# Investigating Signalling Pathways in Rice under Salt Stress Induced by Plant Growth-Promoting Bacteria

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# DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, "**Investigating Signalling Pathways in Rice under Salt Stress Induced by Plant Growth-Promoting**" is based on the results of investigations carried out by me in the Botany Discipline at the School of Biological Sciences and Biotechnology, Goa University under the Supervision of Dr. Siddhi K. Jalmi and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will be not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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# COMPLETION CERTIFICATE

This is to certify that the dissertation report **"Investigating Signalling Pathways in Rice under Salt Stress Induced by Plant Growth-Promoting Bacteria"** is a bonafide work carried out by **Mr. Fedrick Joseph Vas** under my supervision in partial fulfillment of the requirements for the award of the degree of **Master of Science** in the Botany Discipline at the School of Biological Sciences and Biotechnology, Goa University.

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Place: Goa University

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# PREFACE

This dissertation deals with the some of the aspects that surround the interaction of microbes and plants. Rice is the primary staple food of most of the south Asian countries. The cultivation of rice has been widespread in these areas. Due to increasing climate change the sea water level is increasing rapidly, which leads to soil salinization. To add to this problem is the increasing use of fertilizers by farmers which has led to magnification of this problem. Certain varieties of rice are developed through breeding which has demonstrated salinity tolerance, but having all attributes into a single plant is fairly a difficult task. There are varieties of rice which are high yielding but sensitive to different biotic and abiotic stresses. The interaction between microbes and plants is a topic that has gained great momentum in the recent times. This interaction may probably be the basis of enhancement of plant growth in certain areas whereas regression in the other. With the isolation of certain halotolerant bacterial strains from the salt-pans of Ribandar, Goa and some of the preliminary work done on their Plant Growth Promoting effects on two salt resistant rice varieties motivated me to study their probable role in providing salt tolerance to a salt sensitive variety and if this process in advanced through a particular signalling cascade.

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# ABBREVIATIONS

BIK1: BOTRYTIS-INDUCED KINASE 1

CAT: Catalase

EIN4: ETHYLENE INSENSITIVE 4

FLS2: FLAGELLIN SENSING2

LRR: Leucine Rich Repeat

MAPK: Mitogen Activated Protein Kinase

POD: Peroxidase

RIPK: Receptor-interacting Protein Kinases

SERF1: SALT- RESPONSIVE ERF1

SIT1: Salt Intolerance 1

SOD: Superoxide dismutase

TDR: Tracheary element Differentiation inhibitory factor Receptor

ZMB: Zobell Marine Broth

# ABSTRACT

Plant Growth Promoting Bacteria (PGPB) are responsible for various growth promoting attributes towards the development of a plant. Research work has suggested their role in helping salt tolerant varieties and also providing tolerance to sensitive plants. Mitogen activated protein kinase (MAPK) signalling cascade has received immense attention in the recent years. However, their role in mediating signals related to growth promoting bacteria is not widely studied. Here, we investigated the plant growth promoting activity of two strains of bacteria, i.e. *Pseudomonas multiresinivorans* ABSK11 and *Bacillus subtilis* ABSK186 on a salt sensitive rice variety IR64 for its role in alleviating salt stress. The analysis of the plant growth showed that there was an increase in the shoot-root length, dry weight and proline content in inoculated plant samples compared to un-inoculated samples. ROS species which are known to accumulate as a result of high salt concentration are also lowered in inoculated samples. Quantitative real-time analysis on *Pseudomonas multiresinivorans* ABSK11 inoculated plants suggested that *MPK3* and *MPK6* were upregulated in comparison to un-inoculated plants. *In silico* analysis on the various plant receptors was done to ascertain their role in signal transduction through the MAPK cascade and it led to the identification of various MAPK proteins and other kinase domain containing proteins that interacts with the receptors. The results suggest the involvement of MAPK signalling components in transmitting signals of plant growth promoting bacteria in imparting salt tolerance to salt sensitive rice. This research work will form basis for identifying mechanism utilized by the salt tolerant PGPB in providing salinity tolerance to rice crop plant.

***Keywords***: Plant growth promoting bacteria; Rice; MAPK signalling; Salinity, Receptors

# CHAPTER 1

# INTRODUCTION

# 1. Introduction

In the recent years there has been a great increase in the salinity of agricultural land. This is mainly due to unsustainable farming practices, rise in sea level, impact on coastal areas, rise in temperature, and increase in evaporation and water shortages. It has been projected that global dry lands will increase by 23% by the end of the 21$^{st}$ century, with developing nations experiencing 80 percent of this increase. Severe water shortages are currently affecting 27% of the world's population, and this number is expected to rise to 42-68% by 2050 (FAO, 2023). Around 6.727 million ha area in India is salt-affected, of which 2.956 million ha is saline (Arora & Sharma, 2017). All these predictions point towards an increased soil salinity levels, which are a great challenge to the agriculture sector. The plants in salt-affected environments experience two types of stress, first is the osmotic stress which is due to low osmotic potential of water in saline soils which adversely affects water absorption by plants. Another is nutrient stress which is due to both toxicity (Na, Cl, B) and deficiency of plant nutrients (N, Ca, K, P, Fe, Zn) (Kumar & Sharma, 2020). Plants under such stress show different morphological, bio-chemical and genetic changes (Zhao et al., 2021). In saline areas, plant growth and metabolism is negatively affected due to accumulation of $Na^+$ ions in the plant tissues which leads to reactive oxygen species (ROS) accumulation. These are responsible for speeding up the toxic reactions, which may be DNA mutation, protein degradation and membrane damage (Numan et al., 2018).

Plants on their part have developed various mechanisms to overcome salt stress. The salt overly sensitive (SOS) regulatory pathway is one such mechanisms which regulates ion homeostasis through modulating $Na^+/H^+$ antiporters activity during salt stress (Park, Kim & Yun, 2016). Osmolytes, such as proline, polyols, and sugars, accumulate in

substantial quantity and regulate osmotic pressure by lowering the osmotic potential in the cytosolic compartment under salt stress. Protein kinases act as a major transduction system for the downstream pathway. These and other signalling strategies are used by plants to sense the changes in the environment and give a particular response (Zhao et al., 2021).

In addition to these plant inherent mechanisms, certain plant growth promoting bacteria (PGPB) also have specialized mechanism that play key role in salt stress tolerance and plant growth promotion. There have been various advancements in the field of techniques to overcome the problem of salinity and its effects on plants (Arora & Sharma, 2017). Some of which are: cultivation of salt tolerant varieties, sub-surface drainage technology, rainwater harvesting in dugout farm ponds, phytoremediation and bio-remediation. Bio-remediation forms its foundation on plant-microbial interaction, and has received increased attention worldwide for enhancing productivity of salt-affected soils (Backer et al., 2018). Plant growth-promoting bacteria are known to facilitate plant growth and development either indirectly or directly. Indirect methods occur when bacteria prevents or reduces the harmful effects of some sort of stress e.g. salt, and in this way help the plant to grow. The direct methods of plant growth promotion entails fixation of nitrogen, iron and phosphate assimilation, or promoting plant growth by altering plant hormone levels such as auxin, cytokinin and ethylene (Glick, 2014; Olanrewaju et al., 2017). The bacteria are known to follow various mechanisms in providing abiotic stress resistance and resilience to the plant. Among others one of them is the expression of different genes involved in salt stress tolerance in plants (Etesami & Glick, 2020; Kumar et al., 2020).

Mitogen-activated protein kinase (MAPK/MPK) cascades play key roles in the signal perception and transduction of salt stress signalling in plants by sequential

phosphorylation reactions (Gao et al., 2010; Chen et al., 2021). Activated MAPKs target and activate specific downstream substrates, such as other kinases, enzymes and transcription factors. 17 MAPK, 8 MAPKK, and 75 MAPKKK coding genes were specifically found in the rice genome (Hamel et al., 2006; Jia et al., 2016). Lately, several MAPK cascades mediating the rice salt response have been identified. Numerous studies have proven that the MAPK cascade responding to salt stress is closely related to the regulation of salt-responsive genes (Lin et al., 2021). Although different MAPK cascades were shown to regulate salt response in rice, their modulation by microbes and the substrates phosphorylated by MAPK during rice salt tolerance remain largely unknown.

Goa being a state along the west coast of India is greatly acclaimed among tourists as a great place for marine adventure. Apart with tourism, it is also known for its local traditional practices, one of them being the preparation of salt in solar salterns. Currently, there are only 9 villages in Goa that produce salt and the total area under current salt production is about 2,978 ha. A study on the saltpans of Ribandar, Goa has led to the isolation of a number of microorganisms which are beneficial towards plant growth. Estimation of ammonia production, phosphate solubilization, nitrogen fixation, and other studies along with plant growth promoting activity on salt tolerant rice varieties have been done. Two of the most prominent species isolated were *Pseudomonas multiresinivorans* ABSK11 and *Bacillus subtilis* ABSK 186 (Bartakke, 2018). They have shown plant growth promoting activity and thus were chosen for our study to explore the signalling pathway used by rice plant in perceiving this microbe and thus alleviating salt stress in a salt sensitive variety. The rice variety chosen for this study was IR64, which has been used extensively in genetic studies of rice, mainly because of its superior representation of the Indica genetic make-up and other

physiological properties like early maturity. IR64 was developed primarily for irrigated rice production, and abiotic stress resistance was a concern in its developmental process (Mackill & Khush, 2018).

Thus, the aim of our research work was to study the signalling components in rice which are responsible for alleviating salt stress through the interaction with plant growth promoting bacteria.

Our work was thus carried out with the following objectives:

1) To study the bio-chemical and morphological changes in the plant in response to bacterial inoculation.
2) To study the signalling components (*MPK3*, *MPK4* and *MPK6*) in response to bacterial inoculation and salt treatment.
3) To evaluate the interactome or interaction network of the signalling components and their targets in *in silico*.

# CHAPTER 2

# REVIEW OF LITERATURE

# 2. Literature Review

## 2.1. Plant Growth Promoting Bacteria and Signalling

Plant growth promoting bacteria are known to produce a number of compounds which are involved in signalling. These compounds can range from hormones to protein to volatile organic compounds (Ilangumaran & Smith, 2017; Jalmi & Sinha, 2022). A number of hormones have been reported to perform this role. Auxins (Indole-3-acetic acid) are one of the most common hormones. *A. caulinodans*, *B. japonicum*, *R japonicum*, *R. leguminosarum*, *R. meliloti*, *R. phaseoli*, *R. trifolii*, *S. meliloti* are well studies in this regard (Yanni et al., 2001; Naidu et al., 2004; Boiero et al., 2007; Senthilkumar et al., 2009; Chi et al., 2010). Cytokinins, gibberellins and ethylene are also released by microbes (Jalmi & Sinha, 2022). The enzyme, ACC deaminase has also been known to be released by *R. leguminosarum*, *R japonicum*, *R. gallicum*, *B. japonicum*, *B. eklani*, *S. meliloti*, *Variovorax sp*. (Duan et al., 2009; Onofre-Lemus et al., 2009; Gupta & Pandey, 2019; Bessadok et al., 2020). Some volatile organic compounds released by PGPB are alkanes, ketones, terpenoids, alcohols, sulfur compounds like 2-heptanol, 2-endecanone, and pentadecane, cyclodipeptides (CDPs) and lipo-chitooligosaccharide (LCO) (Jalmi & Sinha, 2022). *Bacillus subtilis* GB03 and *Bacillus amyloliquefaciens* IN937a has been found to release 2,3-butanediol and 3-hydroxy-2-butanone, which in turn participates in signalling (Ryu et al., 2004). Flagellin found on microbial surface can also act as a signalling agent (Mani et al., 2023).

These signals from microbes need to be actively sensed by the plant so that further transduction and response is produced. There are certain sensors which sense an

external stimulus and these signals from various agents are perceived by the plant at a particular location, on the membrane, called the receptor (Jones and Dangl, 2006). In the recent years a number of receptors have been identified in plants, which can be the windows for signal transduction into the plant body. Plants are found to recognize effector protein through Nucleotide-Binding Leucine-Rich Repeat (NB-LRR) receptor proteins. The elf18 peptide derivative of the EF-Tu N terminus and Tu elongation factor (EF-Tu) of bacteria are detected by Arabidopsis in the same manner through LRR receptor kinase (Kunze et al., 2004; Zipfel et al., 2006). Zipfel et al. (2004) catogorized the receptor kinase EFR (EF-Tu receptor) as the EF-Tu (elongation factor Tu) receptor based on loss-of-function experiments in *Arabidopsis* and gain-of-function experiments in *N. benthamiana* plants. Arabidopsis receptor kinase MALE DISCOVERER 1-INTERACTING RECEPTOR-LIKE KINASE 2 (MIK2) is known to recognize the conserved signature motif of SERINE-RICH ENDOGENOUS PEPTIDEs (SCOOPs) in bacterial *Comamonadaceae* and starts a signalling cascade (Hou et al., 2021).

It was discovered that flg22–FLAGELLIN SENSING 2 (FLS2) is a LRR Receptor–like Kinase which is involved in the perception of the bacterial elicitor flagellin in *Arabidopsis* (Gómez-Gómez and Boller, 2000; Chinchilla et al., 2006). Trdá et al. also characterized the flagellin sensing system flg22–FLAGELLIN SENSING 2 (FLS2) in grapevine, and analyzed the flagellin perception in the interaction with the endophytic plant growth-promoting rhizobacterium (PGPR) *Burkholderia phytofirmans* (Trdá et al., 2014). FLS2 has also been found in rice, these results indicated that monocotyledonous as well as dicotyledonous rice possesses a flg22 per-ception system (Takai et al., 2008). FLS2 functions as a bacterial flagellin receptor localized on the cell membrane of plants. In Arabidopsis, the co-receptor BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) cooperates with FLS2 to detect the flagellin epitope flg22, resulting in

formation of a signalling complex that triggers plant defense responses. Another co-receptor involved is the BIK1, which further transphosphorylates FLS2 and BAK1 and thus BIK1 is required in flagellin-triggered immunity (Lu et al., 2010). OsSERK2 effectively phosphorylates OsFLS2, which reciprocally phosphorylates OsSERK2, leading to complete activation of OsSERK2 and rapid phosphorylation of the downstream substrate receptor-like cytoplasmic kinases OsRLCK176 and OsRLCK185 (Zhao et al., 2024).

