESI-MS/MS analysis was performed using the same chromatography conditions as described by Chapter 3; Raja Gopalan et al.,2022. The MS/MS conditions were optimized under multiple reaction monitoring mode (MRM) using commercially available SA standard to determine the precursor ion (m/z) and product ion (m/z). The intensity/area of the most abundant product ion was used for quantification. The concentration of SA in the plant sample was extrapolated from the standard curve of "area under the curve" vs concentration (using commercially obtained SA standard). The complete details of MS/MS conditions are provided in the supplementary data (*Appendix 2:* Supplementary Fig. 8 and 9).

4.2.3. Gene expression studies:

For gene expression studies, the seedlings were harvested after two weeks of inoculation and drought induction. The shoot and root were separated, and the shoot tissue of

harvested seedlings was snap-frozen and ground with liquid nitrogen using a mortar and pestle. Total RNA was isolated from the ground tissue using trizol reagent and used for subsequent cDNA synthesis. Prior to cDNA synthesis, the RNA samples were subjected to DNase treatment. The quantity of RNA isolated was measured using a nanodrop. For cDNA synthesis, and 1µg RNA was converted into cDNA using Superscript III reverse transcriptase. The gene expression studies were then performed using quantitative real-time PCR using the gene-specific primers (details provided in the *Appendix 2:* Supplementary data). The gene expression was analyzed by the comparative ΔΔCT method. For the endogenous control gene, β-actin 2 or elongation factor-1 α were used. The gene expression was quantified with respect to non-stressed, non-inoculated control (NS). For PCR primer designing, web-based open-source primer3 software (https://primer3.org/webinterface.html) was used. The complete information about the list of primers used is provided in the supplementary data (*Appendix 2*: Supplementary Table. 1).

4.2.4. Physiological studies with *A. thaliana* mutants:

For these studies, SALK-confirmed homozygous *A. thaliana* mutant plants with a dysfunctional *NPR4* gene (by t-DNA insertion) were used (Pajerowska-Mukhtar et al., 2013; Ding *et al.*, 2018; Liu *et al.*, 2020). Mutant seeds were procured from the Arabidopsis Biological Resource Centre (ABRC), The Ohio State University, Columbus, OH, U.S.A. (accession number SALK_098460C). Prior to the experiments, we verified the homozygosity of the procured mutant line by genotyping. About 20-30 mutant seeds were sown and germinated in a germination tray using the same method discussed above. After germination, seedlings were allowed to grow for 2 weeks (under the same conditions as described above) and then subjected to genotyping. A total of 10 seedlings were randomly selected for genotyping, as it is easier to detect any heterozygous or wild-type seed cross-contamination in the procured seed lot with a considerably large sample size (n=10). Prior to genotyping, the 10

seedlings were segregated into 2 replicate groups, each containing 5 individual plants. Individual seedlings within each replicate group were pooled prior to DNA extraction. The pooled samples were ground in liquid nitrogen and then subjected to DNA extraction , followed by genotyping through PCR using mutant-specific primers (BP- tDNA forward primer and RP- wild type reverse primer) and wild-type specific primers (LP- wild type forward primer- RP- wild type reverse primer). The reverse primer used was the same for both mutant and wild-type DNA samples. The forward primer was designed for wild-type genomic DNA sequence in the case of LP-primer, and in the case of mutants, the tDNA sequence-specific BP-primer suggested by the SALK iselect primer tool (http://signal.salk.edu/isect.2.html) was used. The PCR reactions setup with BP-RP primer pair yielded a clear PCR amplification product for the DNA extracted from homozygous and heterozygous mutant lines. It did not yield any amplification product for the wild-type DNA samples. The LP-RP reactions yield a clear PCR amplification product for the DNA extracted from wild-type and heterozygous seed lines and don't yield any amplification product for the homozygous seed line. Homozygosity was confirmed by the presence of a clear amplification product in BP-RP reactions and no amplification product in the LP-RP reactions. For reliability of results, DNA samples were also extracted from wild-type (col-0) seedlings and used as positive controls in LP-RP reactions and negative control in BP-RP reactions. The PCR product was visualized using a BioRad Gel Doc XR after running the samples used for PCR reactions in agarose gel electrophoresis (using 1% gel). The complete information about the primers used for the genotyping process and the results obtained are given in supplementary data (*Appendix 2:* Supplementary Fig. 10).

4.2.5. Physiological observation of plant immune status using model pathogen *Pseudomonas syringae* DC3000 by syringe infiltration technique:

The *Pseudomonas syringae* DC3000 strain was a kind gift from Dr. Saikat Bhattacharjee, Regional Centre for Biotechnology, Faridabad, India. *A. thaliana* WT plants

were grown and inoculated with *P. putida* AKMP7 using the same method and treatment conditions as mentioned above. After 2 weeks of inoculation and water-withholding, mature leaves of *A. thaliana* seedlings were infiltrated with *P. syringae* DC3000 by syringe infiltration using the method described by (Jacob et al., 2017), with slight modifications. Prior to syringe infiltration, a monoculture of *P. syringae* DC3000 was revived from glycerol stocks and grown overnight in autoclaved LB broth. After overnight incubation, a volume of 2 mL culture was subcultured into a fresh autoclaved LB media (50 mL) and grown to OD_{600nm} of 0.8. Cells were then centrifuged at 5500 RPM, and the supernatant was discarded. The pellet was resuspended in 5 mL of sterile distilled water to an approximate culture load of $1x10^8$ CFU, and 100 µL of the resuspended culture per leaf was used for syringe infiltration. After 72 hours of infection, pictures of infected leaves were taken using the macro-photography. Pathogen-triggered cell death was visualized using confocal laser microscopy by staining the infected leaves with SYTOX green nucleic acid dye (Invitrogen, USA). In order to corroborate the microscopy data, the colony forming units (CFU) of DC3000 were counted by plating the infected *A. thaliana* leaves on King's B medium (modified from Jacob, Panchal and Melotto, 2017). Briefly, 2-days post DC3000 infiltration, *A. thaliana* leaves were harvested, homogenized and subjected to serial dilution in autoclaved water. The suspension was then plated on Petri plate containing King's B medium and incubated overnight at 28 ^oC. Following incubation, the CFU were counted.

4.2.6. Statistical analysis:

Statistical analysis of the results was performed using one-way ANOVA, followed by Tukey's post-hoc HSD test with the level of significance set at $p \leq 0.05$. Data were collected from at least 6 biological replicate plants (one plant per pot), from two different independent experiments.

4.3. Results:

The objective of our study was to identify how *A. thaliana* immune response is regulated by *P. putida* AKMP7, under well-watered vs. water-stress conditions. Since SA is the master regulator of plant immunity, we wanted to understand the impact of *P. putida* AKMP7 on the modulation of SA biosynthesis (SA accumulation and expression pattern of both the ICS and PAL pathway related genes) and SA signaling (expression of *NPR1* and *PR1* genes involved in plant immunity and SAR) in *A. thaliana*, under, stressed and non-stressed conditions.

We also studied the impact of AKMP7 inoculation on the pathogenesis of *P. syringae* DC3000 (a known phyllospheric pathogen of tomato and Arabidopsis) on *A. thaliana*.

4.3.1. Analysis of SA levels using LC-ESI-MS/MS reveals AKMP7 causes a high accumulation of SA in *A. thaliana* leaves under well-watered and water-stress conditions:

As shown in Fig. 11, some very interesting trends were seen in SA levels of *A. thaliana* shoots, after 1st and 2nd week post treatments. 1-week post-treatments (Fig. 11A), no significant change was seen in SA levels with AKMP7 inoculation under well-watered conditions (as opposed to the non-stressed, non-inoculated controls). Water stress induced a slight upregulation in SA levels when compared to the well-watered treatments, and AKMP7 inoculation under water stress further increased the SA levels. Going from week-1 to week-2 (Fig. 11B), the SA levels remained somewhat unchanged in the non-inoculated plants, both, under well-watered (NS) as well as water-stressed (WS) conditions. However, the AKMP7 inoculated plants exhibited an increase in SA levels, both, under well-watered (NSI) and water-stressed conditions (WSI), as compared to week-1. By week-2, therefore, an almost 2-fold

increase was seen in the SA levels of the AKMP7 inoculated plants under well-watered conditions, and an over 2.5-fold increase in the inoculated, water-stressed plants, when compared to their respective, non-inoculated controls (i.e., NS vs. NS+I and WS vs. WS+I). The maximum accumulation of SA was seen in water-stressed plants under AKMP7 inoculation.

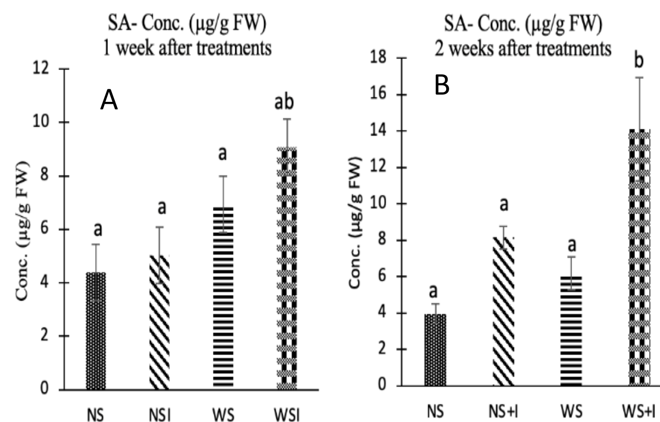


Fig. 11. Concentration of SA in *A. thaliana* shoots under the four different treatments -non-stressed (NS), non-stressed, inoculated (NSI), water-stress (WS), water-stressed, inoculated (WSI) at two different time points (A: 1- week and B: 2- weeks) post inoculation and drought induction. Each bar represents the mean ± SE of 6 biological replicate plants from 2 different experiments. Different letters indicate statistical significance between treatments (p-value set at 0.05).

4.3.2. *P. putida* AKMP7 causes upregulation of SA biosynthetic gene expression in *A. thaliana* under well-watered and/or waters-stressed conditions.

As shown in Fig. 12 (A, B), the *ICS1* and *EPS1* genes were upregulated under all treatments, as compared to the non-stressed, non-inoculated controls, albeit to different degrees. With AKMP7 inoculation under well-watered conditions, ~1.5- fold and 2-fold

inductions were seen in the expressions of *ICS1* and *EPS1*, respectively. In both genes, the highest levels of expression were seen on AKMP7 inoculation under water-stress.

Interestingly, the PAL-mediated SA biosynthetic genes (*PAL1*, *PAL2*, and *PAL3*- Fig. 11C-E) were not upregulated on AKMP7 inoculation under well-watered conditions. The expressions of all 3 genes were upregulated under water-stress. AKMP7 inoculation under water stress, further induced the expressions of *PAL2* and *PAL3*. The expression of *AIM1* was induced only under water-stressed, inoculated conditions (Fig. 11F).

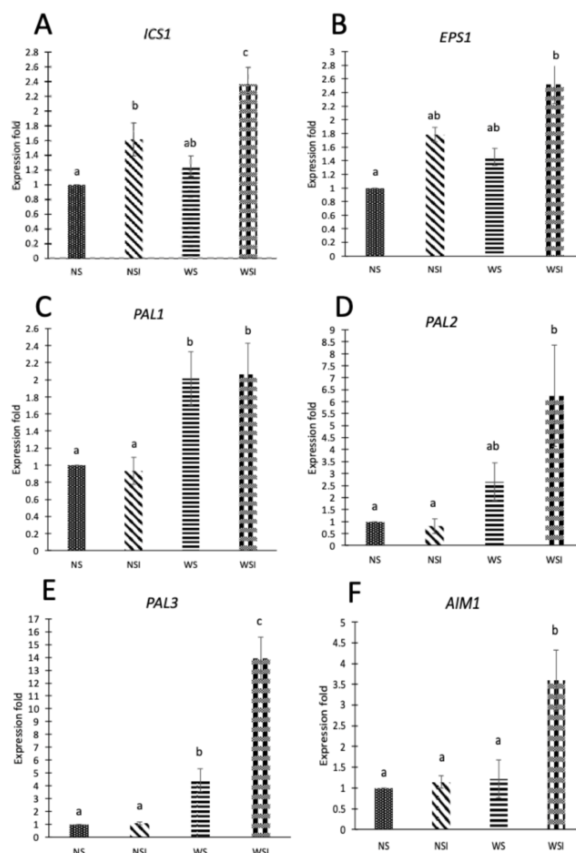


Fig. 12. Gene expression analysis of SA biosynthetic genes in *A. thaliana* shoots. A: *ICS1*, B: *EPS1*, C: *PAL1*, D: *PAL2,* E: *PAL3* and F: *AIM1*, after two weeks of inoculation with *P. putida* AKMP7 and drought induction. Each bar represents the mean ± SE of 6 biological replicate plants from 2 different experiments. Different letters indicate statistical significance between treatments (p-value set at 0.05).

4.3.3. *P. putida* AKMP7 inoculation causes upregulation of *NPR1* and *PR1* gene expression under well-watered conditions while causing a drought-specific downregulation of the same.

As shown in Fig. 13, *P. putida* AKMP7 causes upregulation in the expression of both, *NPR1* as well as *PR1* genes under well-watered, inoculated (NSI) seedlings compared to well-watered non-inoculated plants. Water-stress caused an induction in the expression of *NPR1*, but downregulation in *PR-1* expression. AKMP7 inoculation under water-stressed conditions, caused a down-regulation in the expression of both these genes, as compared to the water-stressed, non-inoculated plants. In fact, the PR1 expression was minimum in the water-stressed, inoculated plants, among all treatments (Fig. 13B).

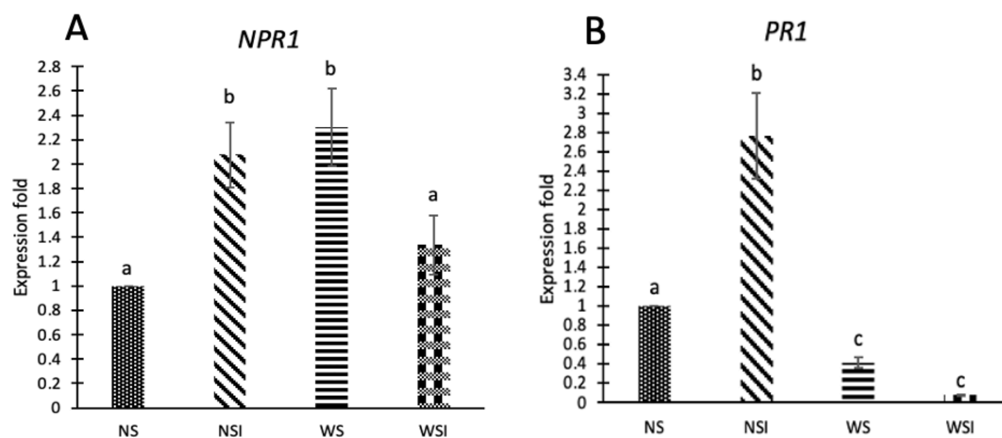


Fig. 13. Gene expression analysis of SA signaling genes in *A. thaliana* shoots. A: *NPR1* and B: *PR1*. Each bar represents the mean ± SE of 6 biological replicate plants from 2 experiments. Different letters (i.e. a and b) indicate statistical significance between treatments (p-value ≤ 0.05).

4.3.4. *P. putida* AKMP7 induces the expression of *NPR4* (a negative regulator of *NPR1*), under water-stressed conditions in *A. thaliana*:

As shown in Fig. 13A, *P. putida* AKMP7 causes upregulation of the *NPR1* negative-regulator, *NPR4,* only under water-stressed conditions. There was also a drought-specific upregulation of the *PR1* negative regulator, *NPR3* in both water-stressed inoculated and non-inoculated seedlings (Fig. 14B).