The studies on soybean (Wei et al., 2020) have yielded that the plant is able to perceive flg22Rso from *Ralstonia solanacearum*, the causal agent of the bacterial wilt disease, and thus trigger a downstream signalling cascade namely MAPK. This also happens with the help of GmFLS2 and GmBAK1 domains. Further investigation revealed that that β-1,3-glucans activates a ROS and MAPK response in *H. vulgare* and *B. distachyon* (Wanke et al., 2020). Another receptor known as the Toll/interleukin-1 receptor (TIR) signalling has a key role in pattern-triggered immunity (PTI) mediated by pattern recognition receptors (Tian et al., 2021). *Arabidopsis* receptor-like cytoplasmic kinase genes PBL2 and RIPK have been reported to provide immunity against *Xanthomonas* infection where they recognize xopAC from the bacteria (Guy et al., 2013).

The ethylene released by bacteria can be detected by plants with the help of ethylene receptors. Plants contain multiple ethylene receptor isoforms. In Arabidopsis, five isoforms have been identified and are referred to as ethylene response 1 (ETR1), ethylene response sensor 1 (ERS1), ETR2, ERS2, and EIN4 (Bleecker et al., 1988; Chang et al., 1993; Hua et al.1995; Sakai et al., 1998). Tintor et al. (2013) further found that loss of ETHYLENE-INSENSITIVE2 (EIN2), a master signalling regulator of the

phytohormone ethylene (ET), lowers sensitivity to both elf18 and flg22 in different defense-related outputs.

## 2.2. MAPK Signalling in Plants

Mitogen-activated protein kinase (MAPK) cascades are highly conserved signalling modules consisting of MAPK Kinase Kinases (MAPKKKs/MEKKs), MAPK Kinases (MAPKKs/MKKs/MEKs), and MAPKs/MPKs, which are widely used to relay and amplify signal from plasma membrane receptors to various downstream responses in eukaryotes (Widmann et al., 1999; MAPK-Group, 2002). The MAPK cascade becomes a link between salt stress sensors and target genes. However, evidence suggesting that the MAPK cascade directly regulates target genes is lacking (Lin et al., 2021). Besides its critical roles in regulating plant growth and development, the MAPK module MKK4/5-MPK3/6 is also essential for plant immunity (Meng & Zhang, 2013; Thulasi Devendrakumar et al., 2018). Beneficial microbes are perceived by modulating MAPK, CDPKs, ROS and hormonal signalling pathways. This result in enhanced expression of stress responsive genes leading to PGPR mediated stress tolerance (Tiwari et al., 2017).

According to the experiments done by Wang et al. (2014) OsMPK3 and OsMPK4 were identified as the strongest downstream target of OsMKK1. By examining the survival rate and $Na^+$ content in shoot, it was found that OsMKK1-knockout (*osmkk1*) mutant was more sensitive to salt stress than the wild type. OsMKK1 activity in the wild-type seedlings and protoplasts was increased by salt stress. Thus, concluding that OsMKK1 is involved in giving salt tolerance to rice. OsMAPKKK63 (Na et al., 2019) has been identified in rice which interacts with this OsMKK1 and regulates salt stress. Kumar and Sinha established the functional role of OsMKK6 in salinity stress by transgenic

approach. It was reported that the over expression of OsMKK6 is responsible for salt tolerance in rice (Kumar & Sinha, 2013).

A study on the OsMKKK10-OsMKK4-OsMPK6 cascade and the OsMPK6 interactors, revealed that the zinc finger transcription factor DST, which was previously shown to regulate drought and salt tolerance was upregulated when rice was exposed to the stress (Huang et al., 2009; Guo et al., 2020) According to a study by Jia et al. (2022), Ideal Plant Architecture 1 (IPA1) is a negative regulator of salt tolerance in rice. The loss of function mutant seedlings showed significantly enhanced salt tolerance. IPA1 is a downstream substrate of OsMPK4, this OsMPK4 phosphorylates it to reduce its stability and thus confer salt tolerance to the plant. OsMPK6 is known to regulate biomass production and help to increase height in rice (Liu et al., 2015). This may be possibly involved in the increase in the shoot length of the rice plants when inoculated with the bacteria. OsMPK4 has shown to be upregulator of *OsDREB2B*, *OsNAC6*, *OsABI5* and *OsMYBS3*, which help in conferring salt tolerance (Wang et al., 2014). MPKK10.2 physically interacted with MPK6 and MPK3, and phosphorylated the two MAPKs *in vivo* (Ma et al., 2017).

OsMPK3 is shown to interact with OsZFP213 and enhance the transactivation activity of $C_2H_2$ ZnF transcription factor (OsZFP213) (Zhang et al., 2018). It is possible that salt-induced ROS activate OsMPK3, and the activated OsMPK3 positively regulates OsZFP213 to enhance the enzyme activity of ROS scavengers, including APX1, GR2, SOD2, and CAT. The OsMKK1 gene was seen to be expressed more than 6-fold after 1 h and then gradually decreased to around 3-fold after 4 h of 200 mM NaCl treatment. Further analysis found that OsMKK1 activates OsMPK3 and OsMPK4, which in turn induced an increase in OsDREB2B and OsMYBS3 which are involved in salt stress tolerance (Cui et al., 2011, Wang et al., 2014) MYC2 (a bHLH TF) was observed to

regulate proline biosynthesis by down regulating P5CS1 gene in *Arabidopsis*. Further investigation on MYC2 yielded that, MKK3 and MYC2 both are necessary for the salt stress-induced activation of MPK6 and MKK3 acts upstream to MPK6 in salt stress (Verma et al., 2020). Studies by Zhou et al. (Zhou et al., 2022) have yielded that PIP3–RLK7 (PAMP-INDUCED SECRETED PEPTIDE 3 and RECEPTOR-LIKE KINASE 7) module activates MPK3/6 signalling in response to salt stress. The MPK3/MPK6 cascade is further involved in salt tolerance regulation. Studies on the probable substrate of MPK3 has brought to light that AZI1, a lipid transfer protein (LTP)-related hybrid proline-rich protein (HyPRP), as a novel target of MPK3. AZI1-overexpressing lines are markedly more tolerant to salt stress (Pitzschke et al., 2014).

SIMKK (a MAPKK in *Medicago*) is an upstream activator of SIMK and MMK3 (ACC-activated MAPKs) that mediates ethylene induced activation of these MAPKs (Ouaked et al., 2003). AtMEK1 (Xing et al., 2007), an Arabidopsis MAPK kinase, is involved in Catalase 1 (CAT1) expression in response to different stresses triggering $H_2O_2$ signal production. BWMK1 is a member of a new family of plant MAPKs BWMK1, which can be classified into family V MAPK and is activated by SA-associated defense signals and by ethylene/JA-associated plant defense signals. The protein is maintained at baseline levels within the cell and is synthesized anew when a pathogen attacks (Cheong et al., 2003). MKK4 was observed to play an important role in osmotic-stress signal transduction by modulating MPK3 (Xing et al., 2015). MKK4 mutants were found to be sensitive to salt stress, whereas MKK4 over-expression lines were seen to demonstrate tolerance to salt. All these are the signal transduction mechanisms which are responsible for the different salt responsive genes that are further regulated in response to salinity stress.

## 2.3. Salt Responsive Genes in Rice

Soil salinity induces both osmotic and toxicity stress in plants, resulting in growth inhibition, developmental changes, metabolic adaptations, and ion sequestration or exclusion (van Zelm et al., 2020). To counteract this, plants have various molecular mechanisms which are triggered by the signalling. A study found that salt tolerance of plants may depend on the HKT (HIGH-AFFINITY K+ TRANSPORTER 1) transporter, which play a key role in regulating $Na^+$ homeostasis because it mediate $Na^+$ -specific or $Na^+$ -$K^+$ transport (Hauser and Horie, 2010). Plants treated with salt have shown to differentially express the *HKT* gene in the roots and shoots (Zhang et al., 2018). Salt overly sensitive 1 (SOS1) is a key systemic determinant of $Na^+$ extrusion from the cytosol to the apoplast, and its functional mutant sos1-1 displays the exceptional sensitivity to salt stress. HKT1 and SOS pathway is essential for modulating $Na^+$ homeostasis in plant cells (Ma et al., 2022). Along with this salt stress induces the expression of ABA-responsive genes and ABA accumulation in the primary root (Huang et al., 2021; Han et al., 2023).

MYC2, a bHLH TF, was observed to regulate proline biosynthesis by down regulating *P5CS1* gene in *Arabidopsis*. Proline biosynthesis may proceed either via glutamate, by successive reductions catalyzed by pyrroline-5-carboxylate synthase (P5CS) and pyrroline-5-carboxylate reductase (P5CR) or by ornithine pathway. When *P5CS* gene was overexpressed in the transgenic tobacco plants, an increased production of proline coupled with salinity tolerance were noted (Kishor et al., 1995). Further investigation on MYC2 yielded that, MKK3 and MYC2 both are necessary for the salt stress-induced activation of MPK6 and MKK3 acts upstream to MPK6 in salt stress (Verma et al.,

2020). MYC2 is also a key transcription factor in the JA pathway and acts as a regulatory hub for several biotic and abiotic stress-related responses (Delgado et al., 2021).

Plasma membrane intrinsic proteins (PIPs) are localized in cellular plasma membrane. The 10 genes were found in rice and were designated as *OsPIP1-1 (OsPIP1a), OsPIP1-2, OsPIP1-3* (*RWC-3I*), *OsPIP2-1* (*OsPIP2a*), *OsPIP2-2, OsPIP2-3, OsPIP2-4, OsPIP2-5, OsPIP2-6,* and *OsPIP2-7*. When treated with salt, the expression levels of most *OsPIP*s were first suppressed to a certain extent and then increased (Guo et al., 2006). *OsPIP1* genes are of great importance for collectively modulating the osmotic potentials in rice under salt tolerance as any mutation in them can negatively trigger a series of physiological changes, and lower rice biomass (Tao et al., 2023).

The dehydration-responsive element (DRE) is a major cis-regulatory element regulating gene expression response to salt stress. GROWTH-REGULATING FACTOR7 (GRF7) binds to the DREB2A (dehydration-responsive element binding protein 2) promoter and interacts with the DREB2A protein. A study on the OsDREB has found that overexpressing OsDREB1A, OsDREB1F and OsDREB2A showed improved salinity tolerance in transgenic rice (Matsukura et al., 2010; Kumar et al., 2013). These systems and their efficiency may be enhanced by the interaction of the plant with certain plant growth promoting microbes.

## 2.4. Bacterial Role in Alleviating in Salt Stress

A study by Liu et al. showed that *Bacillus amyloliquefaciens* W25, a γ-PGA-producing bacteria (Liu et al., 2024) can be used as PGPB to alleviate salt stress by regulating plant physio-biochemical processes. Inoculation with strain W25 could significantly decrease $Na^+$ content in the roots and leaves of salt-treated lettuce. Bacterial inoculation

(*P. aeruginosa* PHL3 and *A. faecalis* BRC3) in paddy (CARI Dhan 3) has shown significant increase in radical length in comparison to those treated only with salt (Kumar et al., 2017).

*Brevibacterium linens* RS16 inoculated 100 mM salt stressed salt sensitive IR29 plant showed statistically significant enhancement in dry mass (Chatterjee et al., 2018). Bacteria inoculation scaled up the catalase activity and carotenoid content with increasing salt concentration. Another study by Kumar et al. (2021) on the effect of *Bacillus pumilus* strain JPVS11 on rice growth with NaCl showed that inoculation improved plant height significantly as compared to non-inoculated at all levels of salt stress. The proline content was also seen to increase by 16.86 %, 20.33 %, 18.57 %, and 22.89 % in the JPVS11 inoculated plants. In addition to this the ROS concentration was seen to decrease in inoculated and salt treated plants in comparison to non-inoculated plants due to SOD and catalase activity

When rice cultivar GJ17 was studied under salt stress and inoculation with *Pseudomonas pseudoalcaligenes* and *Bacillus pumilus*, the adverse effects of salinity stress were reduced (Jha & Subramanian, 2014). It has been reported in wheat that the dry weight (9.31–24.46%) and fresh weight (7.43– 20.48%) of *Bacillus subtilis* HG-15 inoculated plants were significantly increased (Ji et al., 2022). The MDA content of the plants was also seen to decrease. Inoculation with *B. paralicheniformis* L1C5L, *Pseudomonas sp.* L5C14T, *B. rugosus* L1C7T, and *F. helveticus* L2C1L2 significantly reduced $Na^+$ uptake per plant under sodic stress conditions (Gunasekaran et al., 2022). Inoculation of tomato and rice plants with halotolerant *Staphylococcus sciuri* ET101 isolate resulted in higher net photosynthetic rates than un-inoculated plants grown under salt stress (Taj & Challabathula, 2021). *Bacillus halotolerans*, another

halotolerant bacterium, was found to help in alleviating salt stress through ACC deaminase activity and high antioxidant capacity (Kapadia et al., 2022)

The seedlings from *Bacillus siamensis* primed seeds produced significantly higher fresh and dry weights than untreated seeds at 75 mM and 100 mM salt stress concentrations (Brahim et al., 2024). The proline content in bacteria treated seedlings was considerably higher than the untreated seedlings at all salt stress concentrations. *Methylobacterium oryzae* CBMB20 was seen to improve salt resistance in plants (Rice cultivar IR29 and FL478) indicated by decreased stress volatile emission, ACC accumulation, ACO (ACC oxidase) activity, and increased photosynthesis and vacuolar $H^+$ ATPase activity in response to subsequent exposure of plants to salinity stress (Chatterjee et al., 2019). Inoculation of rice seedlings with strains ST.6 and ST.8 (which are close to *Pantoea dispersa* and *Burkholderia cenocepacia*, respectively) promoted rice growth under both normal and salt-stressed conditions (Do et al., 2023). *Variovorax sp*. strain P1R9 inoculated salt stressed wheat plants showed enhanced germination and decreased lipid peroxidation in comparison to un-inoculated plants (Acuña et al., 2024). The two endophytic bacteria, *Bacillus haynesii* 2P2 and *Bacillus safensis* BTL5 and the rhizospheric bacteria used were *Brevibacterium frigoritolerans* W19 and *Pseudomonas fluorescens* 1001 were used by a research team and found that *B. frigoritolerans* W19 inoculated plant gave the highest chlorophyll and proline in rice in comparison to the control (Gupta et al., 2023).

The survival of salt treated rice plants inoculated with consortia of rhizobacteria was higher than the un-inoculated ones. The combined halotolerant rhizobacterial inoculations showed significantly higher chlorophyll retention as well as yield under the maximum NaCl concentration applied compared to application of single species (Sarker et al., 2023). Bacterial endophytes such as *C. oceanosedimentum* SAK1, *C. luteum*

SAK2, *E. ludwigii* SAK5, *B. cereus* SA1, *M. yunnanensis* SA2 and *E. tabaci* SA3 significantly increased the shoot length of plants (Khan et al., 2020). *Bacillus subtilis* strain significantly improved photosynthetic pigments; reduced $H_2O_2$ and MDA content when compared to un-inoculated control plants (Gul et al., 2023). *Bacillus subtilis* ER-08 (BST) isolate has shown efficiency to alleviate the harmful impacts of drought and salt stress on fenugreek plants. Antioxidant enzymes accumulation was reduced significantly following the BST isolate inoculation. BST inoculation also lowered the $H_2O_2$ and MDA concentrations (Patel et al., 2023).

Bacteria have shown enhanced activity when combined with certain chemicals, such as the study on rice by Wang et al. showed that the shoot height, root length, dry weight, fresh weight and relative water content of NaCl treated rice plants enhanced in the combined application of *B. pumilus* JIZ13 and gamma-aminobutyric acid (GABA) (Wang et al., 2023). MDA and $H_2O_2$ levels in rice plants decreased significantly by the combined application. The bacterial strain *Enterobacter* sp. JIV1 and exogenous putrescine (Put) when used in combination effectively mitigated the inhibitory impact of salt stress simulated by 200 mM NaCl on rice (*Oryza sativa* L.) growth (Ji et al., 2024).

PGPB are also known to stimulate changes in gene expression in the plant. Studies by Tiwari et al. (2017) on rice have shown that *Bacillus amyloliquefaciens* NBRI-SN13 inoculated rice seedlings subjected to salt stress along with showing enhanced proline and soluble sugar content also showed increased expression of *DNH* (dehydrin) and *GST* (glutathione S-transferase) genes in salt stressed plants till 3 h with a ~11-fold induction. Under salt stress, *LEA* (late embryogenesis abundant), *NAM* (no apical meristem) and *GRAM* (glucosyltransferases, Rab-like GTPase activators, myotubularin) genes showed highest expression (~4- to ~5.5-fold) in SN13-inoculated seedlings at 3

and 10 h of stress as compared to control. *B. aryabhattai* MS3 isolated from the wetlands of Bangladesh when inoculated on rice grown under 200 mM salt stress were found to be favored by enhanced expression of *BZ8, SOS1, GIG* and *NHX1* genes (Sultana et al., 2020). *OsNHX* genes play a significant role in the adjustment of Na+ and K+ levels in the rice cytoplasm. *OsNHX1* expression was upregulated under moderate salinity stress of 80 mM NaCl but downregulated under high salinity of 160 mM NaCl (Asif et al., 2023).

The inoculation of *Bacillus oryzicola* YC7007 for 14 days increased the weight of salt-treated seedlings under salt stress 2.2-fold. It also improved salt tolerance by controlling $Na^+$ ion homeostasis. Stress responsive genes were upregulated in inoculated plants. Upon conditions of salt stress, YC7007 alleviated the salt hypersensitivity of only *sos2-1* and *sos3-1* mutants, as reflected by an increase in their fresh weight and number of roots. These data suggest that YC7007 might be involved in salt tolerance through SOS1-dependent signalling. The salt tolerance of *sos2-1* and *sos3-1* mutants by YC7007 was increased, suggesting that the functions of two genes in the same pathway to regulate by YC7007, unlike SOS1 function (Baek et al., 2020).

Studies on salt sensitive rice cultivar *Nipponbare* cv. inoculated with *Azospirillum brasilense* have shown that it improves rice growth under high salt-stress conditions. In *A. brasilense* treated salt-stressed plants, *HKT2* was upregulated in expression, but *HKT1* were not differentially expressed. Gene encoding catalases was seen to be up regulated. There were changes in the key transporter genes, which can be correlated to the morphological changes in the plant (Degon et al., 2023).

# CHAPTER 3

# METHODOLOGY

# 3. Methodology

## 3.1 Materials Used

| Type | Materials/ Chemical Names | Source |
|---|---|---|
| Fine Chemicals | Ethanol, Sodium hypochlorite, Sodium chloride, Sulfosalicylic acid, Ninhydrin, Glacial Acetic Acid (GAA), Toluene, L-proline, Ortho phosphoric acid, Trichloroacetic acid (TCA), Potassium phosphate buffer, Potassium iodide, Hydrogen peroxide, RNA-Xpress Reagent, Chloroform, Isopropanol, Diethyl pyrocarbonate (DEPC), Ethidium bromide (EtBr), Agarose, Tris-EDTA | HiMedia; Duchefa Biochemie; Thermo Scientific; Qualigens |
| Media | Zobell Marine Broth | HiMedia |
| Kits | Bio-Rad iScript™ cDNA Synthesis Kit; Bio-Rad SYBR® green master mix | Bio-Rad |
| Primers | Gene specific | Eurofins Genomics |

## 3.2. Methodology

### 3.2.1. Bacterial Inoculums

The bacterial cultures were procured from Biotechnology Discipline, SBSB, Goa University. The cultures were streaked on 1/4$^{th}$ strength Zobell Marine Agar (Marine Broth 2216 from HiMedia). After the bacteria were grown it was inoculated into 80 mL1/4$^{th}$ strength Zobell Marine Broth which is the primary culture. This was kept overnight on rotary shaker at 120 rpm at 28-30°C. After turbidity was seen in the flask, 0.08 mL of primary culture was transferred to another flask with 80 mL1/4$^{th}$ strength ZMB and incubated till sufficient turbidity was seen. The absorbance was checked at 600 nm for an absorbance of 0.7 to 0.8. After the required absorbance was obtained the entire contents were emptied in a sterile centrifuge tube and centrifuged at 8000 rpm for 10 min. After decanting the supernatant the pellet was washed once with sterile distilled water and dissolved (50% of original volume). This suspension was inoculated onto the plants and incubated for 2 days (Lopes et al., 2021).

### 3.2.2. Plant Materials

IR46 rice seeds were surface sterilized in 75% ethanol for 30 sec and then in 2% v/v Sodium Hypochlorite for 1 min and washing with sterile distilled water for 3 times. The seeds were then soaked for 2 days in sterile distilled water. The germinated seeds were then planted in the surface sterilized pots filled with autoclaved potting mix containing soil and compost (1:1) and transferred to the growth room where the temperature was maintained between 20-25°C and 80% humidity. The plants were watered with sterile distilled water and allowed to grow for 7 days. The pots were labeled as: C for Control, C+S for 150 mM NaCl treated, C+$B_1$ for *Pseudomonas multiresinivorans* treated, C+S+$B_1$ for *Pseudomonas multiresinivorans* and 150 mM NaCl treated, C+$B_2$ for

*Bacillus subtilis* treated, C+S+$B_2$ for *Bacillus subtilis* and 150 mM NaCl treated. On the 7$^{th}$ day the seedlings were treated with bacterial inoculums as per the labeling and kept for 2 days. On the 3$^{rd}$ day the seedlings were treated with 150 mM NaCl and harvested at different time points (0 h, 3 h, 6 h and 24 h), after which they were immediately frozen in liquid nitrogen for nucleic acid extraction.

### 3.2.3. Bio-chemical Analysis

#### *a. Proline Estimation*

The proline accumulation in the rice seedlings was estimated according to Bates et al. (1973). 0.5 g fresh rice seedling samples were homogenized in 3% aqueous sulfosalicylic acid, then instantly centrifuged for 10 min at 10,000 g at 4℃. 1 mL of the supernatant was mixed with 2ml glacial acetic acid and 2ml acid ninhydrin (1:1, v/v). The mixture was then incubated in a hot water bath at 90$^{o}$C for 60 min and immediately cooled on ice to halt the reaction. Then 4 mL of toluene was added to each tube and the color was extracted in it by rigorous shaking. The content of the proline was determined with a spectrophotometer at 520 nm using toluene as the blank. The standards curve was plot using 1 mg/mL stock solution of L-proline from HiMedia RM 061.

#### *b. ROS Estimation*

Fresh leaf samples were used for $H_2O_2$ determination. Hydrogen peroxide level was determined according to Sergiev et al. (1997) method. A 0.5 g of fresh samples was homogenized in liquid nitrogen with 5 mL of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000 g for 15 min. Then, 1 mL of the supernatant was added to 0.5 mL of 10 mM potassium phosphate buffer pH 7.0 and 1 mL of 1 M potassium iodide. The supernatant absorbance was measured at 390 nm with UV/vis spectrophotometer. Hydrogen-peroxide contents were calculated using a standard curve.

### 3.2.4. Morphological Analysis

#### *a. Root and Shoot Length*

After 7 days (before bacterial treatment), 10 days (after bacterial treatment), 11 days (1 day after salt treatment), 18 days (7 days after salt treatment) and 25 days (14 days after salt treatment) the length of root and height of shoot of the rice plants were measured. The plants were taken out from the pots with the roots intact and washed with tap-water and measuring was done with a scale.

#### *b. Dry Weight*

For the determination of the dry weight of seedlings, the plants were harvested after 7 days (before bacterial treatment), 10 days (after bacterial treatment), 11 days (1 day after salt treatment), 18 days (7 days after salt treatment) and 25 days (14 days after salt treatment) and 3 plantlets were used to measure the weight in a petriplate. The plantlets were then dried by oven at 90 ℃ for 24 h and maintained at 60 ℃ for 96 h to obtain dry weight.

### 3.2.5. RNA Extraction and cDNA Synthesis

Total RNA was extracted from the various treatments specified from IR64 using the Trizol method. 0.1 g leaf tissue was homogenised in a mortar and pestle using 1ml RNA-Xpress Reagent (HiMedia) following the manufacturer's instructions. This was followed by centrifugation at 13000 rpm for 15 min at 4℃. To permit the phase separation of nucleoprotein complexes 500µL of chloroform was added to the supernatant, mixed vigorously and allowed to stand for 5 min at room temperature. The resulting mixture was centrifuged at 13000 rpm for 10 min at 4℃. The colourless upper phase containing RNA was transferred to a fresh tube and 500 µL of isopropanol was added, mixed and allowed to stand at room temperature for 15min. The mixture was

later centrifuged at 12000 g for 15 min at 4°C to facilitate RNA precipitation. The pellet obtained was then washed using 1 ml 75% ethanol and centrifuged at 7500 g for 5 min at 4°C. The washing was repeated twice, the supernatant was discarded without disturbing the pellet. The RNA pellet was then air dried at room temperature and dissolved in 25 µL DEPC-treated water. This was followed by quantitative estimation of the total RNA using UV-Vis spectrophotometer at 260 nm and 280 nm. Qualitative estimation was performed using 1% (w/v) agarose gel electrophoresis containing EtBr and visualized under UV transilluminator.

The first-strand cDNA was synthesised using the Bio-Rad iScript™ cDNA Synthesis Kit according to the specified protocol. To a nuclease-free PCR tube, 5 µL of 5x iScript reaction mix, 1 µL iScript reverse transcriptase, 2 µL Oligo$(dT)_{20}$ primer, 1 µg equivalent of the isolated RNA was added and the final volume was made to 20µL. The mixture was then mixed gently followed by incubation at 42°C for 60 min for the primers to anneal and begin extension (reverse transcription). The reaction was then terminated by heating at 85°C for 5 min which heat-inactivates the reverse transcriptase. The product of the first strand cDNA synthesis was either used directly for sqRTPCR/qRTPCR or was stored at -20°C until further use.

### 3.2.6. Primer design for real-time PCR analysis

Specific primers were designed for *MPK3*, *4* and *6* genes on the basis of the genomic sequences of *Oryza sativa* based on the sequences available on National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/). All primers were designed using Primer3 Plus software and synthesized by Eurofins Genomics.

*Methodology*

***a. Primer stock solution***

Tris-EDTA (TE) Buffer was prepared dissolving 15.8 mg TE buffer powder (Duchefa Biochemie) in 10 mL sterile distilled water which was used to prepare 100 µM primer stock solutions. The stock solutions were stored at -20°C until future use. The primers were diluted to 10 µM (5 µL of 100 µM primer stock solution in 45 µL sterile distilled water) to use for qRTPCR analysis.

### 3.2.7. Quantitative Real-time PCR analysis

Real-time analysis of gene expression involved in MAPK signalling pathway and salt tolerance (*MPK3*, *MPK4* and *MPK6*) and the housekeeping gene (Actin) was performed using CFX96TM Real-Time System. Real-time PCR was conducted in a 10µL reaction volume containing, 0.5 µL primers (final concentration µM), 3.5 µL Nuclease-free water, 5 µL Bio-Rad SYBR® green master mix and 1 µL of the diluted synthesized cDNA template. The PCR reaction was performed as follows: Initial denaturation at 95°C for 3 min followed by 39 cycles of denaturation at 95°C for 15 sec, annealing at 48°C for 30 sec and extension at 65°C for 5 sec.