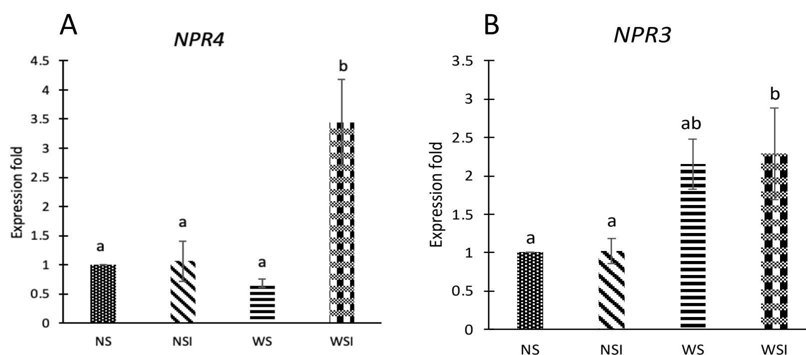


Fig. 14. Gene expression analysis of SA signaling genes in *A. thaliana* shoots. A: *NPR4*, B: *NPR3* in *A. thaliana* seedlings after two weeks of inoculation with *P. putida* AKMP7 and drought induction. Each bar represents the mean ± SE of 6 biological replicate plants from 2 experiments. A different letter (i.e. a and b) represent statistical significance between treatments (p-value ≤ 0.05).

4.3.5. Inoculation studies with *A. thaliana NPR4* mutant shows *NPR4* is required for conditional pathogenesis:

The characteristic phenotype rendered by AKMP7 inoculation to WT *A. thaliana*, i.e., growth promotion under well-watered conditions and stunting under drought conditions has already been described in Chapter 4 and published in Raja Gopalan et al., (2022). The same has been depicted here in Fig. 15A., after 2 weeks of inoculation and drought induction. Also, as can be seen from Fig. 15B, NPR4 mutants show no signs of drought-specific deterioration of growth

(as observed in the wild-type plants) under water stress (WSI) when compared to water-stressed non-inoculated seedlings (WS). Fresh weight data (Fig. 15C) indicates that there is no inoculum-induced drought-specific adverse effect on the growth of *A. thaliana* NPR4 seedlings when compared to water-stressed, non-inoculated seedlings. Similarly, there are no significant changes observed in the inoculated and non-inoculated seedlings under well-watered conditions (NSI and NS). Please note that the quantitative data for growth and physiology of WT *A. thaliana* under all four treatments has already been described in Chapter 4 and published in Raja Gopalan et al., (2022).

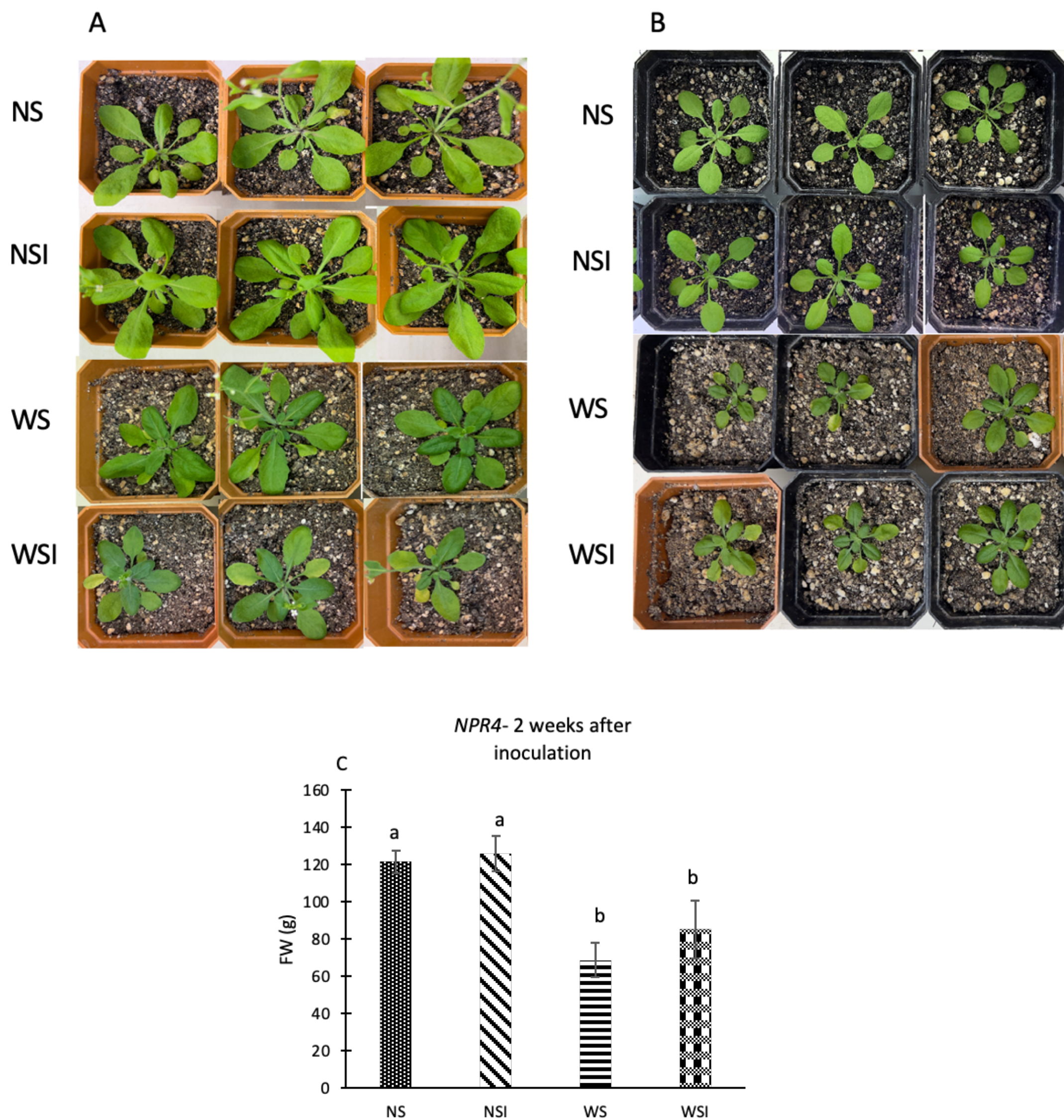


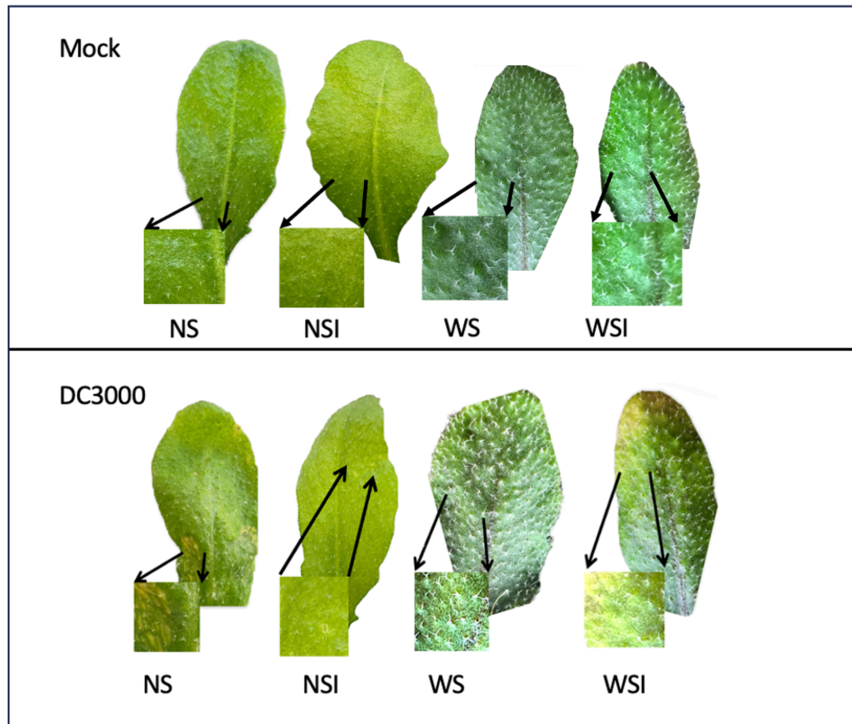
Fig. 15. Representative image of *A. thaliana* seedlings A- Col-0 and B- *npr4* mutant inoculated two weeks post treatments. Representative whole seedling fresh weight data of *A. thaliana npr4*

mutant after two weeks of inoculation with *P. putida* AKMP7 and drought induction. Each bar represents the mean ± SE of 5 biological replicates plants from one experiment. Different letters (i.e., a and b) indicate statistical significance between treatments (p-value ≤ 0.05). The experiment was repeated twice with similar results. Data from one such experiment is shown above.

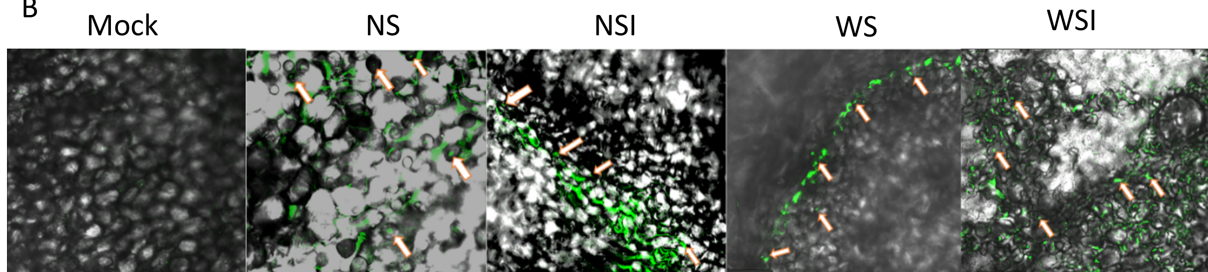
4.3.6. Physiological observation of plant immune status and pathogen susceptibility using *P. syringae* DC3000 infiltration:

As can be seen from Fig. 16A, *P. syringae* DC3000 infiltration shows a characteristic yellowing of leaves after 3 days post infiltration (after 2 weeks of treatments) in all treatments. However, the overall yellowing is significantly reduced in the well-watered inoculated treatments compared to other treatments. Also, the water-stressed inoculated and non-inoculated treatments appear more infected than the well-watered non-inoculated treatment. As shown in Fig. 16B, there is a characteristic pathogen-induced apoptosis (visualized by bright SYTOX green staining of dead cell nucleus) in all the infected leaves in all the treatments except the mock-inoculated leaves. This data is corroborated by Fig. 16C.

A



B



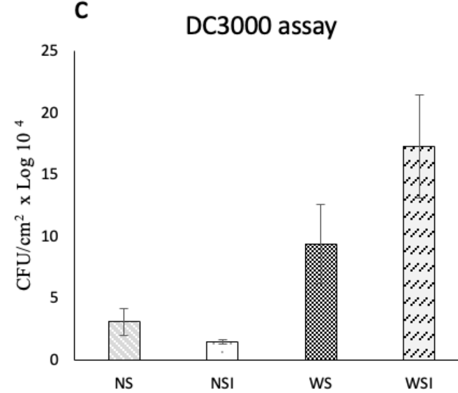


Fig. 16. A- Macro-photograph of *A. thaliana* leaves infiltrated with *P. syringae* DC3000 48 hours after infiltration and two weeks after treatments. B- visualization of pathogen-triggered cell death using confocal laser microscopy under four treatment conditions. A bright green,

fluorescent spot indicates the presence of dead cells in and around the infected area, as indicated by the arrows. The microscopy resolution was kept at 20X magnification. C- Colony Forming Unit (CFU) data of *A. thaliana* leaves infected with *P. syringae* DC3000 strain after two weeks post treatments (AKMP7 inoculation and drought induction) and two days post DC3000 infiltration, plated on King's B agar medium. Each error bar represents the mean ± SE of 3 biological replicates plants from one experiment.

In summary, we found that, *P. putida* AKMP7 causes upregulation of SA biosynthesis in *A. thaliana*, both, under well-watered and water-stress conditions, with highest upregulation seen under water-stressed, inoculated condition. However, the SA signalling genes, *NPR1* and *PR1* were upregulated by the bacterium, only under well-watered conditions, and supressed under water-stress. We also found that *P. putida* AKMP7 can enhance the susceptibility of *A. thaliana* to the model plant pathogen, *P. syringae* DC3000, while, supressing infection under well-watered conditions.

4.4. Discussion:

4.4.1. What could the AKMP7-mediated upregulation of SA levels in *A. thaliana* under water stress, mean for the plants?

The levels of phytohormone SA is highly upregulated in plants undergoing pathogenesis/biotic stress as a defence response (Ding and Ding, 2020; Peng *et al.*, 2021). Also, abiotic stresses like drought and salinity can cause an elevation in the levels of SA in plants (Catinot *et al.*, 2008). Hence, to understand the dynamics of SA perception in plants under non-stressed, inoculated (NSI) conditions (where AKMP7 is beneficial to the plants), and under water-stress inoculated (WSI) conditions (where AKMP7 becomes harmful to the plants), we quantified the levels of SA under our experimental conditions. Apart from this, we have also studied the expression of key SA biosynthesis genes from both *ICS* and *PAL* pathway of SA

biosynthesis. We found that, 2-weeks post-treatments, the SA levels were induced in all treatments (NSI, WS and WSI), as compared to well-watered, non-inoculated controls (NS) (Fig. 11B). Intrestingly, compared to NS, NSI and WS treatments, AKMP7 inoculation under water-stress (WSI) caused the highest accumulation in SA levels. These trends positively correlate with the expression of the SA biosynthetic genes, *ICS1* and *EPS1* (Fig. 12), both belonging to the ICS pathway of SA biosynthesis (Fig. 9). Interestingly, the genes of the other pathway of SA biosynthesis (PAL pathway), i.e. *PAL1*, *PAL2* and *PAL3* were upregulated only under water-stress conditions in both non-inoculated and inoculated seedlings (WS and WSI). Under well-watered conditions, the expression of these genes were not impacted by AKMP7 inoculation (NSI). Interestingly, the expression of another key gene of the PAL pathway, *AIM1* was not induced by water-stress alone. While the expression of *PAL1* was not impacted by AKMP7 inoculation, under water-stress, the same caused significant inductions in the expression of *PAL2,3* and *AIM1*. The combined upregulation of both ICS and PAL pathways by AKMP7 inoculation under drought can be contribuing to the higher SA levels seen in WSI treatment. Our data, thus, corroborates well with the studies mentioned above (Ding and Ding, 2020; Peng *et al.*, 2021) and adds more evidence to the "conditional pathogenicity" of AKMP7 under water-stress. While SA-induced plant immunity helps combat pathogens, it comes at the cost of compromised growth. While it is known that SA levels are elevated under both abiotic and biotic stress conditions, too much SA can severely restrict plant growth (Vanacker *et al.*, 2001; van Butselaar and van den Ackerveken, 2020). In our study, the very high SA levels accumulated under water-stressed, inoculated conditions (Fig. 11) might potentially contribute towards the SA-mediated stunting of plants observed under these conditions (Fig. 15A).