### 3.2.8. Insilico Analysis

The protein sequences of various receptors found in plants and bacterial surface proteins were retrieved from "UniProt" database (https://www.uniprot.org/). Protein BLAST was performed with these sequences in Rice Genome Annotation Project (RGAP) (http://rice.uga.edu/) and NCBI (https://www.ncbi.nlm.nih.gov/) to identify the closest homologs in rice. "ProtPARAM" (https://web.expasy.org/protparam/) tool by Expasy was used to find the chemical composition and iso-electric point of the proteins. The conserved domains in these sequences were identified in NCBI Conserved Domain Database (https://www.ncbi.nlm.nih.gov/cdd/). "STRING" database (https://string-

db.org/) was used to check the interaction with the various MAPKs and other proteins. "STITCH" (http://stitch.embl.de/) database was used to find the ligands that interact with the plant protein receptors. The 3-dimentional structure of the proteins was predicted using "AlphaFold" database (https://alphafold.ebi.ac.uk/).

# CHAPTER 4

# RESULTS AND DISCUSSION

# 4. Results and Discussion

## 4.1. Results

### 4.1.1. Biochemical and morphological analysis

***a. Proline Estimation***

Plants subjected to salt tress induced synthesis and accumulation of proline (Table 4.1). After 2 days of salt treatment the proline content increased in all the plant samples. After 4 days the proline content in C+S was 8.14 µg/ml which is 55% higher than that in C (Fig. 4.1 & 4.2). On the same day proline content spiked up to 11.61 µg/ml in C+S+B1 which is 43% higher than C+S, whereas C, C+B1, C+B2 and C+S+B2 were 5.27, 6.34, 6.82 and 5.92 µg/ml, respectively. After 6 days the proline content in C, C+S, C+B1, C+B2, C+S+B1 and C+S+B2 was 3.77, 3.77, 4.01, 4.17, 12.56 and 4.73µg/ml, respectively. There was further 8% increase in proline in C+S+B1 in comparison with that of 4 days. In comparison to C+S there was two times more increase in the proline content. After 6 days of salt treatment the proline content in C, C+S, C+B1, C+B2, C+S+B1 and C+S+B2 were 5.45, 4.55, 3.35, 4.19, 4.07, and 7.96µg/ml, respectively. There was a 75% increase in proline content in C+S+B2 in comparison with C+S. The proline content in C+S+B1 drastically decreased to 4.07µg/ml from a 12.56µg/ml peak two days prior.

***b. ROS Estimation***

Salinity stress mostly triggers the production of reactive oxygen species (ROS) and modulates plant growth and physiology. ROS accumulation in plants was determined through hydrogen peroxide ($H_2O_2$) estimation in leaf tissue (Table 4.2). After 1 day of salt treatment the $H_2O_2$ content in C+S increased by 16% in comparison to C (Fig. 4.3

**Table 4.1.: Proline accumulation in PGPB and salt treated IR64**

| Treatments | Proline Concentration ($\mu$g/mL) | | | |
|---|---|---|---|---|
| | Day 2 | Day 4 | Day 6 | Day 10 |
| **C** | 4.97±0.0316 | 5.27±0.0542 | 3.77±0.0687 | 5.45±0.0525 |
| **C+S** | 4.79±0.0274 | 8.14±0.0011 | 3.77±0.0409 | 4.55±0.0334 |
| **C+B1** | 3.77±0.0065 | 6.34±0.0017 | 4.01±0.0207 | 3.35±0.0271 |
| **C+B2** | 5.03±0.0164 | 6.82±0.0011 | 4.07±0.0710 | 4.19±0.0698 |
| **C+S+B1** | 6.04±0.0104 | 11.61±0.0017 | 12.56±0.0565 | 4.07±0.0254 |
| **C+S+B2** | 4.07±0.0085 | 5.92±0.0017 | 4.73±0.0132 | 7.96±0.0178 |

**Table 4.2.: $H_2O_2$ accumulation in PGPB and Salt treated IR64**

| Treatments | $H_2O_2$ Concentration ($\mu$g/ml) | | | | |
|---|---|---|---|---|---|
| | Before bacteria | After bacteria | 1 day after salt | 7 days after salt | 14 days after salt |
| **C** | 0.15±0.001 | 0.46±0.0049 | 10.40±0.028 | 13.41±0.010 | 9.04±0.011 |
| **C+S** | 0.46±0.0035 | 0.61±0.0057 | 12.66±0.001 | 19.88±0.016 | 14.31±0.015 |
| **C+B1** | 1.06±0.0036 | 1.81±0.0103 | 11.30±0.000 | 17.17±0.015 | 8.14±0.016 |
| **C+B2** | 0.30±0.0025 | 3.02±0.0138 | 12.05±0.028 | 21.09±0.012 | 7.08±0.017 |
| **C+S+B1** | 0.30±0.0028 | 2.26±0.0115 | 12.81±0.009 | 16.57±0.006 | 7.68±0.022 |
| **C+S+B2** | 0.91±0.001 | 2.41±0.0118 | 11.00±0.004 | 21.09±0.005 | 11.30±0.014 |

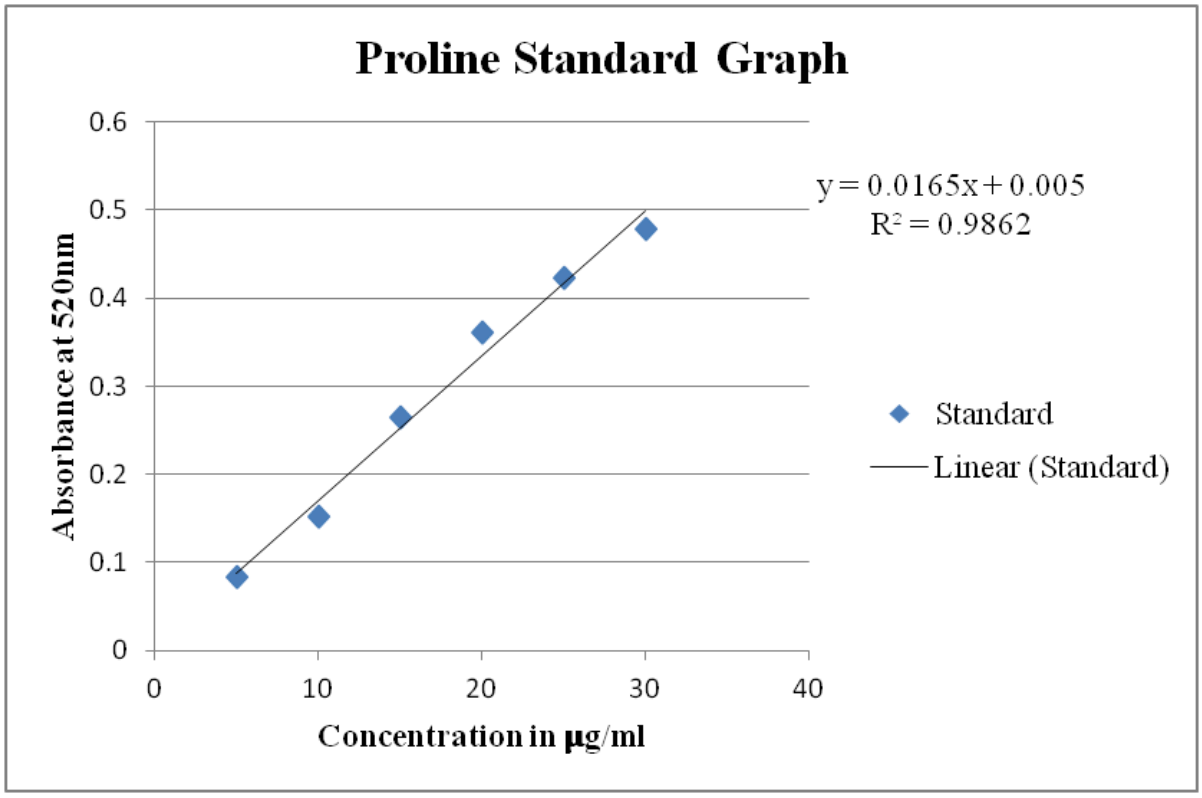



**Fig. 4.1: Standard graph for total proline content**

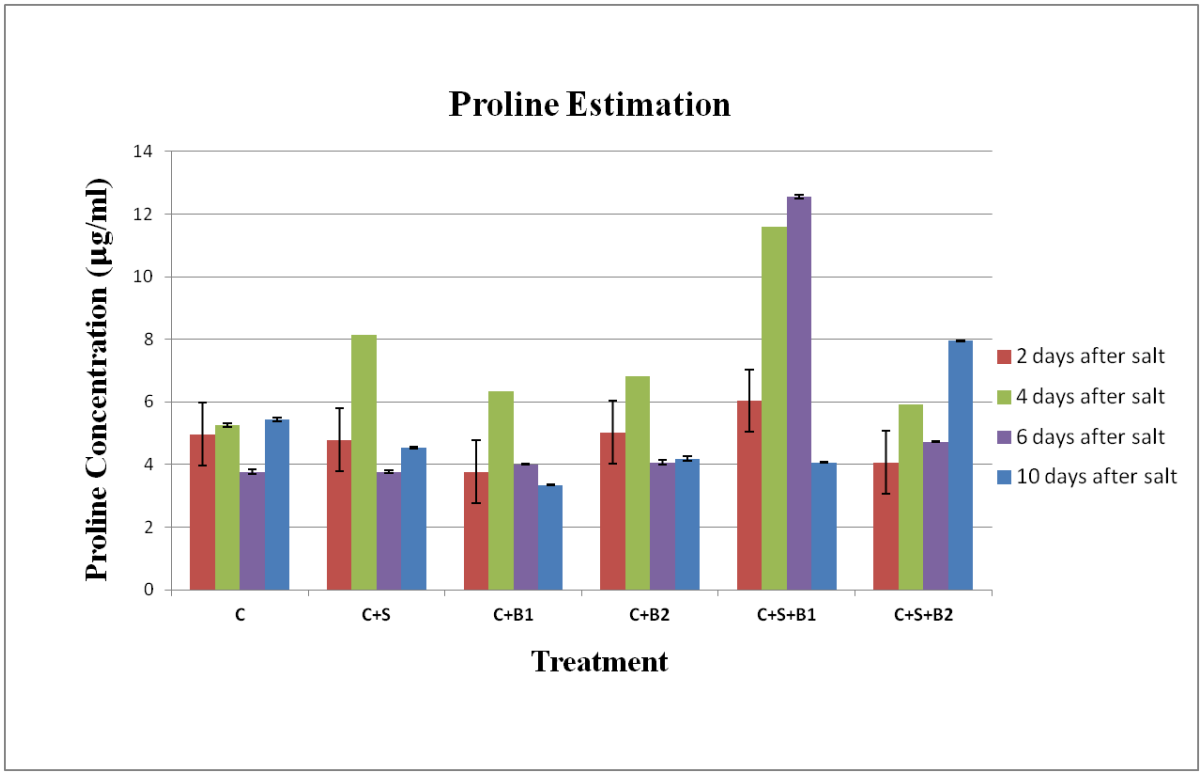



**Fig. 4.2: Relative accumulation of proline in PGPB and salt treated IR64.** Differential accumulation of proline is observed in the treatments where inoculated plants have shown considerable increase in proline content.