4.4.2. *P. putida* AKMP7 enhances plant immunity under well-watered conditions while suppressing it under water-stress (despite high SA accumulation) by negative regulation of *NPR* genes:

In this study, we tried to understand the regulation of plant immune response during the onset of conditional pathogenesis. We have studied the regulation of SA mediated signalling genes, *NPR1* and *PR1* involved in SAR. As mentioned earlier, accumulation/upregulation of SA leads to activation of these genes (Fu *et al.*, 2012; Ding *et al.*, 2018). As discussed above, we have reported higher levels of SA in all the treatments (NSI, WS and WSI) compared to non-stressed, non-inoculated controls (NS) (Fig. 11). Ideally, similar trends are expected in the expression patterns of *NPR1* and *PR1* genes. As can be seen from Fig. 13, under well-watered inoculated conditions (NSI), the expression of *NPR1* gene was upregulated and this correlates well with high SA levels, obeserved in this treatment (Fig. 11). Similarly, under water-stressed non-inoculated conditions (WS), *NPR1* expression was upregulated, correlating well with high SA levels under water-stress. However, under water-stressed inoculated conditions (WSI), despite higher SA levels, there is a supresssion of *NPR1* expression.

While *NPR1* is primarily involved in plant immune response, the induction in levels of *NPR1* under water-stress, non-inoculated conditions (WS) might be due to the fact that *NPR1* also plays an additional role in combating the negative impacts of oxidative and heat stress in plants (Zavaliev *et al.*, 2020). Hence, *NPR1* induction might also be helping the plants sustain the adverse effects of drought stress. Therefore, supression of *NPR1* under water-stressed, inoculated conditions (WSI) by AKMP7 can potentially contribute to early wilting of plants, compared to WS plants, where *NPR1* is upregulated.

As can be seen from Fig.13, there is an upregulation in *PR1* expression under well-watered inoculated conditions (NSI), correlating well with high *NPR1* levels (Fig. 12). Contrastingly, under water-stress conditons without inoculation (WS), *PR1* gene was downregulated despite higher levels of *NPR1* expression (Fig. 12). A possible reasons for this could be, the plants are downregulating *PR1* to prevent an autoimmune response in the absence of a pathogen. Intrestingly, under water-stress inoculated conditions (WSI), the *PR1* expression was further downregulated when compared to all the other treatments (NS, NSI and WS). Such a downregulation is not seen in *NPR1* under water-stressed, inoculated conditions. In fact, *NPR1* expression is similar under NS and WS+I conditions.

To understand the negative correlation in the expression pattern of the SA signalling genes (*NPR1* and *PR1*) with high SA levels, under water-stressed, inoculated conditions, we studied the expression pattern of *NPR4* (a negative regulator of *NPR1*) and *NPR3* (a negative regulator of *PR1*) (Fu *et al.*, 2012; Ding *et al.*, 2018). As can be seeen from Fig.14, from this study, we have identified a drought-induced upregulation of *NPR3* expression in both water-stressed inoculated (WSI) and non-inoculated seedlings (WS), which correlates with low *PR1* levels. Additionally, there is an upregulation of *NPR4* only under water-stress inoculated conditions (WSI), which correlates with the suppression of *NPR1* expression and further depleted levels of *PR1* under water-stress. We use the term suppression because there is a non-induction of *NPR1* gene expression that negatively correlates with an elevated SA accumulation inside the plant.

While there are a lot of studies on the modulation of host defense during pathogenesis, but to our knowledge, there are no reports on the negative regulation of *NPR1* as a mechanism of pathogenesis by a rhizobacterium. A lot of research in the past decade points towards the importance of SA-mediated *NPR1* induction as a mechanism of plant defense upon pathogenesis to prevent disease progression and mount a successful defense response (Cao *et*

al., 1997; Backer et al., 2019). Apart from defense response, *NPR1* also prevents effector-induced cell death upon infection (caused by hypersensitive response) (Zavaliev *et al.*, 2020). In our previous study, we reported accelerated cell-death in water-stressed, AKMP7 inoculated seedlings, which correlates well with the supressed *NPR1* levels in these plants (Chapter 4; Raja Gopalan et al., 2022). It is known that SA can promote autophagy via *NPR3* and *NPR4* while NPR1 can promote cell survival by preventing the same (Wang *et al.*, 2016, Zavaliev *et al.*, 2020). Based on our current data and evidence from literature, we hypothesize that induction of *NPR1* under water-stress in non-inoculated seedlings might prevent the stress-induced cell damage, while on the contrary, a combined induction of *NPR3* and *NPR4*, thereby suppressing *NPR1* expression, is possibly causing severe autophagy in water-stress AKMP7 inoculated seedlings, leading to an overall devastating effect on plant survival. Also, in our previous study, we reported an accelerated stress-induced cell death in AKMP7 inoculated seedlings under water-stress (Chapter 4; Raja Gopalan et al., 2022). This correlates well with our current findings. As mentioned in the "Introduction", we have previously identified that AKMP7 releases phytohormones such as zeatin, indole acetamide (an auxin derivative), and auxin amino-acid conjugates into the growth medium (Chapter 4; Raja Gopalan et al., 2022), among which, indole-acetamide and trans-zeatin play a crucial role in aiding the severity of drought-specific adverse effects of *P. putida* AKMP7 on *A. thaliana* seedlings. There is a possibility of a correlation between the immune suppression that we have observed in our present study and the adverse impact of these bacterial released chemicals that we have reported in our previous study (Chapter 4; Raja Gopalan et al., 2022). It is possible that immune suppression could be enhancing the toxic impact of these chemicals, or vice-versa. Further studies need to be done to establish the exact correlation between these events.

Hence, based on our previous data from Chapter 4; Raja Gopalan et al., 2022, and current findings, we strongly believe, low *NPR1* levels and high *NPR3/NPR4* expression causes

the enhanced growth deterioration and wilting seen under water-stress conditions in AKMP7 inoculated seedlings. To validate our hypothesis, we have done physiological stuides with *NPR4* homozygous mutants (as AKMP7 induces this gene only under water stress) of *A. thaliana* and the results of which are discussed in detail below.

4.4.3. *NPR4* is required for conditional pathogenesis:

Since *NPR4* is a negative regulator of *NPR1*, we hypothesized that high *NPR4*, which is potentially responsible for low *NPR1* in water-stressed, inoculated plants is responsible for conditional pathogenesis. Hence, we performed physiological studies with *NPR4* knock-out mutants of *A. thaliana*. These mutants also constitutively express high *NPR1* and *PR1* (Zhang *et al.*, 2006; Fu *et al.*, 2012). Physiological studies with *A. thaliana (NPR4)* mutants reveal that the drought-specific adverse effects exhibited by *P. putida* AKMP7 treatment were not observed after 2 weeks of treatments, and the inoculated seedlings do not appear different from water-stress non-inoculated seedlings (Fig.14). Also, a slight but not statistically s
ignificant growth enhancement was observed in water-stress inoculated seedlings. In the case of well-watered inoculated seedlings, no distinctive growth promotion was observed, as seen in wild type, seedlings under the same conditions after 2 weeks of inoculation. This might be due to slow and delayed growth exhibited by *NPR4* mutant seedlings (possibly due to constitutive immune gene expression) compared to wild type seedlings.

4.4.4. A possibility of enhanced disease susceptibility towards other pathogens cannot be ruled out in conditional pathogenesis:

Our results from defense gene expression showed an alarmingly low expression of important genes involved in plant defense. Several studies show that plants with compromised/low expression of *NPR1* and *PR1* were more susceptible to a wide range of pathogens (Johansson et al., 2006; Stein *et al.*, 2008). Concerningly, *P. putida* AKMP7 inoculated seedlings (that exhibit low expression of both *NPR1* and *PR1* under water stress

conditions) and, as evidenced by our preliminary study (Fig. 15), exhibit differential susceptibility (NSI-resistance vs WSI-susceptibility) to the model pathogen, *P. syringae* DC3000. This indicates that conditional pathogenesis could turn the tide against plants and favour other opportunistic plant pathogens, leading to an overall devasting effect on plant growth and survival. More research is needed to confirm the negative effects of *P. putida* AKMP7-mediated compromise in plant immunity.

4.4.5. A call for caution for PGPR application for plants:

From an agronomical point of view, *P. putida* AKMP7 is a rhizobacterium isolated from maize rhizosphere, and it's proven to be beneficial to wheat, and it can ameliorate the adverse effects of heat stress (Zulfikar Ali *et al.*, 2011). Our studies with the same bacterium in the model plant *A. thaliana* prove to be otherwise. Over the years, much information has been uncovered on mechanisms by which PGPR positively regulate plant growth and immune/stress response, leading to better growth and stress tolerance in plants (Mantelin and Touraine, 2003; Beneduzi et al.,2012). What is not known is whether the same bacteria provide the same kind of benefits under all environmental conditions or in all host plants they associate themselves with. The potential of a PGPR to become detrimental to the host plant due to changes in the environment or due to incompatibility is not well studied. In our previous studies, we have identified one such instance, where the generally beneficial plant-associated rhizobacterium, *P. putida* AKMP7, causes severe drought-specific growth retardation when inoculated onto *A. thaliana* seedlings under water-stress conditions (Chapter 4; Raja Gopalan et al., 2022). Hence, based on our current observation and previous studies, we suggest/highlight that the beneficial aspect of any plant-microbe relation can be purely contextual and very specific/limited to a particular type of host or environment. Also, it may not always translate well under all environmental conditions or with all host plants in the field. What's even worse is that when there is a change in the host or environmental conditions, there is a possibility that the

application of a seemingly harmless and beneficial microbe can turn harmful/pathogenic. Even though positive outcomes are expected, and negative impacts of PGPR can be very rare, we strongly advocate exercising caution and thoroughly studying the behavior of a PGPR strain under varying conditions (to avoid unexpected losses in yield) before large-scale application in the field for commercial benefits. Through our current study, we have identified some of the important molecular mechanisms involved in the conditional pathogenesis of the PGPR strain *P. putida* AKMP7, when inoculated onto *A. thaliana* seedlings under drought/water-stress conditions. While this study focusses on certain aspects of the molecular mechanisms leading to conditional pathogenesis, further studies need to be done to get a comprehensive understanding of this phenomenon and its universality. The possibility of involvement of other phytohormones such as ethylene and JA and associated signaling pathways can be explored to get a deeper understanding of this phenomenon of conditional pathogenesis. Moreover, similar studies need to be done using other rhizobacterial-plant combinations to establish common mechanisms/pathways involved in conditional/opportunistic pathogenesis of seemingly beneficial microbes.

4.5. Conclusions from chapter 4: Based on our study we can conclude that, *P. putida* AKMP7:

- Causes conditional pathogenesis in *A. thaliana* only under water-stress, by downregulating SA-mediated immunity within the plant.
- Upregulates SA biosynthesis in *A. thaliana*, both, under well-watered and water-stressed conditions, with greater upregulation of SA under water-stress.
- Induces the expression of the SA-mediated defence signalling genes, *NPR1* and *PR1* under well-watered conditions, while, suppressing the expression of the same, under water-stress, in *A. thaliana* seedlings.
- Can cause enhanced disease susceptibility of *A. thaliana* to other pathogens only under water-stress.

Chapter 5

Functional Genome Analysis of *P. putida* AKMP7.

5.1. Introduction:

To understand the phenomenon of conditional pathogenesis from a genomics perspective, we have performed whole genome sequencing (WGS) and functional genome annotation of *P. putida* AKMP7. Our study aims to address important gaps in understanding the conditional/opportunistic pathogenesis of AKMP7 using a genomics approach. We have submitted our genome sequencing results to the GenBank database which can be accessed using the accession ID: CP124529. We have performed a comparative genome analysis of AKMP7 with some other *P. putida* strains (30 different strains of clinical, environmental and plant origin) using average nucleotide identity (ANI) analysis to identify closer relatives of this strain. Finally, we have analyzed some of the genes involved in bacterial virulence, xenobiotic resistance, phytohormone production etc., and some genes that impart stress tolerance and growth promotion to plants. WGS was performed by MiBiome Therapeutics LLP, Mumbai, India.

5.2. Materials and methods:

5.2.1. Bacterial Genomic DNA isolation:

A glycerol stock of AKMP7 was used to streak a Petri plate containing cetrimide agar medium and incubated overnight at 28° C. Following incubation, a single colony of AKMP7, was revived in 50 mL of Luria Bertani (LB) broth and grown overnight in a shaking incubator at 28° C and shaken at 120 RPM. 2 mL of overnight culture was subcultured into LB broth media and grown to an OD_{600nm} of 0.8. Genomic DNA isolation was performed from this culture using Machery-Nagel Nucleospin ® Microbial DNA kit. Bacterial DNA was extracted from 30 mg (wet weight) of a pellet of the microbial cell culture described above. Highly pure DNA was eluted using elution buffer provided in the kit and the collected DNA was used for further analysis.

5.2.2. DNA integrity check

The extracted genomic DNA was quantitated using a NanoDrop (Thermo Fisher Scientific) by measuring the absorbance at 260 nm and a Qubit3 fluorometer (Thermo Fisher Scientific, USA) using the Qubit dsDNA HS Assay Kit. Subsequently, the quality of extracted genomic DNA was assessed by subjecting the isolated DNA sample to 1% agarose gel electrophoresis and a genomic DNA screentape analysis on Tapestation 4200 (Agilent Technologies, USA). The results obtained are provided in the supplementary data (*Appendix 3:* Supplementary Fig. 14 and Supplementary Table. 2).

5.2.3. Whole genome sequencing and functional annotation

The bacterial DNA sample was subjected to whole genome sequencing using Illumina and Oxford Nanopore Platforms (ONT). Illumina Novaseq 6000 was used to sequence paired end short reads at a coverage of 2065X, while single end long read sequencing was performed on ONT MinION platform at a coverage of 40X. The hybrid de-novo assembler of CLC Genomics Workbench (version 21.0) was used to assemble the genome using both the short and long reads. The functional annotation of the assembled genome was performed using "Rapid Annotation using Subsystem Technology (RAST)" annotation pipeline (https://rast.nmpdr.org/rast.cgi) and Pathosystems Resource Integration Center-Bioinformatics Resource Centre (PATRIC-BRC) annotation pipeline (Appendix 3: Supplementary data sheet 1) (https://www.bv-brc.org/) as described by Aziz et al. (2008) and Wattam AR et al. (2017).

5.2.4. Average nucleotide identity and comparative genome analysis

Bacterial genome similarity was calculated from the average nucleotide identity parameter using JSpecies Web Server, by comparing the AKMP7 genome against the reference genome database as described by Richter et al. (2016). The comparative functional genome

analysis was performed by comparing the *P. putida* AKMP7 genome against the genomes of reference microbes using the comparative genome tools provided in RAST and PATRIC-BRC annotation pipelines.

5.3. Results:

5.3.1. General features of *P. putida* AKMP7 genome:

From the whole genome sequencing data, as can be seen in Table 4, we have identified that the total genome length of *P. putida* AKMP7 is around 5764016 base pairs, and the GC content of the genome is 62.93%, which is typical for *P. putida* strains. As can be seen from Fig. 17, using PATRIC-BRC genome annotation database, we have identified 5355 protein-coding genes based on PATRIC cross-genus family (PGfam) assignments and 5305 protein-coding genes with PATRIC genus-specific family (PLfam) assignments. We have also identified 1865 genes with subsystem assignment, 965 genes with metabolic pathway assignments, 1085 protein-coding genes with Gene Ontology (GO) assignment and 1267 genes with Enzyme Classification (EC) number assignments. Overall, from the PATRIC -RC functional annotation pipeline, we have identified 4486 functional protein-coding genes and 1119 hypothetical protein-coding genes. Similarly, as shown in Table 5, based on the RAST annotation pipeline, we have identified that the genome has 5605 coding sequences, 80 repeat regions, 71 tRNA genes and 22 rRNA genes. This analysis has identified 4487 functional proteins and 1118 hypothetical proteins. Also, as shown in Fig. 18, phylogenetic tree analysis classified this strain under *P. putida* species (with a P value of- 0.03).