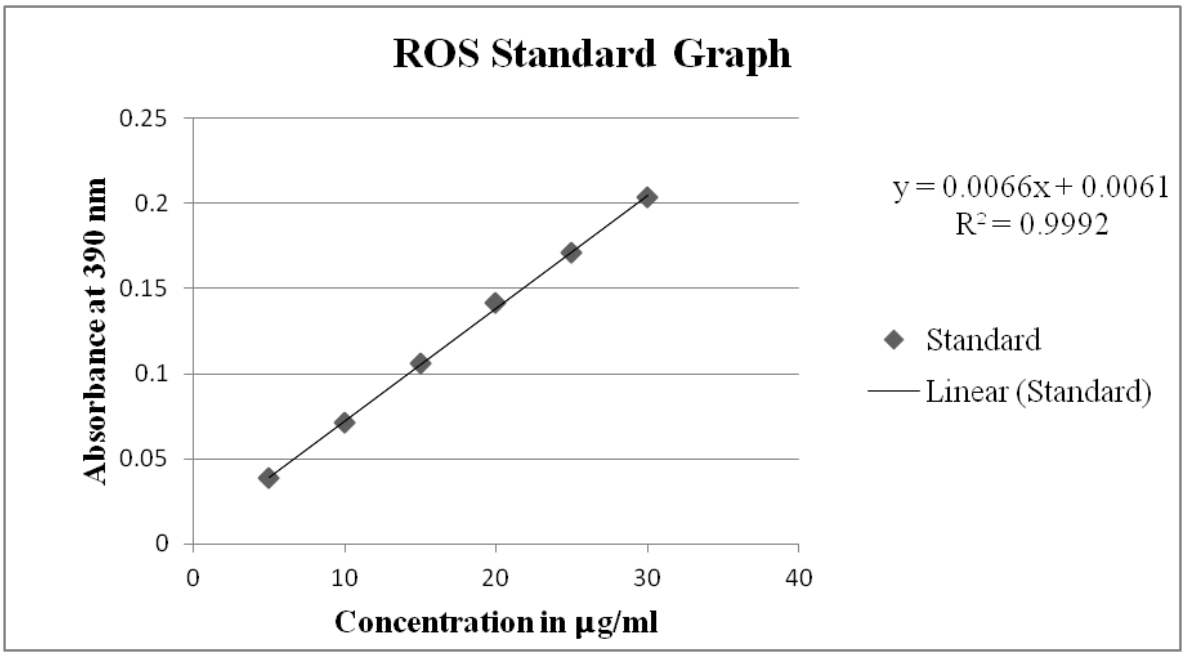



**Fig. 4.3: Standard graph of total H2O2 content**

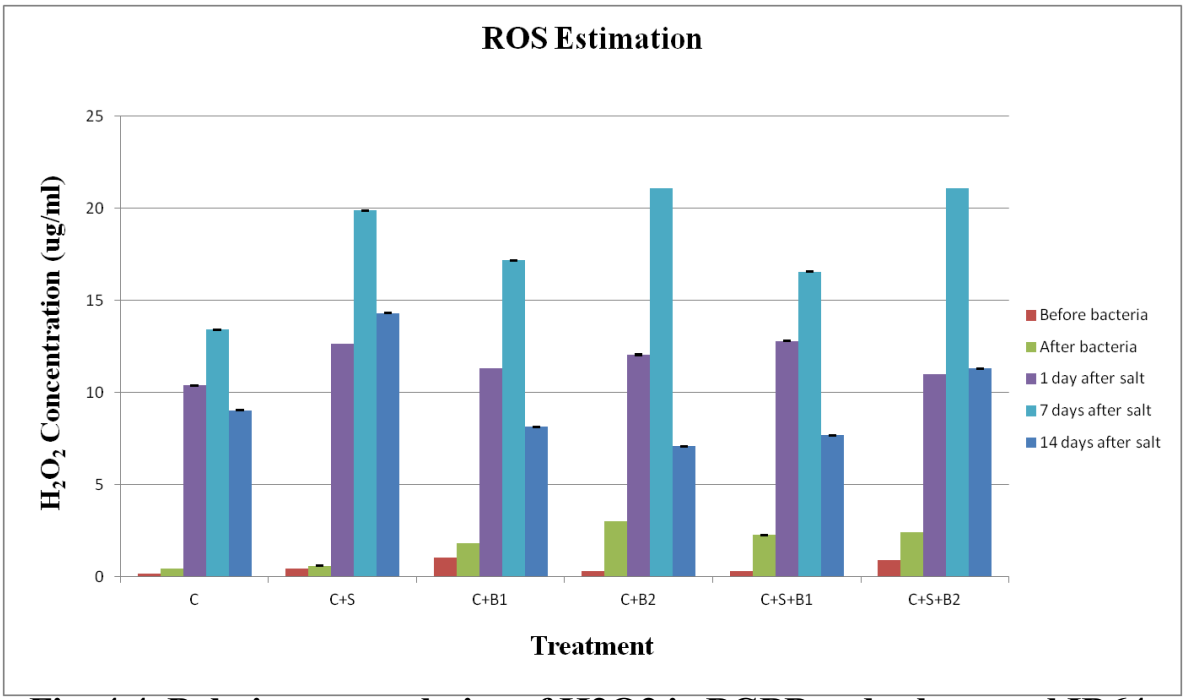



**Fig. 4.4: Relative accumulation of H2O2 in PGPB and salt treated IR64.**

The concentration of ROS decreased in inoculated plant samples after 14 days of salt treatment.

& 4.4). There was 23% and 6% increase in $H_2O_2$ in C+S+B1and C+S+B2, respectively, in comparison to C. After 7 days of salt treatment the $H_2O_2$ content in C+B2 and C+S+B2 was 21.09µg/ml which is unexpectedly 6% higher than that in C+S. There was 17% reduction in $H_2O_2$ content in C+S+B1 in comparison to C+S. After 14 days $H_2O_2$ content significantly decreased in bacteria treated plants where, C+S+B1 was 7.68µg/ml and C+S+B2 was 11.30µg/ml which is 46% and 21%, respectively, less than C+S.

***c. Shoot-Root Length***

The shoot-root length are and indicative of plant growth parameters and biomass. The shoot-root length was seen to be consistent till the inoculation with bacteria. After the inoculation of bacteria the length of shoot and root increased in comparison with the un-inoculated control (Fig. 4.9). After one day of salt treatment there was no significant difference in the length (Table 4.3 & 4.4). After 7 days of salt treatment there was 16% and 11% decrease in root and shoot length, respectively, in comparison with C (Fig. 4.5 & 4.6). The shoot length of C+S+B1 increased by 15% and the root length increased by 8% in comparison with C+S. The shoot and root length of C+S+B2 increased by 18% in comparison to C+S. Shoot and root length of C+B1 increased 5% and 2%, respectively, whereas in C+B2 the shoot and root length decreased by 5% and 1% respectively. After 14 days of salt treatment, the shoot and root length of C+S+B1 increased by 13% and 8% respectively in comparison with C+S. The shoot and root length of C+S+B2 increased by 3% and 15%, respectively in comparison with C+S. The shoot and root length of C+B1 increased by 4% in comparison with C whereas, there was no significant difference observed in C+B2.

***d. Dry Weight***

There were no significant differences in the dry weight of the plants in the initial phases of growth and bacterial inoculation (Table 4.5). After 7 days of salt treatment, C+S+B1

## Table 4.3.: Shoot length in PGPB and Salt treated IR64

| Treatments | Shoot Length in cm | | | | |
|---|---|---|---|---|---|
| | Before bacteria | After bacteria | 1 day after salt | 7 days after salt | 14 days after salt |
| **C** | 14.3±3.09 | 25.6±2.28 | 25.8±2.18 | 28.5±1.70 | 29.2±2.15 |
| **C+S** | 16.5±3.22 | 23±2.47 | 21.3±2.38 | 23.8±1.69 | 24.5±2.25 |
| **C+B1** | 22.8±3.46 | 27.3±2.35 | 26.3±1.17 | 29.8±1.03 | 30.4±1.91 |
| **C+B2** | 17±3.71 | 25.2±2.46 | 25.3±1.04 | 27±0.95 | 29.3±1.57 |
| **C+S+B1** | 21.5±4.38 | 28.2±2.5 | 28.1±1.34 | 27.3±1.36 | 27.8±1.28 |
| **C+S+B2** | 23.1±3.56 | 26.4±2.83 | 25.6±1.77 | 28.1±2.07 | 25.2±1.75 |

## Table 4.4: Root length in PGPB and Salt treated IR64

| Treatments | Root Length in cm | | | | |
|---|---|---|---|---|---|
| | Before bacteria | After bacteria | 1 day after salt | 7 days after salt | 14 days after salt |
| **C** | 3.1±0.55 | 8.2±1.35 | 8.2±0.90 | 9.3±0.86 | 9.2±0.91 |
| **C+S** | 3.3±0.59 | 7.9±1.42 | 6.7±0.87 | 8.3±0.89 | 7.8±0.96 |
| **C+B1** | 3.5±0.57 | 7.3±1.47 | 6.8±0.80 | 9.5±0.93 | 9.6±0.61 |
| **C+B2** | 3.9±0.36 | 9.8±1.37 | 8.6±0.50 | 9.2±0.74 | 9.1±0.64 |
| **C+S+B1** | 3.8±2.79 | 10.8±1.47 | 7.7±0.70 | 9±0.67 | 8.4±0.37 |
| **C+S+B2** | 3.9±2.47 | 9.8±1.29 | 9.1±0.61 | 9.8±1.30 | 9±0.25 |

## Table 4.5.: Dry weight in PGPB and Salt treated IR64

| Treatments | Dry weight in grams | | | | |
|---|---|---|---|---|---|
| | Before bacteria | After bacteria | 1 day after salt | 7 days after salt | 14 days after salt |
| C | 0.0136± 0.0020 | 0.0236± 0.0011 | 0.0239± 0.0017 | 0.0566± 0.0030 | 0.0629± 0.0019 |
| C+S | 0.0133± 0.0011 | 0.0279± 0.0026 | 0.0279± 0.0026 | 0.0323± 0.0085 | 0.0333± 0.0342 |
| C+B1 | 0.0146± 0.0015 | 0.0173± 0.0046 | 0.0176± 0.0049 | 0.0656± 0.0047 | 0.0730± 0.0130 |
| C+B2 | 0.0176± 0.0015 | 0.0249± 0.0045 | 0.0249± 0.0045 | 0.0509± 0.0026 | 0.0543± 0.0080 |
| C+S+B1 | 0.0149± 0.0009 | 0.0266± 0.0040 | 0.0266± 0.0040 | 0.0446± 0.0015 | 0.0569± 0.0062 |
| C+S+B2 | 0.0223± 0.0005 | 0.0259± 0.0039 | 0.0259± 0.0039 | 0.0439± 0.0085 | 0.0556± 0.0049 |

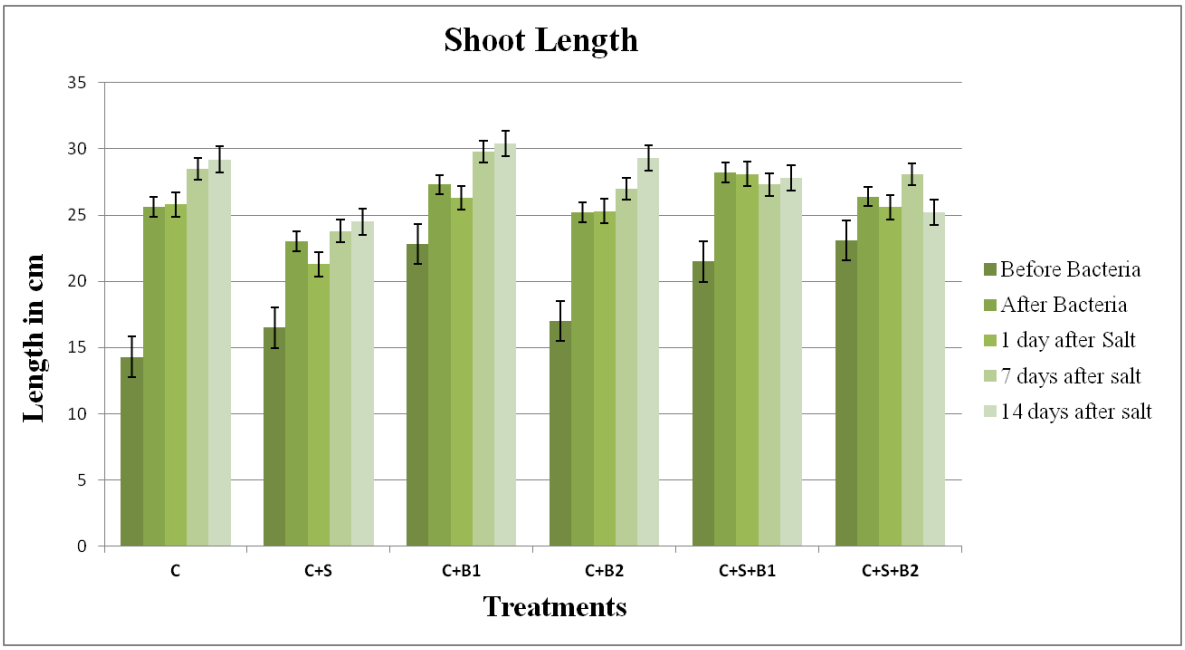



**Fig. 4.5: Relative shoot length in PGPB and salt treated IR64.**
The shoot length of the inoculated plants is higher then that of un-inoculated plants. C+S shows the shortest length.

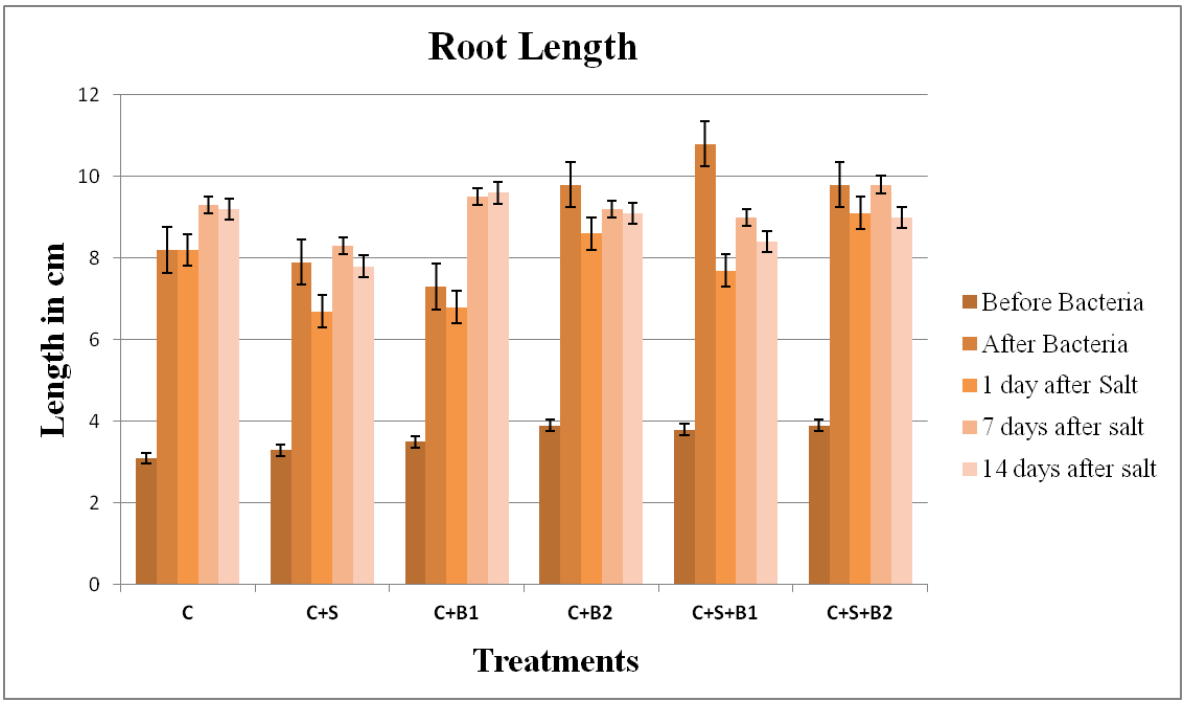



**Fig. 4.6: Relative root length in PGPB and salt treated IR64.**
The root length of the inoculated plants is higher then that of un-inoculated plants.