Table. 4: Genome assembly data

Contigs	1
GC Content	62.93%
Plasmids	0
Genome Length	5,764,016 bp
N50	5,764,016 bp

Table. 5: Genome annotation features

CDS	5605
Repeat Regions	80
tRNA	71
rRNA	22
Partial CDS	0
Miscellaneous RNA	0

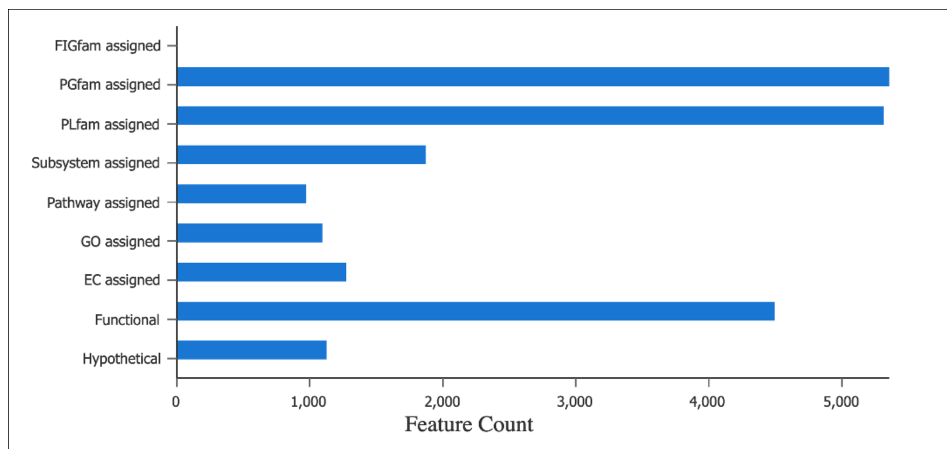


Fig. 17. General features of *P. putida* AKMP7 obtained from PATRIC-BRC annotation pipeline.

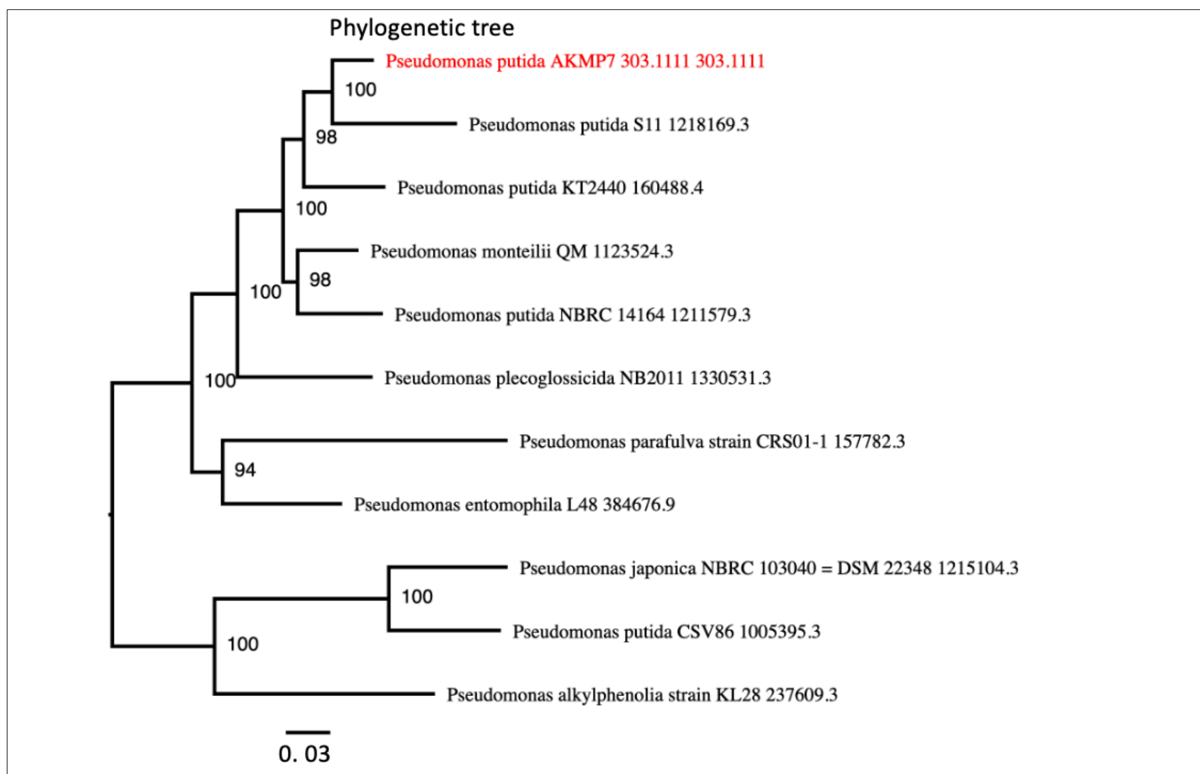


Fig. 18. Phylogenetic tree based on *P. putida* AKMP7 genome and taxonomic position of AKMP7 with closely related genus.

5.3.2. Subsystem features of *P. putida* AKMP7 genome:

As shown in Fig. 19, we have identified different genes in the AKMP7 genome for various cellular processes like cell wall biosynthesis, DNA metabolism, protein metabolism, stress tolerance, virulence factors, antibiotic resistance, etc. The distribution of these genes in the genome is represented as a pie chart. The genomic dendrogram in Fig. 20, represents the location and position of key genomic sequences in the AKMP7 genome.

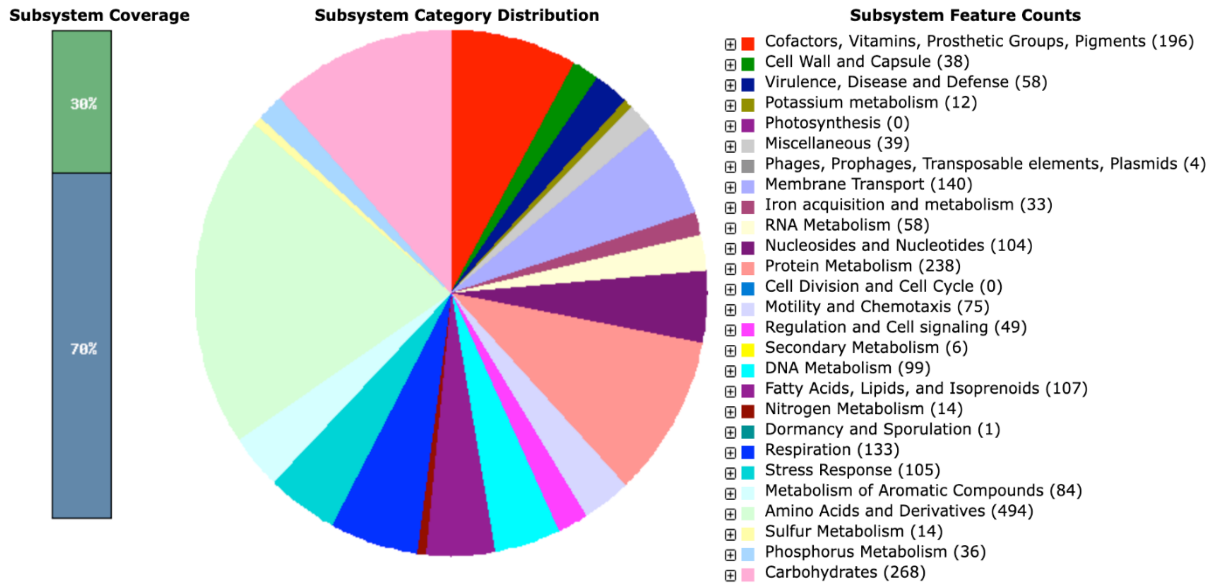


Fig. 19: Distribution of genomic subsystem features of *P. putida* AKMP7 genome.

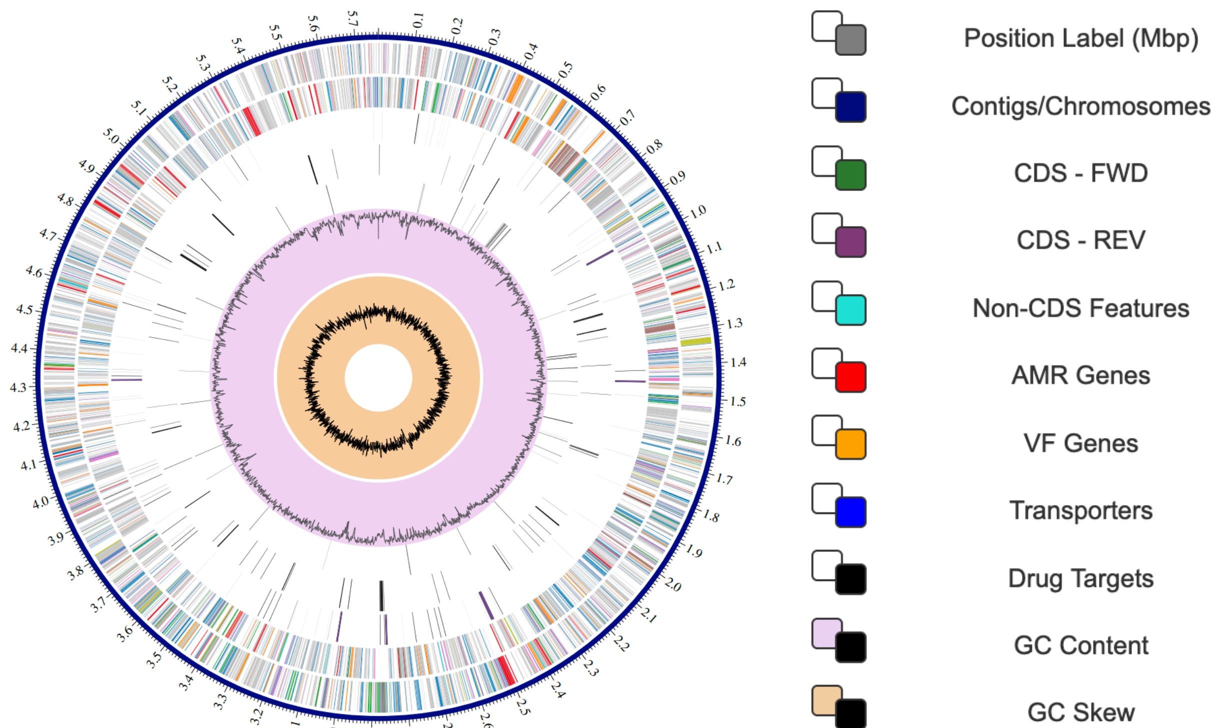


Fig. 20. Representative genomic dendrograms of *P. putida* AKMP7 highlighting key genome features and position of different genomic regions of interest. Abbreviations: CDS-FWD stands

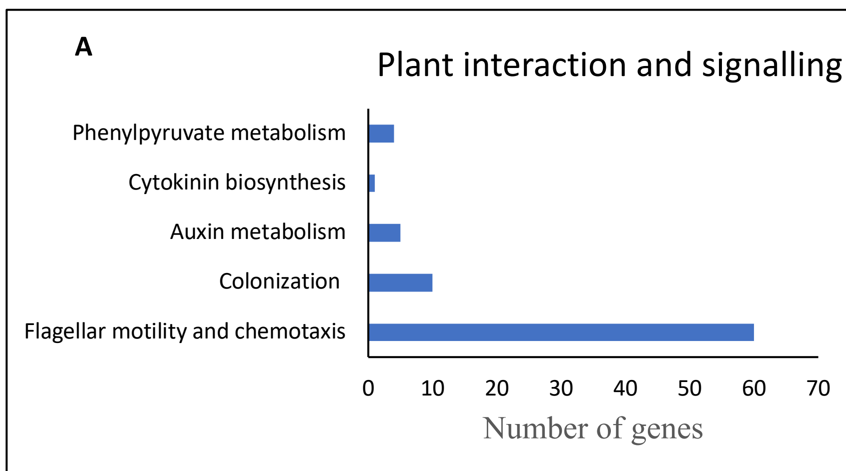
for coding sequences forward, CDS-REV stands for coding sequence reverse, AMR stands for antimicrobial resistance, VF stands for virulence factors, and Mbp stands for megabase pairs.

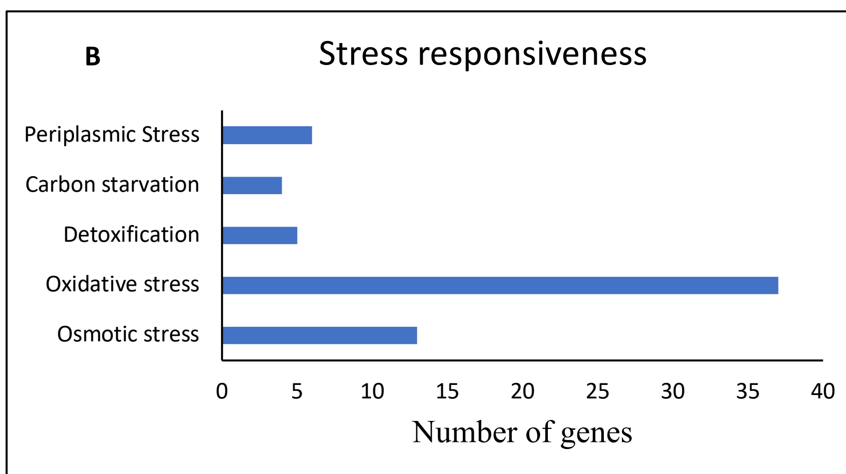
5.3.3. Functional genome analysis of key genes involved in plant-microbe interactions:

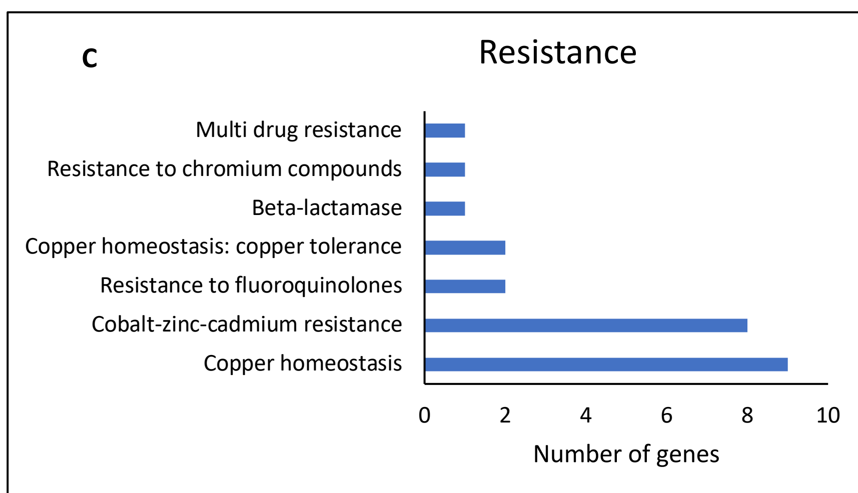
As can be seen in Fig. 21A, from the annotated genome of AKMP7, we have identified different genes involved in plant-microbe interactions, such as those involved in phenylpyruvate metabolism, phytohormone biosynthesis (auxin derivatives and cytokinin metabolism), root colonization and chemotaxis. We have identified a tryptophan synthase gene, responsible for tryptophan biosynthesis from serine and indole-3-glycerol phosphate. We have also identified an important gene called aspartyl-tRNA (Asn) amidotransferase subunit A or indole-acetamide hydrolase, which converts tryptophan into indole acetamide. We have previously reported that the bacterium produces indole acetamide and trans-zeatin in relatively high concentrations under water stress (Chapter 4; Raja Gopalan et al., 2022). In the same publication, we have described how these chemicals aid in the conditional pathogenesis of *P. putida* AKMP7 in *A. thaliana*, potentially by enhancing the bacterium's virulence under water stress. Based on RAST annotation, we have identified a tRNA dimethyl transferase gene responsible for the biosynthesis of phytohormone zeatin. For efficient root colonization, the bacterium possesses a widespread colonization island with Type-2/4-Secretion System (T2SS/T4SS) apparatus that aids in adherence to host cell and colonization (i.e., Tight adherence locus A, B and C genes involved in bacterial pilus assembly) (Tomich et al., 2007).