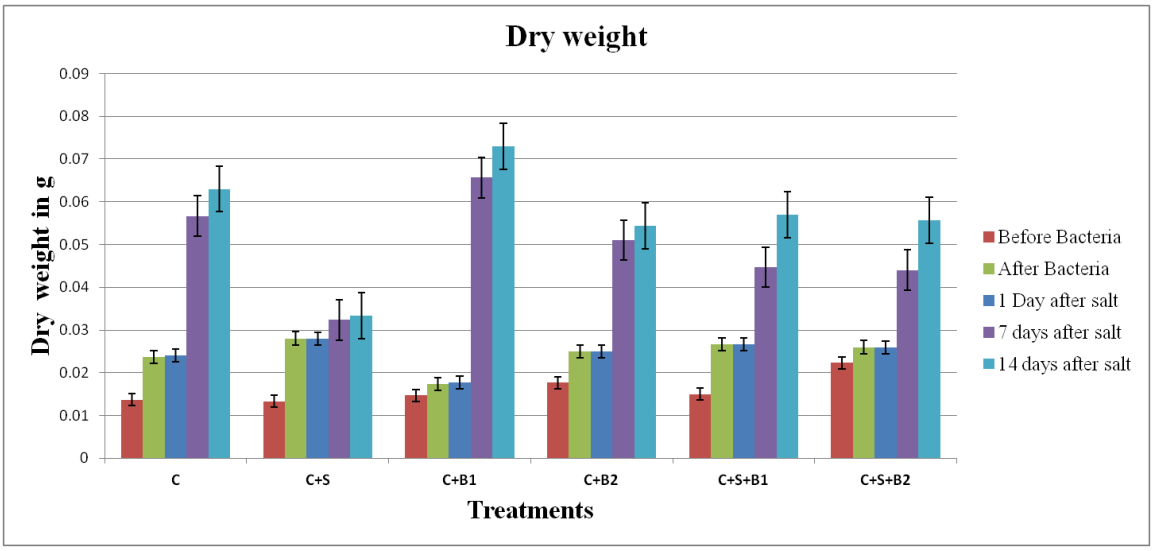



**Fig. 4.7: Relative dry weight in PGPB and salt treated IR64.** Dry weight of inoculated plants is higher in comparison to un-inoculated plants.

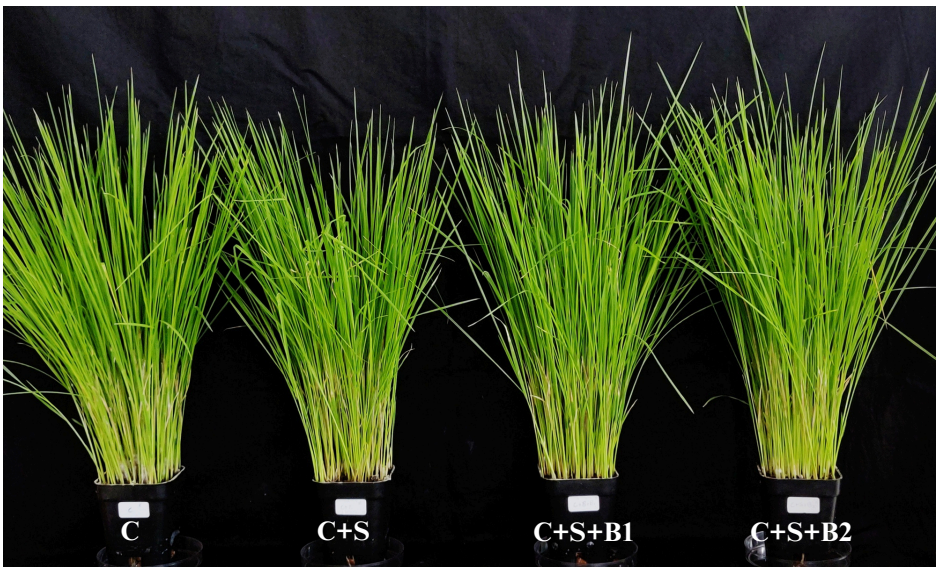



**Fig. 4.8: Growth of IR64 Rice seedlings in PGPB and salt treated IR64**

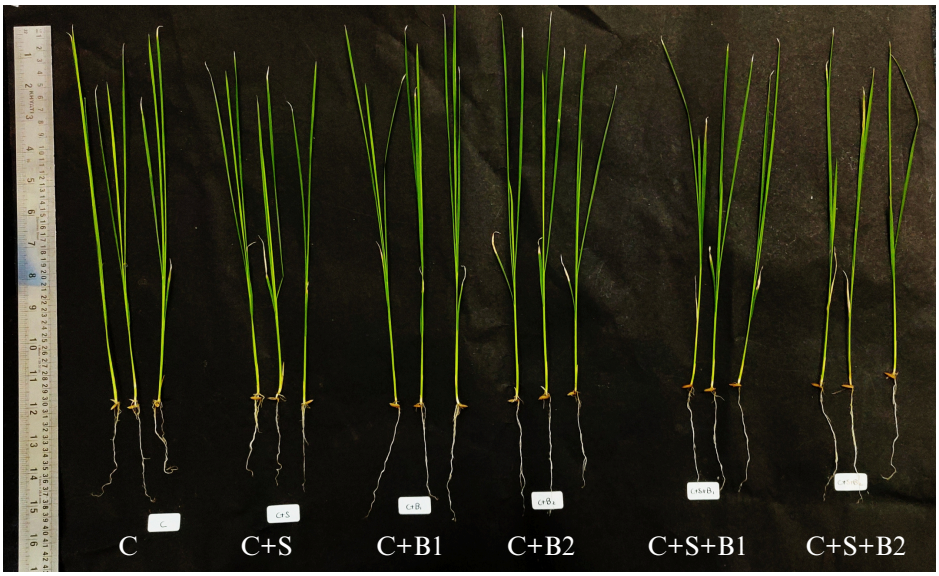



**Fig. 4.9: Shoot and root length of IR64 Rice seedlings in PGPB and salt treated IR64**

showed 38% increase in dry weight in comparison with C+S and 21% decrease in comparison with C. C+S+B2 showed 36% increase in dry weight in comparison with C+S and 22% decrease in comparison with C (Fig. 4.7). After 14 days of salt treatment, C+S+B1 showed 71% increase in dry weight in comparison with C+S and 10% decrease in comparison with C. C+S+B2 showed only 2% increase in dry weight in comparison with C+S and 12% decrease in comparison with C.

### 4.1.2. Gene expression analysis

In order to understand the role of MAPKs in alleviating salt stress in rice the gene expression analysis was done. The rice samples treated with ABSK11 were selected based on its superior plant growth promoting activity.

For this analysis the isolated RNA which showed clear and intact bands for 28S, 18S and 5.8S (Fig. 4.10) upon agarose gel electrophoresis, indicating that the RNA was not degraded and suitable for cDNA synthesis. The synthesized cDNA was then used for the gene expression analysis (Fig. 4.11). The gene expression analysis gave us an idea into the signalling process in which takes place in the plant relative to the different treatments.

C+S+B1 0h showed 6.25 and 6.73 times increase in *MPK3* and *MPK6* (Fig. 4.12 A & C), respectively, whereas there was only 2.67 times increase in *MPK4* in comparison to the control (Fig. 4.12 B). After 3h hours of salt treatment C+S 3h showed 3-fold increase in *MPK3* (Fig. 4.12 A), 6-fold increase in *MPK4* (Fig. 4.12 B) and 8-fold increase in *MPK6* (Fig. 4.12 C). In C+S+B1 3h it was observed that there was 3.53 times increase in *MPK3* expression in comparison C+S 3h; 0.89 times decrease in *MPK4* and 1.15 times increase in *MPK6*. After 6h of salt treatment C+S+B1 6h showed equilibrium with that of C+S 6h for *MPK3*, whereas there was 1.66 times decrease in

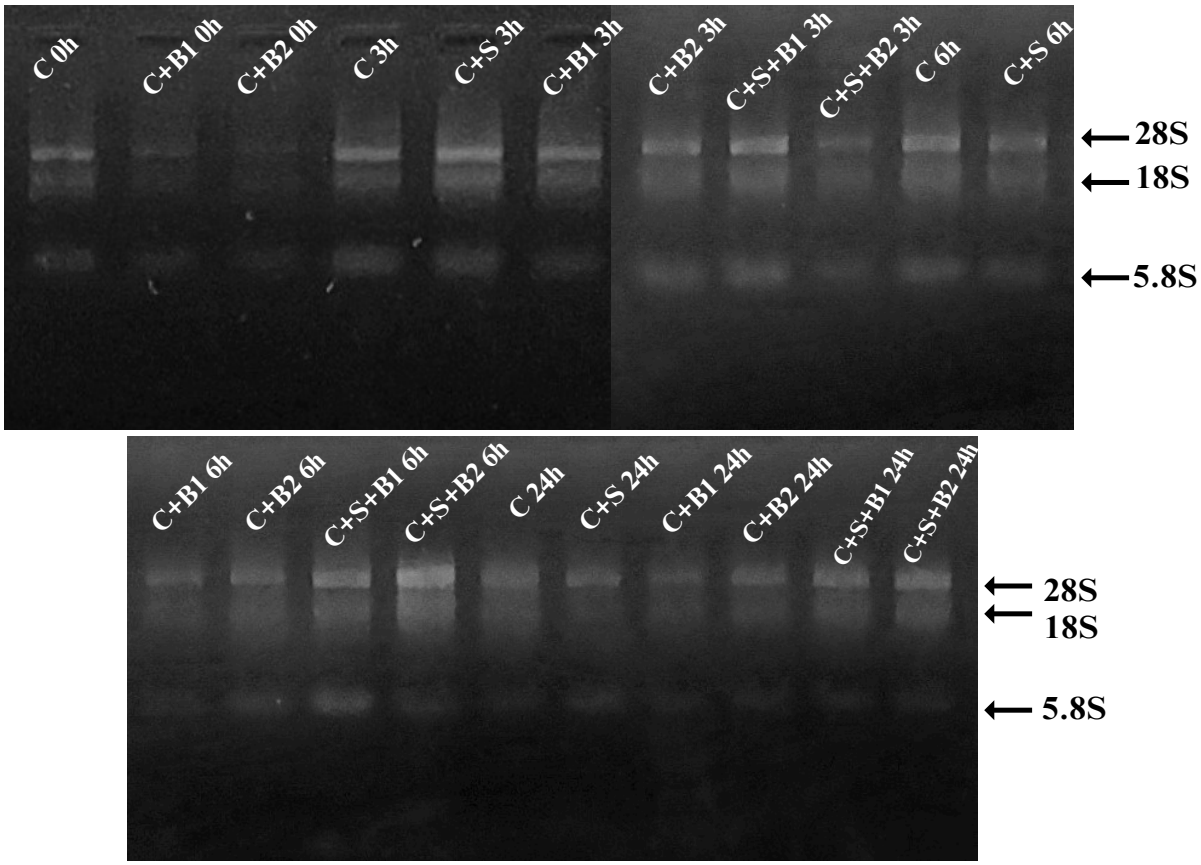



**Fig. 4.10: Agarose gel electrophoresis of total RNA from IR64.** Clear separation of 28S, 18S and 5.8S RNA is seen which is indicative of undigested RNA.

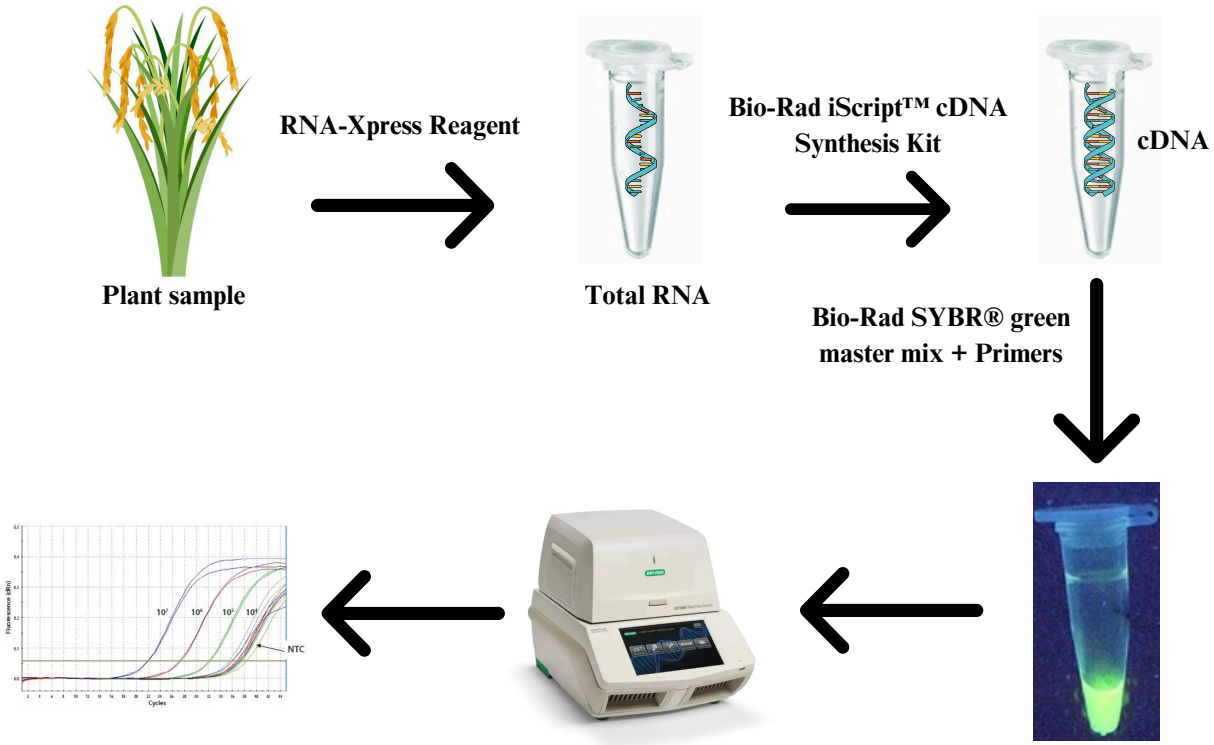



**Fig. 4.11: Work flow for gene expression analysis.** Total RNA is extracted using RNA-Xpress Reagent, then cDNA is synthesized using Bio-Rad iScript™ cDNA Synthesis Kit. The cDNA is used as template for qRTPCR analysis using Bio-Rad SYBR® green master mix along with various primers. The reading is taken using Bio-Ras CFX96TM Real-Time System