AKMP7 can grow under osmotic stress, colonize the root, and survive under water-deprived conditions (Shah et al., 2017; Raja Gopalan et al., 2022). As shown in Fig. 21B, the bacterium possesses stress-responsive genes that mitigate oxidative stress, water deprivation and osmotic stress. Also, this bacterium possesses genes responsible for survival under carbon

starvation. As can be seen from Fig. 21C, we have identified some important genes conferring resistance to antibiotics, which correlates well with the fact that this bacterium is resistant to antibiotics/antimicrobials such as rifampicin and cetrimide. Apart from genes required for survival, we have also identified genes involved in plant growth promotion. As mentioned above, this bacterium can significantly promote growth in *A. thaliana* seedlings under well-watered conditions and increases the overall plant biomass compared to non-inoculated seedlings. As can be seen from Fig. 21D, the plant growth promotion ability can be attributed to the ability of this bacterium to solubilize inorganic phosphate present in the soil, enhance iron uptake in roots and nitrogen fixation.







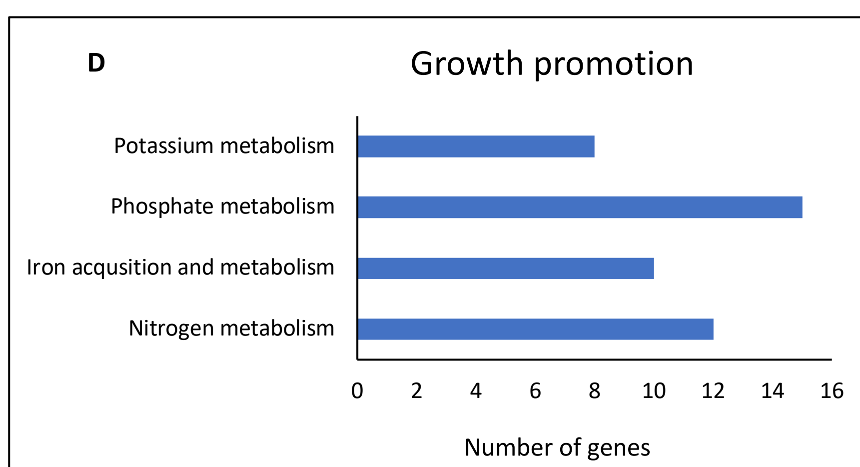


Fig. 21: An overview of important genes in *P. putida* AKMP7 genome involved in A. plant interactions and signaling, B. stress responsiveness to different stress factors, C. resistance to antibiotics and heavy metals and D. Plant growth promotion.

5.3.4. Comparative functional genome analysis of *P. putida* AKMP7 with non-pathogenic *P. putida* strains and pathogenic rhizobacterial strains:

As can be seen from Fig. 22A-D, we compared the genome of AKMP7 with other harmless *P. putida* strains KT2440, W619, F1 and GBI based on functional genes present in the genome. From that, we identified AKMP7 shares 1752 functional genes with KT2440 and

has 111 functional genes which are not present in KT24440 genome. Similarly, AKMP7 shares 1615, 1622 and 1599 genes with W619, F1 and GB1 strains. AKMP7 also has 122, 132 and 113 functional genes which are not present in W619, F1 and GB1 strains. As can be seen from Fig. 21 E-H, we compared the genome of AKMP7 with pathogenic rhizobacterial strains such as *P. aeruginosa* PA14, PA7 and PAO1 and *Agrobacterium tumefaciens* GV3101. From that we identified AKMP7 shares 1545, 1548 and 1674 functional genes with PA14, PA7 and PAO1 strains. It also shares, 1202 functional genes with *Agrobacterium tumefaciens* GV3101. AKMP7 also has 182, 178 and 174 genes absent in PA14, PA7 and PAO1 strains. Similarly, AKMP7 has 503 functional genes absent in GV301. From the virulence factors analyzed, we identified AKMP7 shares a functional gene encoding Type-4-secretion system (T4SS) with conjugative transfer functionality with all pathogenic rhizobacterial strains we have analyzed. Interestingly, the T4SS encoding gene is absent in all the beneficial/harmless *P. putida* strains we have analyzed.

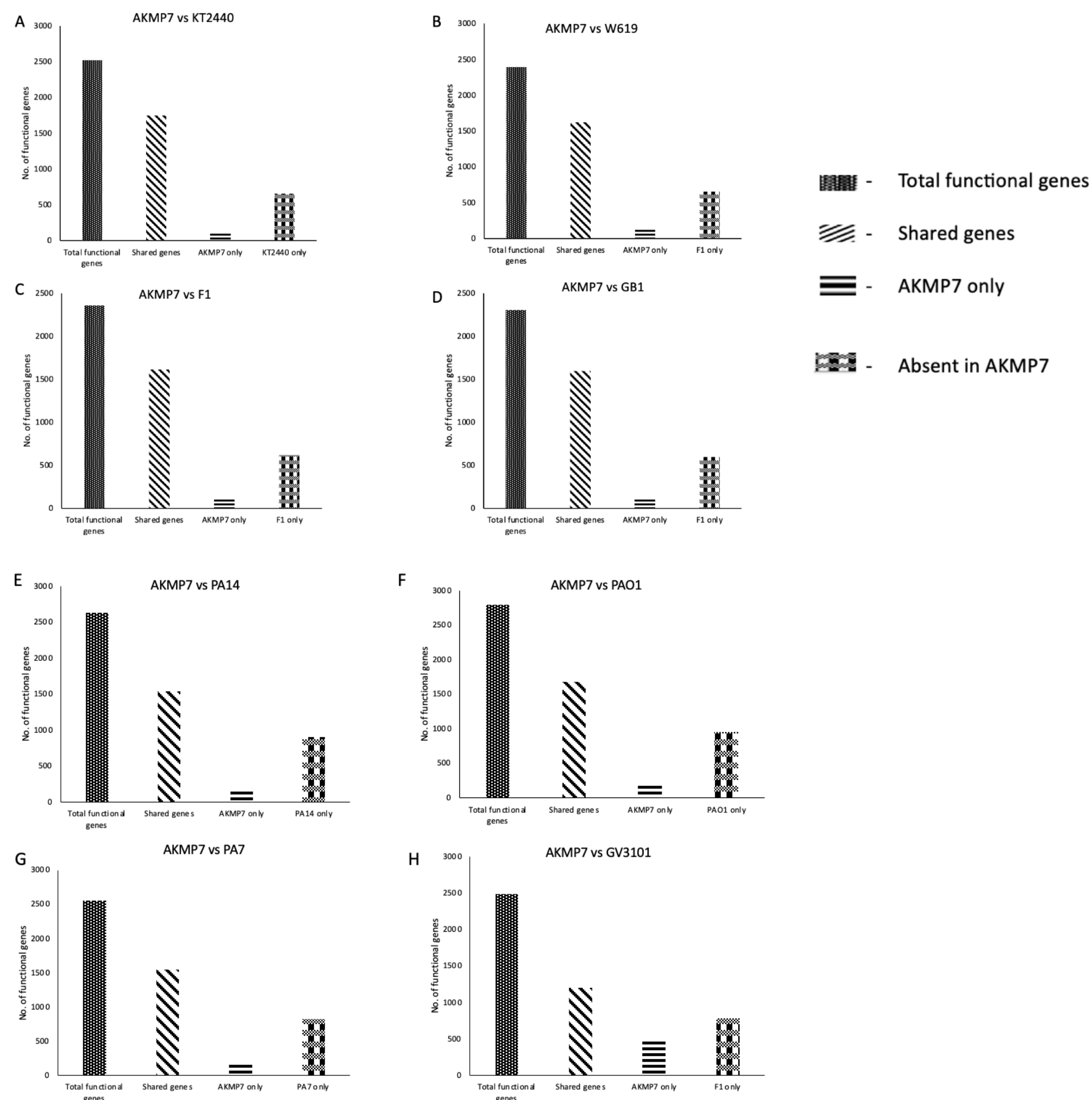


Fig. 22: Comparative functional genome analysis: Panels A-D represents a functional gene feature comparison of non-pathogenic/beneficial *P. putida* strains vs conditionally pathogenic *P. putida* AKMP7 and panels E-H represents a functional feature comparison of pathogenic rhizobacterial strains vs *P. putida,* AKMP7.

5.3.5. Comparative genomics based on Average Nucleotide Identity and functional gene features:

We performed a comparative Average Nucleotide Identity (ANI) analysis of *P. putida* strains and other soil, environmental and clinical isolates from *Pseudomonas* spp., closely

related to *P. putida* AKMP7 and found that the conditionally pathogenic rhizobacterium has a high ANI with *P. putida* CP9, a pathogenic strain of *P. putida* isolated from a clinical sample (*Appendix 3:* Supplementary Table. 3). The *P. putida* CP9 can infect insects, mouse, and mammalian cells (Fernández et al., 2015). This indicates that pathogenic *P. putida* strains might have evolved from a common ancestor and adapted to infect different hosts. Initially, to identify the virulence factor responsible for *P. putida* AKMP7 mediated conditional pathogenesis in *A. thaliana* seedlings under water stress, we have performed comparative genomics with harmless *P. putida* strains to screen additional genes present in AKMP7 genome that is possibly contributing to its virulence. From that, we have identified, *P. putida* AKMP7 possesses a Type-IV Secretion System (T4SS), absent in the other four nonpathogenic strains we analyzed (Fig. 22 A-D, and *Appendix 3:* Supplementary data sheet 2). T4SS system is a protein machinery/virulence factor involved in conjugative transfer of genetic material in prokaryotes. Apart from conjugation, it is involved in transfer of proteins and effectors into the host cell, and it has also been shown to be important in pathogenesis of many clinical pathogens (Costa et al., 2021). To elucidate the importance of T4SS in conditional pathogenesis of AKMP7, we have done comparative genomics with other pathogenic strains from *Pseudomonas* spp. and identified that all the four pathogenic strains we have analyzed possess T4SS. Two strains were pathogenic rhizobacteria, and the other two were clinically pathogenic *P. putida* strains. The T4SS in the *P. putida* AKMP7 genome has a conjugative transfer functionality. This virulence factor might have been transferred to the *P. putida* AKMP7 genome through the exchange of genetic material via conjugative bacterial gene transfer from a pathogenic *Pseudomonas* spp., in the rhizosphere.

5.4. Discussion:

5.4.1. Genome features, phylogeny, and functional annotation of *P. putida* AKMP7 genome

As mentioned in the results above, from the whole genome sequence of *P. putida* AKMP7, we have identified important protein coding regions and hypothetical protein coding regions in the AKMP7 genome. We have also identified number of tRNA and rRNA genes in the AKMP7 genome. As can be seen from Fig. 20, we have identified the position of different regions in AKMP7 genome such as CDS, genomic regions contributing to bacterial virulence and antibiotic resistance and regions encoding cellular transporters. From the phylogenetic analysis of AKMP7 genome, we have confirmed that AKMP7 belongs to the *P. putida* species. We have also identified close relatives of this strain, such as *P. putida* S11, *P. putida* KT2440 and *P. monteilii* QM 1123524. As can be seen from Fig. 19 and 21, through functional genome annotation of AKMP7, we have identified the distribution of essential genes present in the AMKP7 genome that are involved in different aspects of biological metabolism, growth, virulence, and survival.

As mentioned earlier, we have reported that the conditionally pathogenic rhizobacterium AKMP7 is able to promote plant growth under well-watered conditions (Chapter 4; Raja Gopalan et al., 2022). This ability of the bacterium can be attributed to the presence of important genes involved in nitrogen fixation, phosphate solubilization and phytohormone production. We have also reported that the bacterium is able to colonize the plant roots well and grow well under water-stress conditions which could be attributed to the presence of a "widespread colonization island" present in the AKMP7 genome with a Type-2 Secretion System (T2SS) apparatus. The ability of the bacterium to withstand severe water-stress can be attributed to the presence of osmoregulatory genes such as aquaporin Z and glycerol uptake facilitator protein. The bacterium also possesses genes such as cytochrome

c551 peroxidase and superoxide dismutase [Fe], involved in protection of cellular membrane damage caused by oxidative stress. The presence of important genes involved in biosynthesis and metabolism of osmoregulatory compounds such as glycine-betaine and glutathione, also helps the bacterium in stress tolerance. Apart from genes involved in plant growth and stress tolerance, we have identified several genes involved in bacterial virulence and pathogenicity (discussed in detail below). The presence of these genes might be contributing to the conditional pathogenesis caused by AKMP7 under water stress in *A. thaliana*.

5.4.2. Comparative genome analysis of *P. putida* AKMP7 genome

Since *P. putida* strains in general are non-pathogenic in nature, we compared the AKMP7 genome with 4 other harmless *P. putida* strains (Fig. 22 A-D) to understand the unique features of the AKMP7 genome and to correlate them with its unusual behavior. We have identified that these strains have many similarities such as genes encoding osmotic stress tolerance, phytohormone production and phosphate solubilization etc. We have also compared the genome of AKMP7 with other pathogenic rhizobacterial strains from closely related species to identify potential virulence factors present in AKMP7 (Fig. 22 E-H). From this study, we have identified that AKMP7 possess an unusual set of genes encoding T4SS with conjugative transfer ability. The T4SS apparatus was found to be absent in all the 4 harmless reference strains of *P. putida* that we analyzed, including the widely studied strain KT2440. However, we have identified that T4SS is present in all the four reference pathogenic strains that we have analyzed. While more emphasis is given to Type-3 Secretions System (T3SS) in pathogenesis, there are also some reports on clinical pathogenesis of a T3SS negative strain *P. aeruginosa* PA7 (Elsen et al., 2014). Interestingly, from our analysis we have identified that PA7 strains also possess a T4SS functionality in its genome. From this we hypothesize that, T4SS present in AKMP7 genome might potentially be contributing to its virulence/conditional pathogenesis.

Further studies need to be carried out to identify the precise role of T4SS in AKMP7 mediated conditional pathogenesis.

5.4.3. T4SS, a possible contributor of virulence in pathogenic rhizobacteria/microbes

Based on our preliminary results, comparative analysis of virulence factors among different plant-pathogenic rhizobacteria and clinically pathogenic microbes revealed that all these microbes have T4SS has a common feature in their genome. While all these microbes are, in general, pathogenic in nature, they infect different hosts such as plants, insects, and human cell lines. Based on these facts, it can be hypothesized that, many pathogenic microbes, irrespective of the nature of their hosts can cause pathogenesis through a T4SS mediated virulence machinery. Another interesting aspect of this analysis is that the non-pathogenic microbes that we analyzed didn't have the T4SS feature in their genome. This further highlights the fact that T4SS can be perceived as a potential marker for pathogenesis in otherwise beneficial soil microbes. Hence, apart from identifying the potential virulence factors in conditional pathogenesis of AKMP7, our study also throws a light on a possible genomic marker for pathogenesis in plant-associated rhizobacteria.

5.5. Conclusions from chapter 5:

Our data presented in this study, helps in decoding the molecular mechanisms of conditional pathogenesis caused by *P. putida* AKMP7, from a genomics perspective. Overall, from this study, we have identified:

1) Key functional genes of the conditionally pathogenic rhizobacterium *P. putida* AKMP7 involved in various processes such as phytohormone production, plant growth promotion, virulence, and stress tolerance.

2) Common genome features between AKMP7 and some non-pathogenic as well as pathogenic rhizobacterial strains
3) A T4SS gene cluster in AKMP7, which could be responsible for its conditionally pathogenic behavior.
4) Presence of T4SS can be perceived as a genomic marker for pathogenesis in some seemingly harmless rhizobacterial strains (i.e., such as AKMP7), certain pathogenic rhizobacteria (i.e., such as PA7) and clinical pathogens (i.e., such as PC9).

Summary and conclusions:

In this study, we have probed into the mechanisms involved in the unique phenomenon of conditional pathogenesis, in which, an abiotic stress tolerant, beneficial soil microbe, *P. putida* AKMP7, promotes plant growth under well-watered conditions in *A. thaliana* seedlings while deteriorating under water-stress conditions. To understand the molecular mechanisms underlying this unusual phenomenon of conditional pathogenesis, we have studied the role of bacterial phytohormones (and their derivatives) in this interaction. Apart from this, we have studied the regulation of plant immune response during conditional pathogenesis. We have also performed functional and comparative analyses of the AKMP7 genome to identify important genomic markers related to conditional pathogenesis. To summarize, through the proposed objectives of this research, we have identified several important molecular cues leading to conditional pathogenesis of a beneficial, abiotic stress-tolerant rhizobacterium, *P. putida* AKMP7 on *A. thaliana*, under water-stress conditions. The objective-wise conclusions from this study are provided in details below.

Objective 1: To understand the role of bacterial phytohormones in conditional pathogenesis by *P. putida* AKMP7 on *A. thaliana*.

From a comprehensive screening of phytohormones in AKMP7 cell-free extracts, we have identified that AKMP7 releases phytohormone trans-zeatin and phytohormone derivatives such as indole-acetamide, indole-acetyl-L-alanine, indole-acetyl-L-phenylalanine, and indole-acetyl-L-aspartate into the growth media under both non-stressed and osmotic stressed conditions. Among the phytohormones and derivatives released by AKMP7, auxin derivative indole-acetamide and phytohormone trans-zeatin play a crucial role in aiding the drought-specific conditional pathogenesis exhibited by AKMP7 under water-stress conditions. Apart from the detection/quantification of phytohormones in AKMP7, our improvised method

can also be used for the detection of major phytohormones such as IAA, ABA, GA, SA and tZ in various microbial extracts of bacterial and fungal origin. This will help study the role of all these major phytohormones in plant-microbe interactions.

Objective 2: Mechanistic insights into the modulation of *A. thaliana* immune-response by *P. putida* AKMP7.

In this objective, we have studied the regulation of plant-immune response by AKMP7 during the onset of conditional pathogenesis. Based on the results from our study, we have identified that AKMP7 differentially regulates plant immune response to cause conditional pathogenesis. Analysis of immune-responsive phytohormone salicylic acid revealed that AKMP7 upregulates SA biosynthesis in *A. thaliana*, both under well-watered and water-stressed conditions, with greater upregulation of SA under water stress. However, AKMP7 induces the expression of the SA-mediated defence signalling genes, *NPR1* and *PR1,* under well-watered conditions while, suppressing the expression of the same, under water stress, in *A. thaliana* seedlings. The differential regulation of plant immune response by AKMP7 can cause enhanced disease susceptibility of *A. thaliana* to other pathogens only under water stress.

Objective 3: Functional genome analysis of *P. putida* AKMP7 genome.

Our data presented in this study helps in decoding the molecular mechanisms of conditional pathogenesis caused by *P. putida* AKMP7 from a genomics perspective. Overall, from this study, we have identified key functional genes of the conditionally pathogenic rhizobacterium *P. putida* AKMP7 involved in various processes such as phytohormone production, plant growth promotion, virulence, stress tolerance, etc. We have also identified that AKMP7 shares many common genome features with some non-pathogenic as well as pathogenic rhizobacterial strains. From the comparative genome analysis, we have identified that AKMP7 possess a T4SS gene cluster, which is absent in non-pathogenic *Pseudomonas*

strains but present in pathogenic rhizobacterial strains. The presence of the T4SS virulence factor as a genomic feature could be responsible for its conditionally pathogenic behavior under water stress. Additionally, the presence of T4SS can be perceived as a genomic marker for pathogenesis in some seemingly harmless rhizobacterial strains (i.e., such as AKMP7), certain pathogenic rhizobacteria (i.e., such as PA7) and clinical pathogens (i.e., such as PC9).

Overall, our work lays a solid foundation for further understanding the conditional pathogenesis phenomenon in greater detail.

Specific contributions:

- First report on the mechanisms of conditional pathogenesis in plant-rhizobacterial interaction
- First report on the presence of auxin conjugates indole-acetyl-L-alanine and indole-acetyl-L-phenylalanine in microbial extract
- A simple, cost-effective method for extracting and quantifying major phytohormones was developed.
- First report on the role of auxin conjugate indole-acetamide and phytohormone trans-zeatin on conditional pathogenesis of beneficial microbe under adverse environmental conditions
- First report on differential regulation of salicylic acid-mediated plant-immune response leading to conditional pathogenesis of beneficial microbes.
- First report on the presence of Type-4-secretion system (T4SS) as a shared genomic feature among pathogenic pseudomonas of plant and clinical origins that distinguishes them from non-pathogenic strains.

Future scope of work:

Our work lays a solid foundation in understanding the precise molecular mechanisms involved in conditional pathogenesis of a beneficial rhizobacterium *P. putida* AKMP7 under adverse environmental conditions.

- The precise molecular mechanisms by which phytohormones produced by *P. putida* AKMP7 affect plant growth differentially under well-watered and water-stress conditions can be further studied.
- The in-depth molecular mechanisms by which *P. putida* AKMP7 suppresses plant immune response can be further studied.
- The role of virulence factors such as T4SS in the conditional pathogenesis of AKMP7 can be further elucidated.
- The phenomenon of conditional pathogenesis can be further extended and tested in other crops and bacterial systems.

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APPENDICES

Appendix 1:

Supplementary data for Chapter 4:

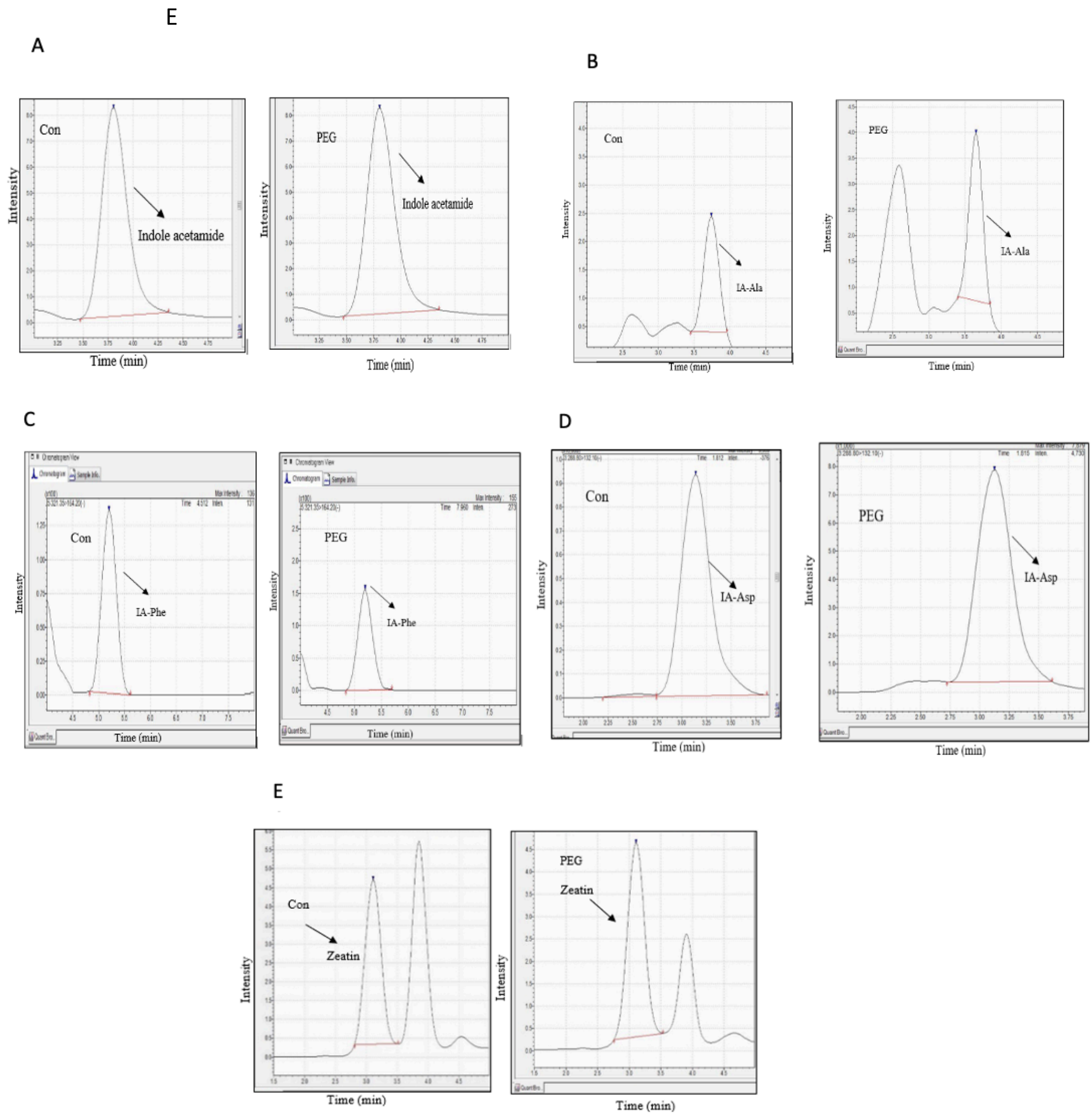
Supplementary data:

Method View - Compound Table

Integration | Identification | Quantitative | Compound | Group | Performance

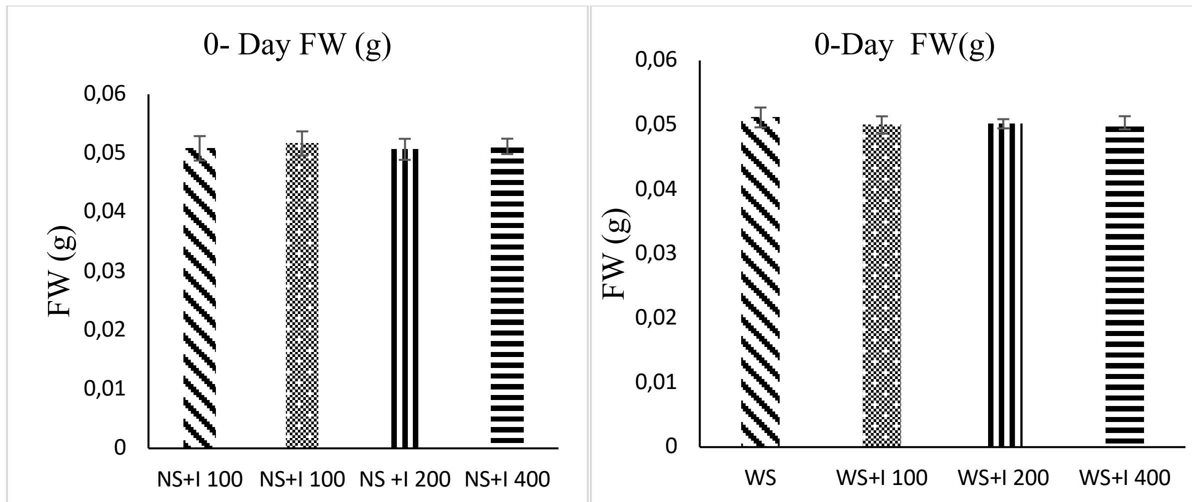
ID#	Name	Type	m/z	Ret. Time	Conc.(1)
1	IA ala	Target	245.25>88.20	3.91	0
2	Zeatin	Target	220.00>202.10	3.62	0
3	Chlorampheni	ISTD	321.05>152.05	5.12	0
4	IA PHE	Target	321.35>164.20	5.15	0
5	IA ASP	Target	288.80>132.10	3.17	0
6	IA	Target	175.10>130.10	3.80	

Supplementary Fig. 1: Optimized MRM ion compund table obtained from commercially obtained phytohormone standards.



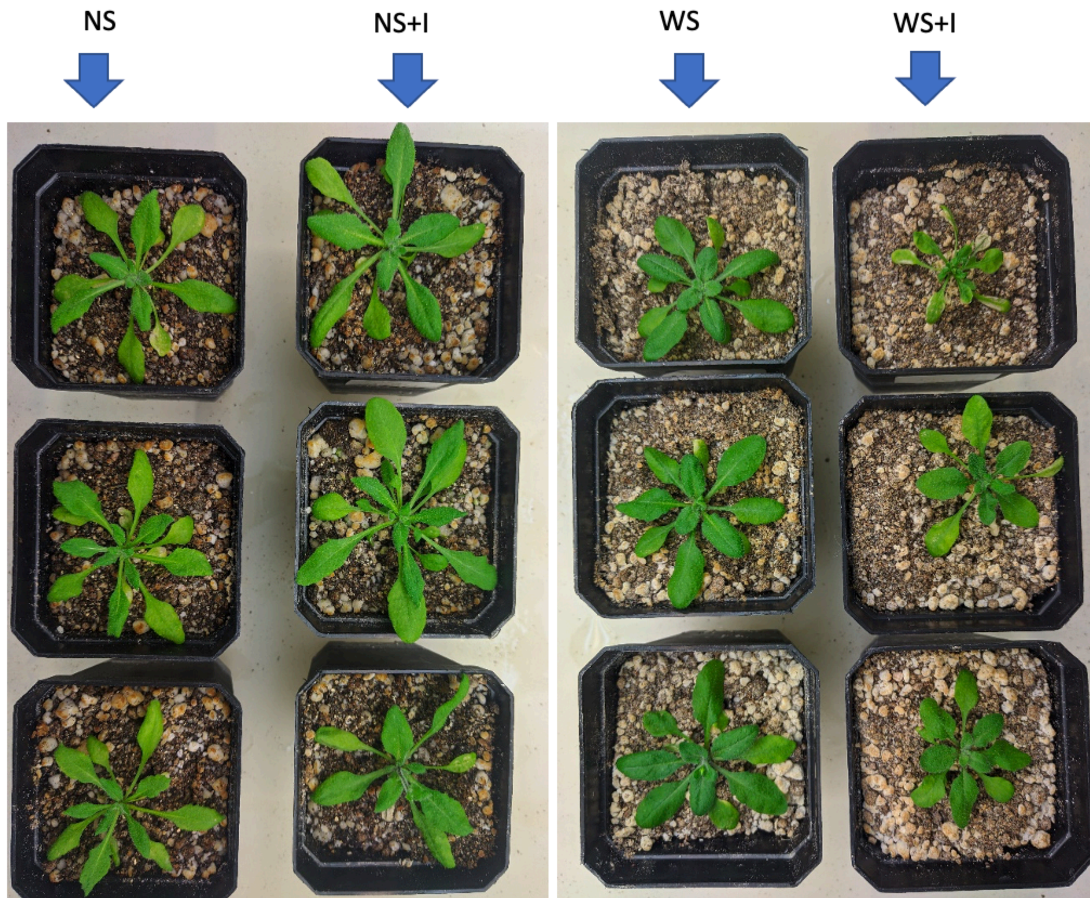
Supplementary Fig. 2: Representative MRM chromatograms of auxin derivatives (A: IA, B: IA- Ala, C: IA-Phe, D: IA-Asp) and (E) Zeatin in Con and PEG samples of *P. putida* AKMP7.