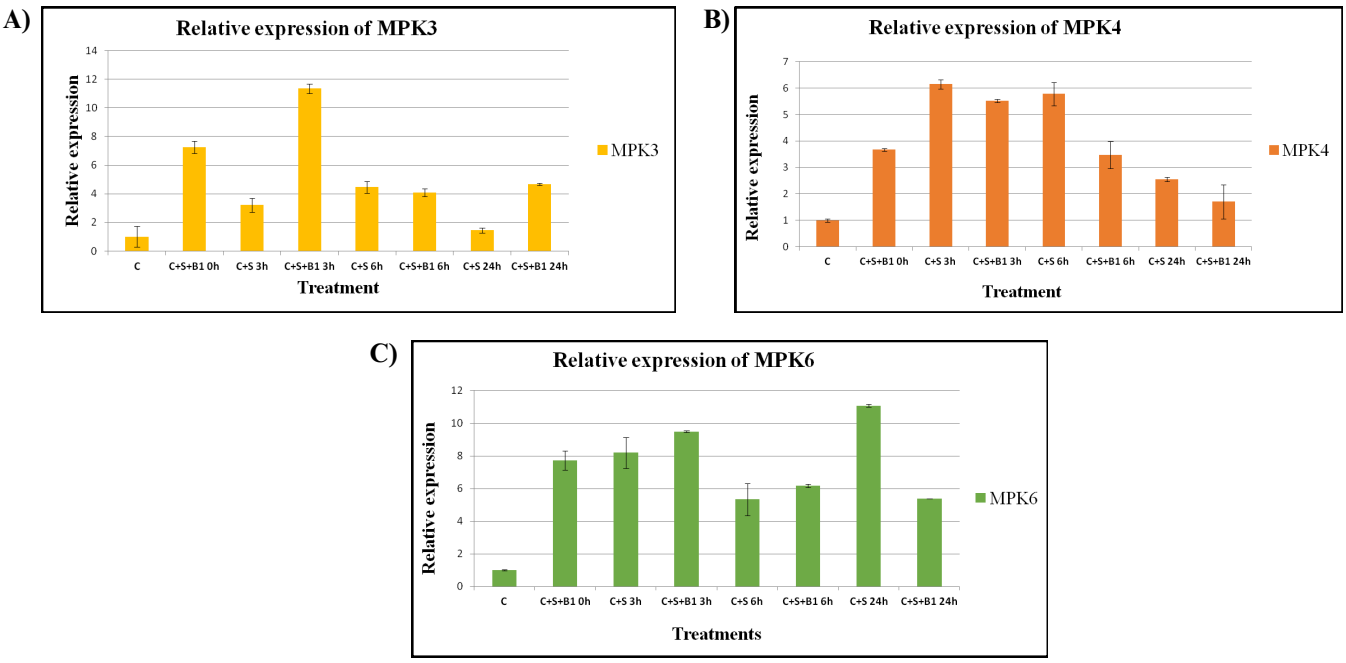



**Fig. 4.12: Comparison of relative expression of A) *MPK3*, B) *MPK4* and C) *MPK6* under different treatments relative to actin in rice.** *MPK3* and *MPK6* show enhanced expression in inoculated plants.

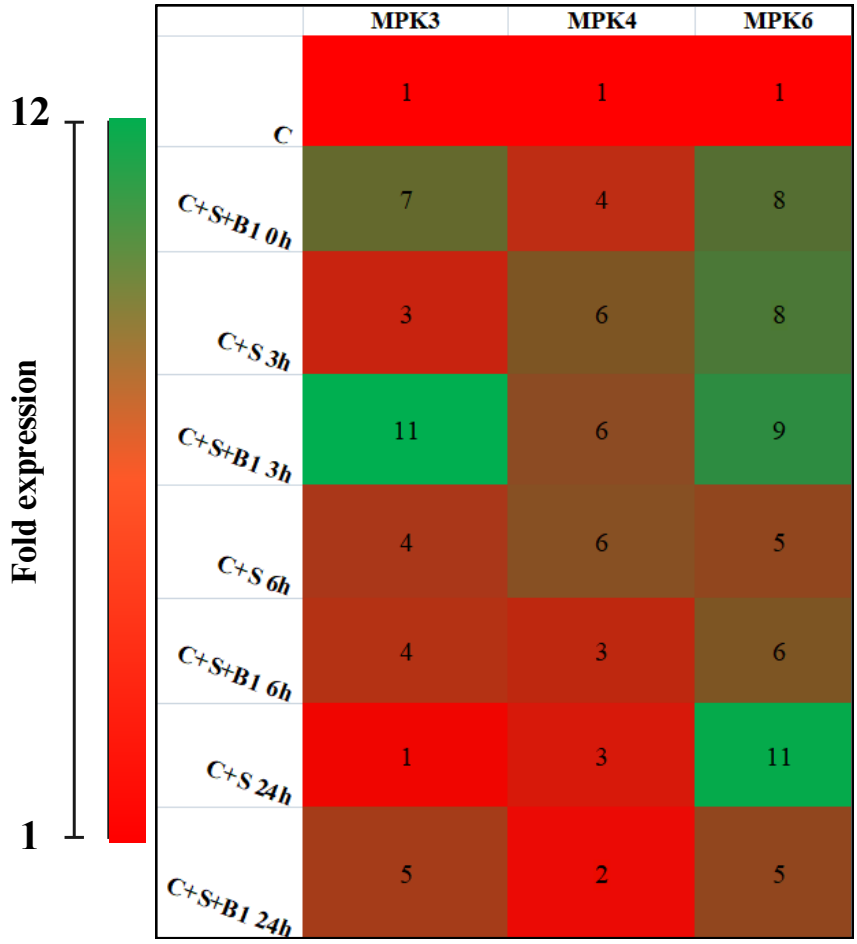



**Fig. 4.13: Heat map of the relative gene expression**

C+S+B1 6h for *MPK4* and 1.15 times increase in C+S+B1 6h for *MPK6*. After 24h of salt treatment, in comparison to C+S 24h, there was 3.20 times increase in C+S+B1 24h for MPK3; 1.49 times decrease for *MPK4* and 2.06 times decrease for *MPK6*.

The highest expression was found for *MPK3* in C+S+B1 (Fig. 4.12 A). For *MPK3* there was an increase in expression after bacterial treatment which went high in comparison to the control. After salt treatment there was a peak attained after 3h of treatment which was seen to decrease after 24h of treatment. The difference in the inoculated and un-inoculated plants was prominently visible. For *MPK4* the expression increased in C+S plants and reached a peak at 3h and decreased close to the initial expression level. In C+S+B1 there was slightly lesser expression observed in comparison to C+S. For *MPK6* there was an increase in expression for un-inoculated plants till C+S 3h and then decreased to surprisingly peak at C+S 24h. In the inoculated plants there was an increase in expression as seen in C+S+B1 3h and then decreased.

### 4.1.3. *Insilico* analysis

The data from previous research on receptor proteins has given knowledge about their sequences. Along with this our analysis on the signalling cascade has also given data with regards to its expression levels. This verifies that MAPK is one of the signalling components in these plants. To receive the signals from extracellular environments the rice plant will have certain receptors on its surface. The plant receptor proteins in *Oryza sativa* and *Arabidopsis thaliana* were identified from present literature and searched in the "UniProt" database (https://www.uniprot.org/) (Table 4.6). These retrieved sequences were then used for protein BLAST in RGAP and NCBI to find their homologs in *Oryza sativa*, where a total of 26 proteins were identified (Table 4.6). The homologs with lowest *E*-value and highest similarity percentage were identified and

## Table 4.6.: Homologs of receptors proteins in Rice

| Sr. No. | Receptor Protein | Accession no. in UniProt | Homologs in RGAP | Homologs in NCBI |
|---|---|---|---|---|
| **1** | BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 | Q94F62 | LOC_Os04g38480 | NP_001409376.1 |
| **2** | Chitin elicitor receptor kinase 1 | A8R7E6 | LOC_Os08g42580 | EEC83957.1 |
| **3** | Chitin elicitor-binding protein | Q8H8C7 | LOC_Os03g04110 | BAE95828.1 |
| **4** | Ethylene response sensor 1 | Q38846 | LOC_Os03g49500 | NP_001405436.1 |
| **5** | Ethylene-insensitive protein 2 | Q9S814 | LOC_Os07g06130 | AAQ95276.1 |
| **6** | G-type lectin S-receptor-like serine/threonine-protein kinase | 9LPZ9 | LOC_Os07g36590 | XP_015613801.1 |
| **7** | Leucine-rich repeat receptor-like protein kinase PEPR1 | Q9SSL9 | LOC_Os08g34640 | KAB8108705.1 |
| **8** | Leucine-rich repeat receptor-like protein kinase PEPR2 | 9FZ59 | LOC_Os08g34640 | KAB8108705.1 |
| **9** | Leucine-rich repeat receptor-like protein kinase TDR | Q9FII5 | LOC_Os08g05290 | EAZ41508.1 |
| **10** | LRR receptor kinase SERK2 | Q67X31 | LOC_Os06g12120 | XP_015643547.1 |
| **11** | LRR receptor-like serine/threonine-protein kinase EFR | C0LGT6 | LOC_Os12g42520 | XP_015620319.1 |
| **12** | LRR receptor-like serine/threonine-protein kinase FLS2 | Q9FL28 | LOC_Os04g52780 | EEC78020.1 |

| | | | | |
|---|---|---|---|---|
| **13** | LysM domain receptor-like kinase 3 | F4IB81 | LOC_Os01g53840 | EEC71457.1 |
| **14** | LysM domain receptor-like kinase 4 | O64825 | LOC_Os02g09960 | EAY84835.1 |
| **15** | MDIS1-interacting receptor like kinase 2 | Q8VZG8 | LOC_Os02g34790 | BAF25976.2 |
| **16** | Nodulation receptor kinase | Q8L4H4 | LOC_Os07g38070 | BAF21940.1 |
| **17** | Probable serine/threonine-protein kinase PBL2 | O49839 | LOC_Os03g07430 | XP_015630811.1 |
| **18** | Probable serine/threonine-protein kinase PBL3 | O49840 | LOC_Os03g07430 | XP_015630811.1 |
| **19** | Protein BRASSINOSTEROID INSENSITIVE 1 | O22476 | LOC_Os01g52050 | KAB8083235.1 |
| **20** | Protein EIN4 | Q9ZTP3 | LOC_Os04g08740 | NP_001389262.1 |
| **21** | Protein LYK5 | O22808 | LOC_Os02g09960 | XP_015623069.1 |
| **22** | Receptor like protein 23 | O48849 | LOC_Os08g42580 | EEC83957.1 |
| **23** | Senescence-induced receptor-like serine/threonine-protein kinase | O64483 | LOC_Os05g44990 | EAY98753.1 |
| **24** | Serine/threonine-protein kinase BIK1 | O48814 | LOC_Os05g02020 | KAB8097796.1 |
| **25** | Serine/threonine-protein kinase RIPK | Q9ZUF4 | LOC_Os03g08170 | XP_015630876.1 |
| **26** | Toll/interleukin-1 receptor-like protein | Q9SSN3 | LOC_Os07g37950 | No significant similarity found |

**Table 4.7.: Conserved domains in receptor protein homologs and their chemical properties**

| Sr. No. | Receptor Protein | Conserved Domains | No. of Amino Acids | Molecular Weight | pI |
|---|---|---|---|---|---|
| **1** | BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 | Protein Kinases, catalytic domain<br>Leucine rich repeat N-terminal domain | 624 | 68701.53 | 5.88 |
| **2** | Chitin elicitor receptor kinase 1 | Protein Kinases, catalytic domain | 640 | 68890.60 | 5.72 |
| **3** | Chitin elicitor-binding protein | Lysin Motif | 382 | 40080.58 | 8.47 |
| **4** | Ethylene response sensor 1 | GAF domain<br>hybrid sensory histidine kinase BarA | 636 | 70683.28 | 6.54 |
| **5** | Ethylene-insensitive protein 2 | Natural resistance-associated macrophage protein | 1281 | 138203.54 | 5.92 |
| **6** | G-type lectin S-receptor-like serine/threonine-protein kinase | B_lectin<br>Protein Kinases, catalytic domain<br>PAN/APPLE-like domain<br>S-locus glycoprotein domain | 813 | 90204.47 | 5.72 |
| **7** | Leucine-rich repeat receptor-like protein kinase PEPR1 | Leucine-rich repeat receptor-like protein kinase | 1112 | 121376.84 | 6.24 |

| | | | | | |
|---|---|---|---|---|---|
| **8** | Leucine-rich repeat receptor-like protein kinase PEPR2 | Leucine-rich repeat receptor-like protein kinase | 1112 | 121376.84 | 6.24 |
| **9** | Leucine-rich repeat receptor-like protein kinase TDR | Leucine-rich repeat receptor-like protein kinase | 1001 | 105477.61 | 6.63 |
| **10** | LRR receptor kinase SERK2 | Leucine-rich repeat receptor-like protein kinase<br>Protein Kinases, catalytic domain | 616 | 67721.28 | 5.74 |
| **11** | LRR receptor-like serine/threonine-protein kinase EFR | Leucine-rich repeat receptor-like protein kinase | 1054 | 114581.48 | 5.94 |
| **12** | LRR receptor-like serine/threonine-protein kinase FLS2 | Leucine-rich repeat receptor-like protein kinase | 1139 | 121052.46 | 6.50 |
| **13** | LysM domain receptor-like kinase 3 | Protein Kinases, catalytic domain<br>Lysin Motif | 687 | 73397.73 | 6.29 |
| **14** | LysM domain receptor-like kinase 4 | Protein Kinases, catalytic domain<br>Lysin Motif | 689 | 71466.93 | 5.81 |
| **15** | MDIS1-interacting receptor like kinase 2 | Leucine-rich repeat receptor-like protein kinase | 1092 | 120248.48 | 7.60 |
| **16** | Nodulation receptor kinase | Protein Kinases, catalytic domain | 609 | 66791.66 | 6.72 |
| **17** | Probable serine/threonine-protein kinase PBL2 | Protein Kinases, catalytic domain | 423 | 46511.04 | 9.56 |

| | | | | | |
|---|---|---|---|---|---|
| **18** | Probable serine/threonine-protein kinase PBL3 | Protein Kinases, catalytic domain | 423 | 46511.04 | 9.56 |
| **19** | Protein BRASSINOSTEROID INSENSITIVE 1 | protein phosphatase 1 regulatory subunit 42<br>leucine-rich repeat receptor-like kinase | 1121 | 120250.14 | 5.76 |
| **20** | Protein EIN4 | GAF domain<br>Histidine Kinase A domain<br>Histidine kinase-like ATPase domain<br>phosphoacceptor receiver (REC) domain | 763 | 84832.23 | 5.96 |
| **21** | Protein LYK5 | Protein Kinases, catalytic domain<br>Lysin Motif | 689 | 71436.86 | 5.81 |
| **22** | Receptor like protein 23 | Protein Kinases, catalytic domain | 640 | 68890.60 | 5.72 |
| **23** | Senescence-induced receptor-like serine/threonine-protein kinase | Malectin-like domain<br>leucine-rich repeat receptor-like protein kinase<br>Protein Kinases, catalytic domain | 912 | 99571.03 | 6.96 |
| **24** | Serine/threonine-protein kinase BIK1 | Protein Kinases, catalytic domain | 395 | 43210.21 | 9.63 |
| **25** | Serine/threonine-protein kinase RIPK | Protein Kinases, catalytic domain | 425 | 46318.94 | 9.39 |
| **26** | Toll/interleukin-1 receptor-like protein | ---------- | ---------- | -------- | ------ |