Supplementary Fig. 3: Representative picture of three weeks old *Arabidopsis* seedlings just before inoculation and drought induction (day-0).



Supplementary Fig. 4: Representation of whole seedling fresh weight of *A. thaliana* seedlings prior to inoculation with *P. putida* AKMP7. Each bar represents mean ± S.E. of 6 replicate pots from 2 experiments.

Supplementary Fig. 5: Representative picture of two weeks old *Arabidopsis* seedlings used for phytohormone co-treatment studies just before inoculation and drought induction (day-0).



Supplementary Fig. 6: Representative image of *A. thaliana* seedlings inoculated with of 400 µL volume of *P. putida* AKMP7 after two weeks of inoculation and drought induction exhibiting dose-dependent growth promotion under well-watered conditions (NS+I) and dose-dependent growth retardation under water-stressed (WS+I) conditions. NS- Non stressed, WS- Water stressed.

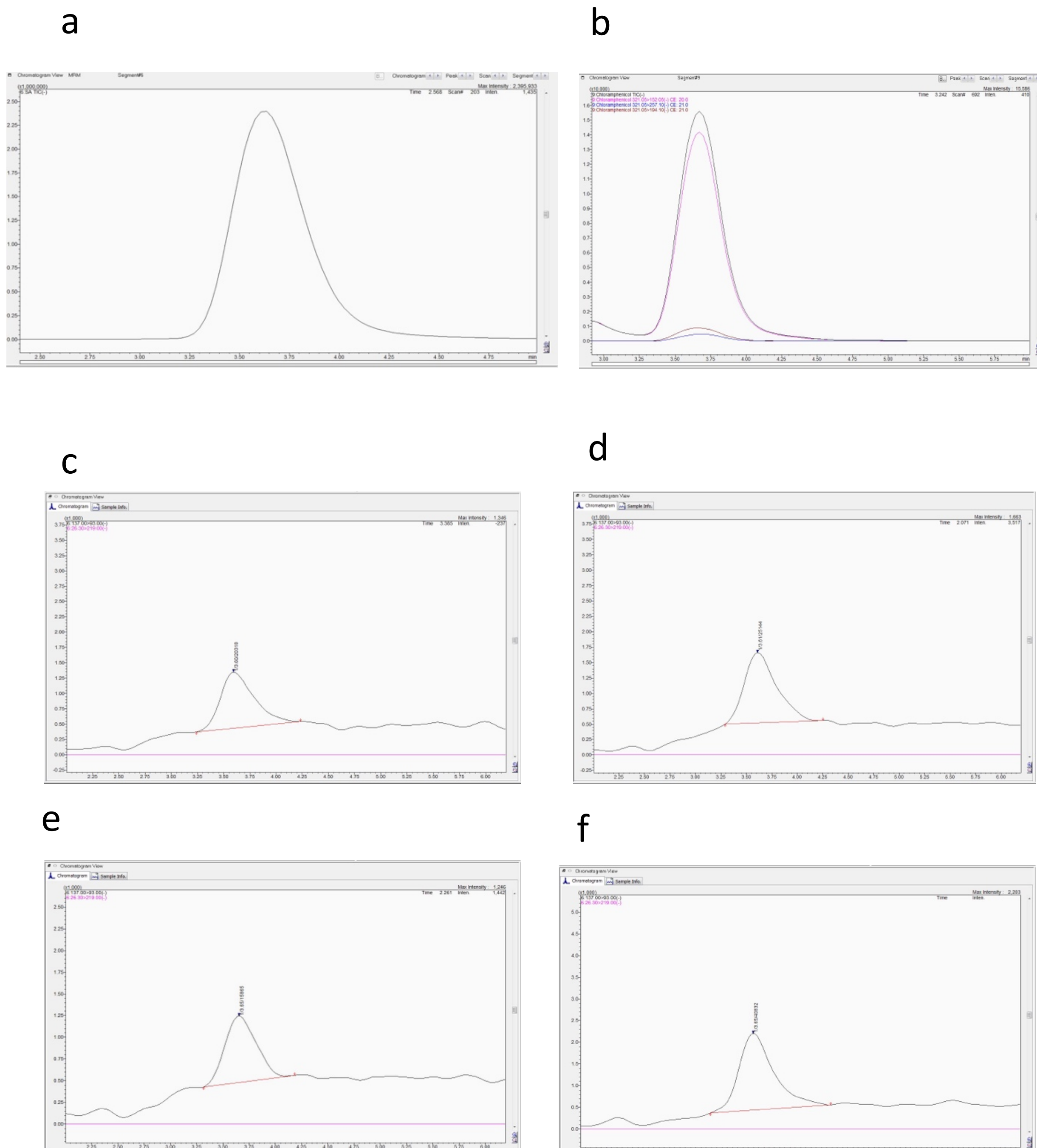
Appendix 2:

Supplementary data for Chapter 5:

Supplementary table 1: Details of primers used for gene expression studies. F- represents forward primer, and R- represents reverse primer.

S.no	**Gene/ ID**	**Sequence of primer (F)**	**Sequence of primer (R)**
1	*ICS1*- AT1G74710.2	CTGTTTTATCTCCGGCAGCC	CGACGAGAGAAGAAACAGCG
2	*EPS1*- AT5G67160.1	TCTGCGTCTGAACCTTCTGT	TGGAGCTTGTGGACCTGTAG
3	*PAL1*- AT2G37040.1	AGGAGGAGTGGACGCTATGT	TTGAGATCGCAGCCACTTGT
4	*PAL2*- AT3G53260.1	AATTGGGGTTTAGCAGCGGA	TCACACCGGCTCTTGAAGTC
5	*PAL3*- AT5G04230.1	TTGTCACTAGGGTTGCCGTC	CAGAAAGCTCCACTGTCGGT
6	*AIM1*- AT4G29010.1	GGATCTCATGGCGGTTGGAA	AATCCCGTGTCCAGCCAAAT
7	*NPR1*- AT1G64280.1	GAATCCGTCTTTGACTCGCC	GCGGTGTTGTTGGAGTCTTT
8	*NPR4*- AT4G19660.2	GGAGACTCACTAGGCCGAAA	GCAACGTCCATAGCCACATT
9	*NPR3*- AT5G45110.1	TGATCCGAAAGTTGTTGCCG	CGCCCTTTAGCTGTTTTGGT
10	*PR1* AT2G14610.1	TGCTCTTGTTCTTCCCTCGA	CTAACCCACATGTTCACGGC
11	EF1- AT1G07940.1	CCAAGTACTCCAAGGCCAGG	GCCTCAAGGAGAGTTGGTCC
12	ACT- AT3G18780.2	TGCCAATCTACGAGGGTTT	TTCTCGATGGAAGAGCTGGT

Supplementary Fig. 7: Representative day-0 pictures of *NPR4* mutants prior to inoculation and drought induction

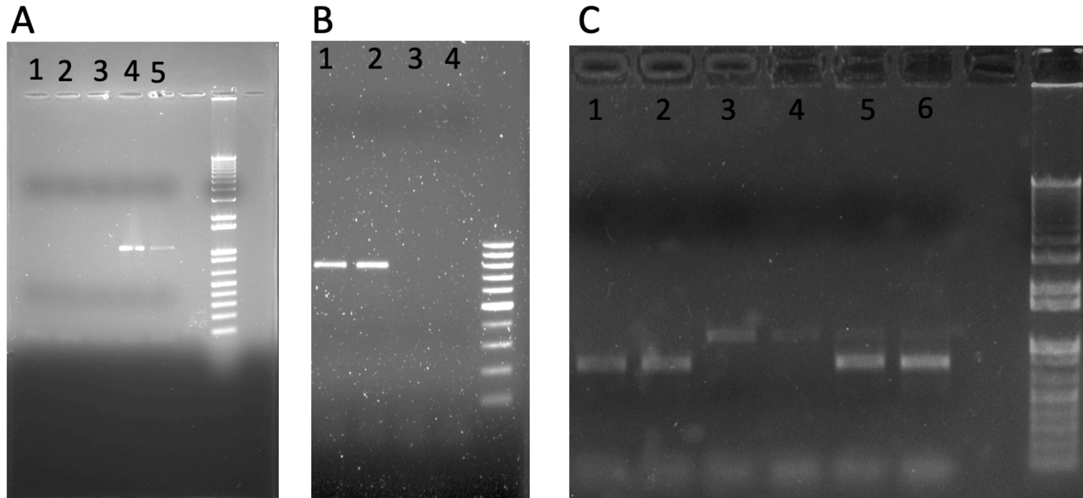


Supplementary Fig. 8: Representative MS/MS chromatogram of plant hormone SA under different treatments two weeks post-inoculation and drought induction. Panel “a” represents the MS/MS chromatogram of commercially available SA standard, and Panel “b” represents the MS/MS chromatogram of commercially procured internal standard (chloramphenicol). Panel “c, d, e, f” represents MS/MS chromatogram of SA in NS, NSI, WS and WSI samples.

a

b

Supplementary Fig. 9: Representative method file parameters used in LC-MS/MS quantification. Panel “a” represents MRM parameters used for quantification, and Panel “b” represents chromatography conditions used for the separation of SA.



D

Note:

N - Difference of the actual insertion site and the flanking sequence position, usually 0 300 bases
MaxN - Maximum difference of the actual insertion site and the sequence, default 300 bps
pZone - Regions used to pick up primers, default 100 bps
Ext5, Ext3 - Regions between the MaxN to pZone, reserved not for picking up primers
LP, RP - Left, Right genomic primer
BP - T-DNA border primer LB - the left T-DNA border primer
BPos - The distance from BP to the insertion site

LB - Left border primer of the T-DNA insertion:
>LBb1 of pBIN-pROK2 for SALK lines
GCGTGGACCGCTTGCTGCAACT
>**LBb1.3** (Newly used by Salk Genotyping Project and with better results)
ATTTTGCCGATTTCGGAAC
>LBa1 of pBIN-pROK2 for SALK lines
TGGTTCACGTAGTGGGCCATCG
>LB_6313R for SALK lines
TCAAACAGGATTTTCGCCTGCT

>LB1 for SAIL lines C/418-451 of pCSA110-pDAP101_T-DNAs
GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC
> >LB2 for SAIL lines C/390-423 of pCSA110-pDAP101_T-DNAs
GCTTCCTATTATATCTTCCCAAATTACCAATACA
>LB3 for SAIL lines C/350-383 of pCSA110-pDAP101_T-DNAs
TAGCATCTGAATTTCATAACCAATCTCGATACAC

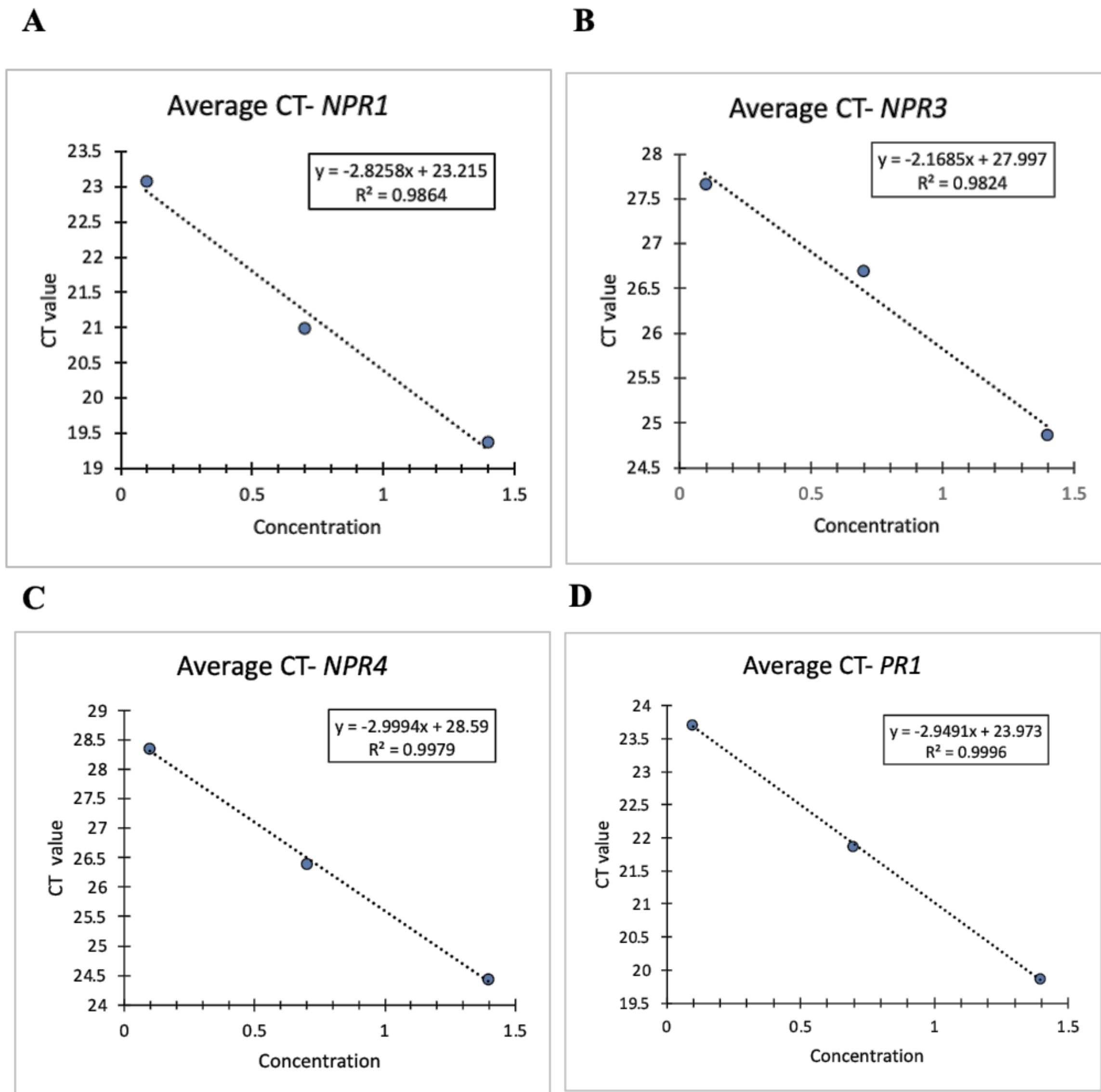
To download SAIL pCSA110 & pDAP101 T-DNAs.

E

```
Primer results for NPR4:
SALK_098460.35.75.x  PRODUCT_SIZE 1124  PAIR_ANY_COMPL 0.00  PAIR_3'_COMPL
0.00  DIFF_TM 0.05  LP GCATTTCTGCATTTCTTGAGC  Len 21  TM 56.7  GC 42.86
SELF_ANY_COMPL 0.05  3'_COMPL 0.00 RP CTGCTGGGAAGAACAACTGAG  Len 21  TM
58.9  GC 52.38  SELF_ANY_COMPL 0.05  3'_COMPL 0.00  Insertion chr4 10697258
BP+RP_PRODUCT_SIZE 584-884
```

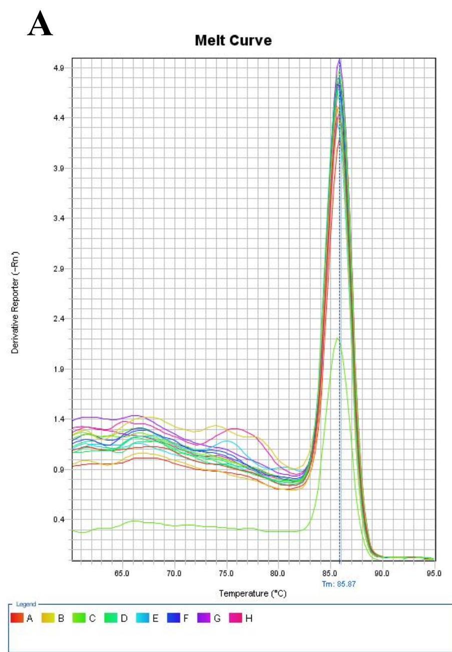
Supplementary Fig. 10: Representative genotyping data of *NPR4* seedlings prior to treatment. Panel A represents LP-RP reactions (to detect intact wild-type DNA): lane 1 is no template control (NTC), lane 2-3 is *NPR4* mutants, lane 4-5 is WT plants and 1KB+ DNA ladder was used to measure the size of PCR product. The expected amplicon size in LP-RP reaction is 1100bp for WT DNA. Absence of specified product can be observed in mutant seedlings. Panel B represents BP-RP reactions (to detect tDNA insertion lines): lane 1-2 is *NPR4* mutants, 3-4 is WT plants and 100BP DNA ladder was used to measure the size of PCR product. Panel C represents the LP+BP+RP reaction: lane 1-2 *NPR4* homozygous mutant, lane 3-4 WT plants, lane 5-6 *NPR4* heterozygous mutants and 1KB+ DNA ladder was used to measure the size of PCR product. Panel D represents the list of t-DNA-specific BP primers displayed on the SALK website. Panel E represent the LP-RP primer pair suggested by the SALK iselect primer tool.

Supplementary Fig. 11: Macro-photograph of *A. thaliana* leaves infiltrated with *P. syringae* DC3000 48 hours after infiltration and two weeks after treatments.

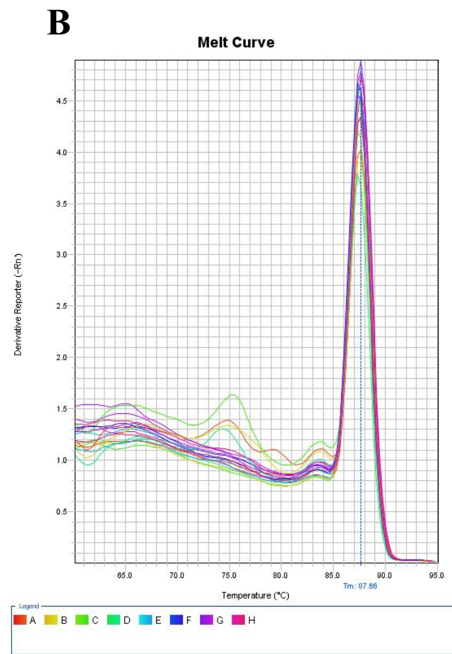


Supplementary Fig. 12: Representative Standard curve for qRT-PCR primer pairs generated with different concentrations of cDNA. A:

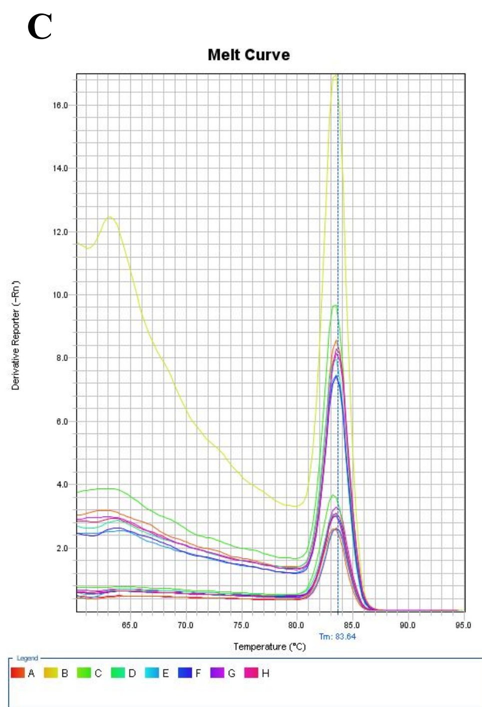
A



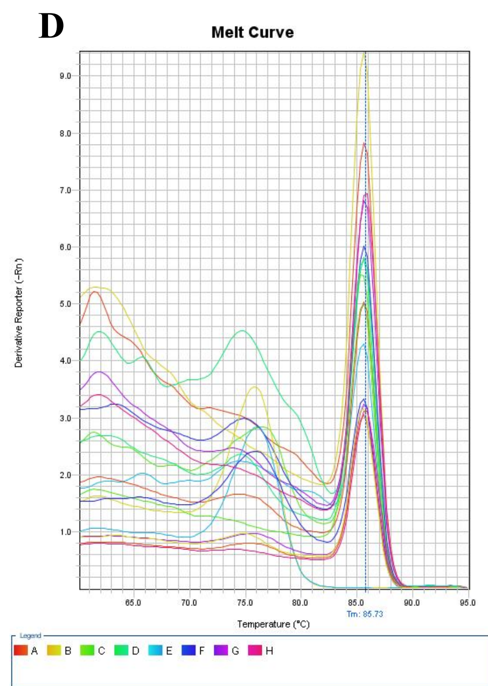
B



C



D



Supplementary Fig. 12: Representative qRT-PCR melt curve plot for A) *NPR1,* B) *PR1,* C) *ICS1* and D) *EPS1* genes.

Appendix 3:

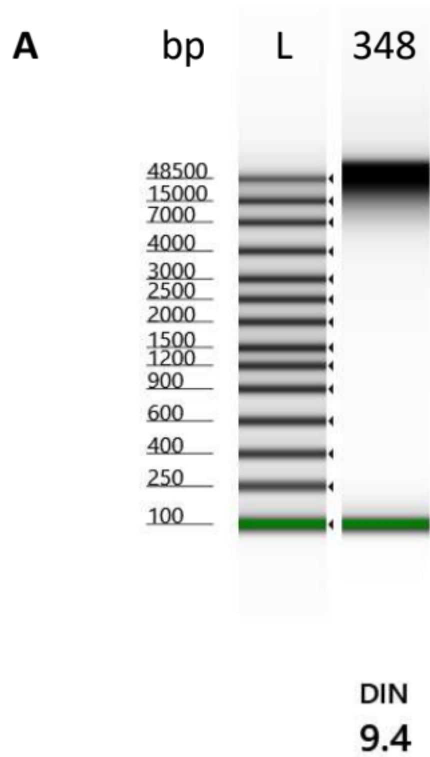
Supplementary data for Chapter 6:

1. **DNA QC summary**

Two DNA samples received from the client were given miBiome internal IDs and quantified on Qubit using standard as a control (Table 1). The DNA integrity (DIN) was analyzed on a genomic screentape using Agilent Tapestation 4200 (Fig 1). Based on the DIN values and the DNA fragment lengths in the range of 15 Kb to >48Kb, samples have passed QC.

Supplementary table 2: DNA QC summary

Internal MiBiome ID	Client ID on tube	Qubit ng/µl
MT/2022/00348	DNA A	488



B

Well	DIN	Conc. [ng/µl]	Sample Description
A1 (L)	-	79.8	Ladder
B1 (348)	9.4	48.8	MT_2022_00348

Supplementary Fig. 14: Tapestation profile of DNA samples: (A) Gel images; (B) Electropherogram

Supplementary table. 3: Average Nucleotide Index blast (ANIb) results of *P. putida* AKMP7 whole genome sequence.

Microorganism	ANIb [%]	Aligned [%]	Aligned [bp]	Total [bp]
Pseudomonas putida **HB3267 PC9**	92.98	80.9	4663322	5764016
Pseudomonas putida **KG-4**	92.81	81.4	4691827	5764016
Pseudomonas taiwanensis **SJ9**	92.78	73.76	4251754	5764016
Pseudomonas shirazica **VM14 [T]**	92.76	79.71	4594236	5764016
Pseudomonas putida **HB13667**	92.73	80.11	4617369	5764016
Pseudomonas putida **S11**	92.7	81.22	4681286	5764016
Pseudomonas inefficax **JV551A3 *[T]***	92.7	81.74	4711709	5764016
Pseudomonas monteilii **SB3101**	92.69	80.23	4624496	5764016
Pseudomonas plecoglossicida **NyZ12**	92.68	80.63	4647712	5764016
Pseudomonas putida **S16**	92.68	79.42	4577967	5764016
Pseudomonas monteilii **SB3078**	92.67	80.38	4633170	5764016
Pseudomonas putida **DLL-E4**	92.67	80.27	4626638	5764016
Pseudomonas putida **ATCC 12633 *[T]***	88.86	76.59	4414400	5764016
Pseudomonas putida **NBRC 14164 *[T]***	88.85	76.56	4413173	5764016
Pseudomonas putida **NCTC10936 *[T]***	88.85	76.53	4411101	5764016
Pseudomonas putida **IPO3752 [T/**	88.85	76.03	4382172	5764016
Pseudomonas putida **S13.1.2**	88.77	76.71	4421461	5764016
Pseudomonas putida **Simmons01**	88.7	75.13	4330378	5764016
Pseudomonas putida **JQ581**	88.59	75.34	4342739	5764016
Pseudomonas putida **KT2440**	88.58	75.18	4333534	5764016
Pseudomonas putida **H**	88.54	74.64	4302343	5764016
Pseudomonas putida **JLR11**	88.53	74.55	4297303	5764016
Pseudomonas putida **BIRD-1**	88.52	74.75	4308734	5764016
Pseudomonas putida **INSali382**	88.48	74.33	4284153	5764016
Pseudomonas putida **H8234**	88.36	74.45	4291477	5764016
Pseudomonas putida **LF54**	88.25	71.92	4145727	5764016
Pseudomonas putida **SJ3**	87.89	63.85	3680269	5764016
Pseudomonas putida KB9	86.94	67.84	3910142	5764016
Pseudomonas sp. **TJI-51**	86.92	67.16	3870829	5764016

Supplementary datasheet 1:

Due to large number of datasets and complexity of data tables, the following data is hosted online and can be made available from the following link: https://docs.google.com/spreadsheets/d/15HMZDnOrZqcGCv5fQGZ0D5QBqC2lL2Kj/edit?usp=share_link&ouid=107348610744180851121&rtpof=true&sd=true

Supplementary datasheet 2:

Due to large number of datasets and complexity of data tables, the following data is hosted online and can be made available from the following link:

https://docs.google.com/spreadsheets/d/15bzBxY7sS3hjF87Y3yyvbQc-s4RGCGf8/edit?usp=share_link&ouid=107348610744180851121&rtpof=true&sd=true

(Note: Alternatively, for ease of access the following files can be made available upon request by sending an email to the author of this thesis: rajagopal1608@gmail.com)

List of publications:

Research Papers:

1. **Raja Gopalan N. S.**, Raunak Sharma, Sridev Mohapatra. 2022. Probing into the unique relationship between a soil bacterium, Pseudomonas putida AKMP7 and Arabidopsis thaliana: a case of "conditional pathogenesis". Plant Physiology and Biochemistry. 183:46-55. https://doi.org/10.1016/j.plaphy.2022.05.003 **(Impact factor: 6.5)**

2. Sowmya V, **N. S. Raja Gopalan**, Maruthi P, Baskar M, Umadevi G, Ladha Lakshmi D, Koteshwar P, Jesudasu G, Santhosha Rathod, Srinivas Prasad M, Rajanikant P, Sundaram RM, Sridev Mohapatra and Kannan C. 2023. "Native bio-control agents from the rice fields of Telangana, India: Characterization and unveiling the potential against stem rot and false smut diseases of rice." World Journal of Microbiology and Biotechnology. Accepted on 28th September 2023 (in press). **(Impact factor: 4.1)**

3. Raja Gopalan N.S., Yegna Priya S, Sridev Mohapatra. 2023. The drought-tolerant rhizobacterial strain, *Pseudomonas putida* AKMP7, causes conditional pathogenesis in Arabidopsis thaliana by suppressing plant immunity via negative regulation of salicylic acid signalling, under water stress. Plant Physiology and Biochemistry. **(Impact factor: 6.5)**

4. Nenmeli Sampath Kumar, R.G., Behera, A. K., Srivatsava, A., Murugkar, V., Arigela, C., Dasgupta, N., Das, G., Grover, M. and Mohapatra, S. 2023. Functional genome analysis of a conditionally pathogenic rhizobacterial strain, *Pseudomonas putida* AKMP7. Current microbiology. Accepted: Pre-proof. [Impact Factor: 2.6]

Book Chapters:

1. Dhar, N., **Gopalan, N.R.**, Nikhil, P.T. and Mohapatra, S., 2022. Role of Phytohormones in Plant-Microbial Interaction. In Auxins, Cytokinins and Gibberellins Signaling in Plants (pp. 313-336). Cham: Springer International Publishing.

2. **Gopalan, N.R**., Nikhil, P.T., Sharma, R. and Mohapatra, S., 2023. The use of microbes as a combative strategy for alleviation of abiotic and biotic stresses. In Unravelling Plant-Microbe Synergy (pp. 175-193). Academic Press

List of conferences:

Abstracts in Conference Proceedings: (Oral presentation)

1. **Raja Gopalan N. S.** Title. "Conditional pathogenesis: understanding why potentially beneficial rhizobacteria turn pathogenic under certain environmental conditions" at the international conference on "Physiological and molecular mechanisms for abiotic stress tolerance in plants" held at the University of Calicut from 26th October-29th October 2022.

Biography of the Supervisor

Prof. Sridev Mohapatra, Associate Professor in Biological Sciences Department, has been working with Birla Institute of Technology and Science, Pilani, Hyderabad Campus, Hyderabad, Telangana, India from 2012 onwards. He was awarded Ph.D. degree in Plant Biology from University of New Hampshire, U.S.A. in the year 2008. He completed his M.Sc. in Botany from Utkal University, Bhubaneswar, India in the year 2001. After his Ph.D., he has worked as a Post-Doctoral research associate in the Texas Tech. University, Lubbock, Texas, U.S.A. and University of Texas at Arlington, Arlington, Texas, U.S.A.. Prof. Sridev Mohapatra primarily focus on research in the area of plant-rhizobacterial interactions for abiotic stress tolerance in plants. Post Ph.D., Prof. Sridev Mohapatra has about 15 years of research and academic experience in the field of Plant Biology. He has supervised and graduated two Ph.D. students and is currently supervising 6. He has already completed three funded projects as Principal Investigator, one each from Science and Engineering Research Board, Department of Biotechnology (both part of Ministry of Science and Technology, Govt. of India) and BITS-Pilani and has one ongoing grant as PI from Council of Scientific and Industrial Research (Ministry of Human Resource Development, Govt. of India). Prof. Mohapatra has published several research papers with good number of citations in reputed international journals and conferences. He also serves as a reviewer for many international journals and is a review editor for the journal, Frontiers in Plant Sciences.

Biography of the Candidate

Mr. Raja Gopalan is a full time Ph.D. student in the department of Biological Sciences at BITS-Pilani, Hyderabad Campus, Hyderabad, Telangana, India. He is working under the supervision of Prof. Sridev Mohapatra in the Department of Biological Sciences. He has obtained his Master's degree (M.Sc.) in Biotechnology from, Dwarka Doss Gowardan Doss Vaishnav college, University of Madras, India in the year 2017. He started his research as a junior research fellow in the November, 2018 in Prof. Sridev Mohapatra's laboratory and enrolled in the Ph.D. programme of BITS-Pilani in August, 2019. He is proficient in the areas of microbiology, plant-microbe interactions, molecular biology and analytical chemistry. He has published his work in reputed international publications. He has participated/presented his work in several national and international conferences. Currently, his career interests are focused on understanding the molecular mechanisms involved in plant-microbe interactions and the signalling mechanisms by which plants attract surrounding microbiome. After submitting his Ph.D. dissertation, he will join as a Research Scientist/Postdoctoral Researcher at Plant Hormone Biology Laboratory, University of Amsterdam, Netherlands.