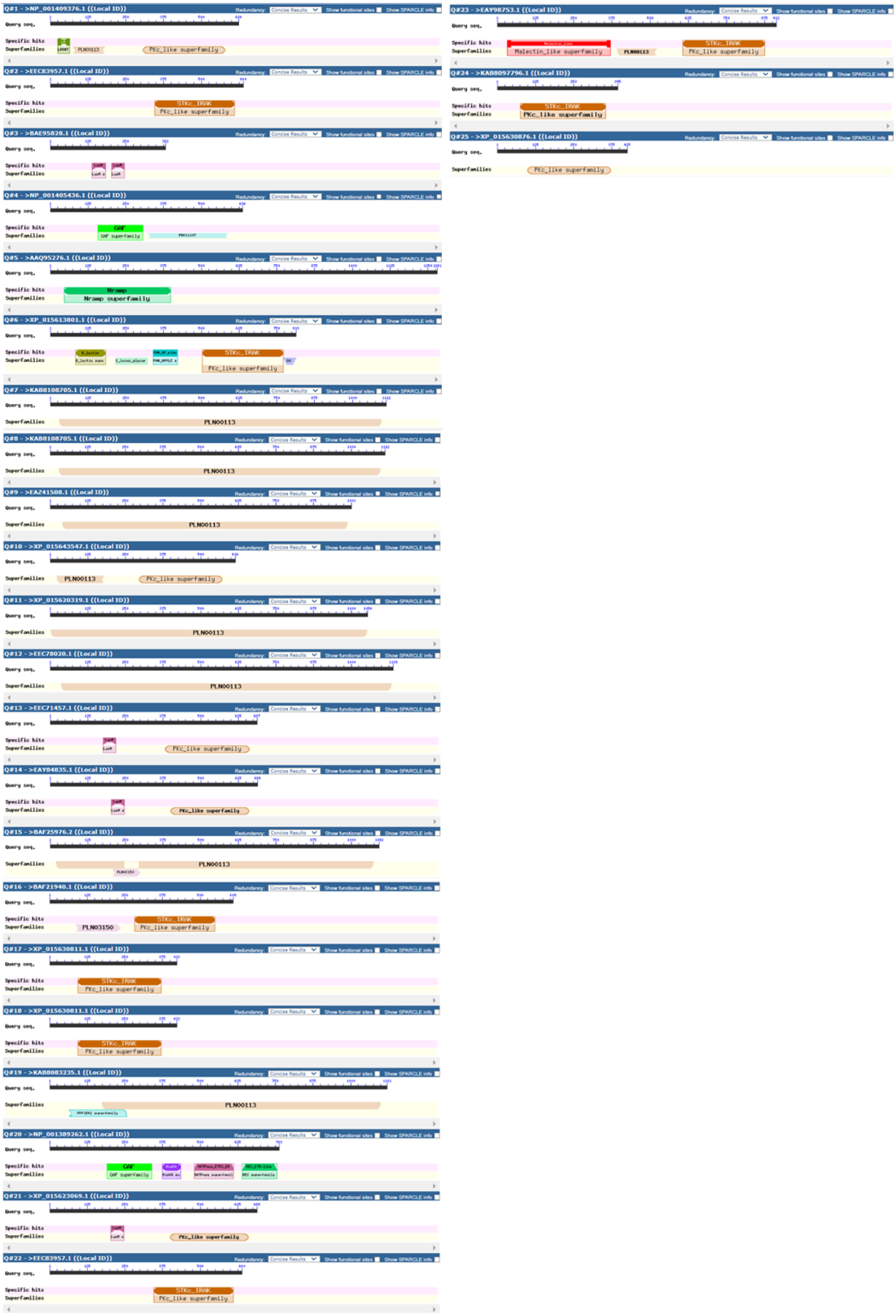



**Fig. 4.14: Conserved domains in the receptor protein homologs in Rice**

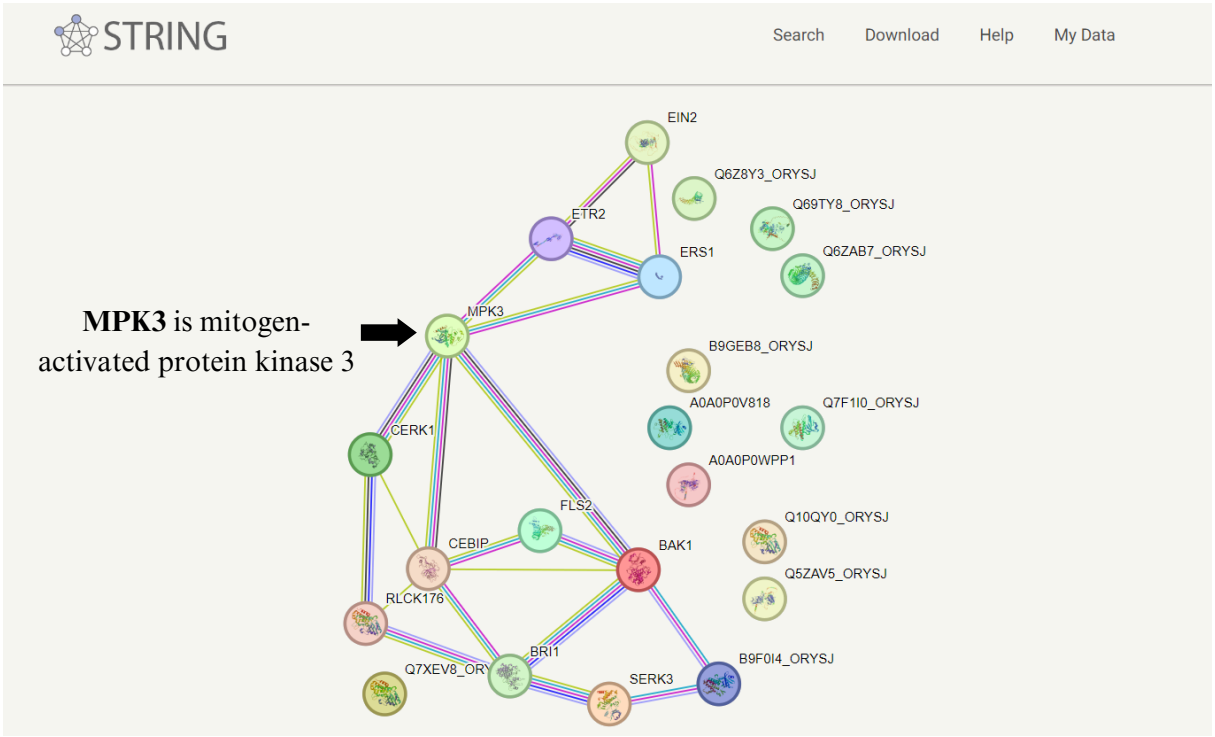



**Fig. 4.15: Protein-Protein interaction of OsMPK3 with different receptor proteins in Rice**

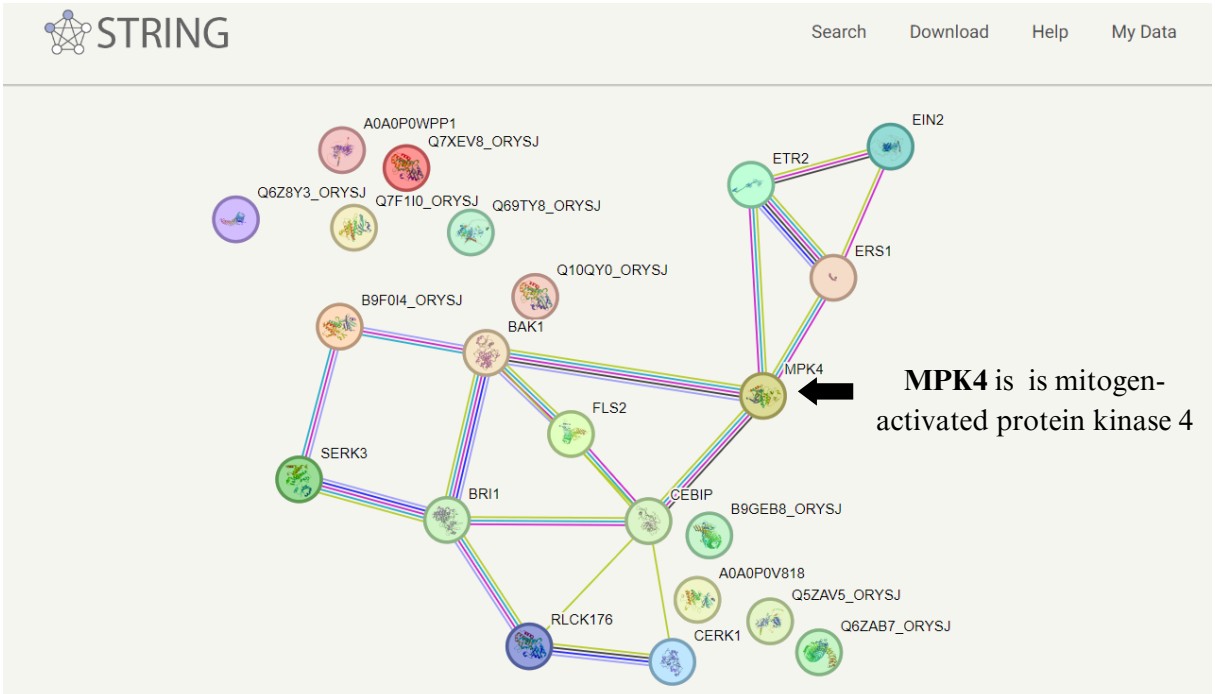



**Fig. 4.16: Protein-Protein interaction of OsMPK4 with different receptor proteins in Rice**

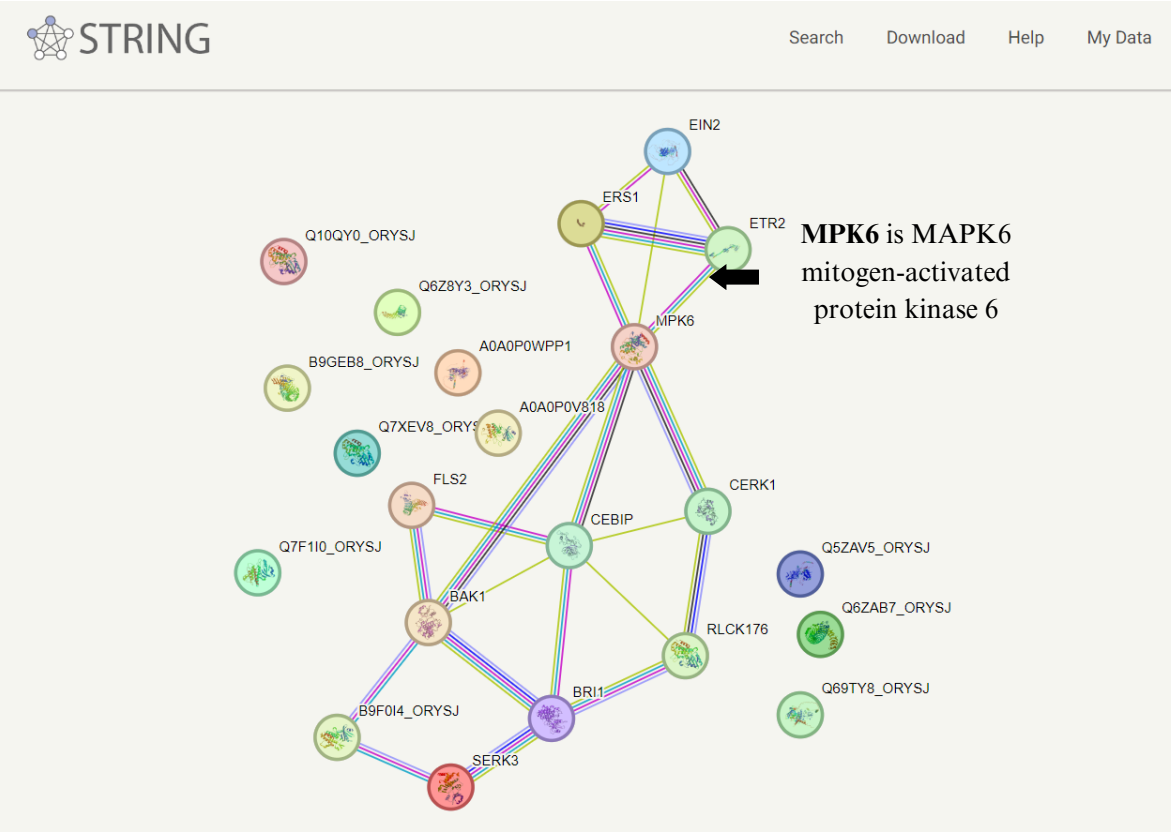
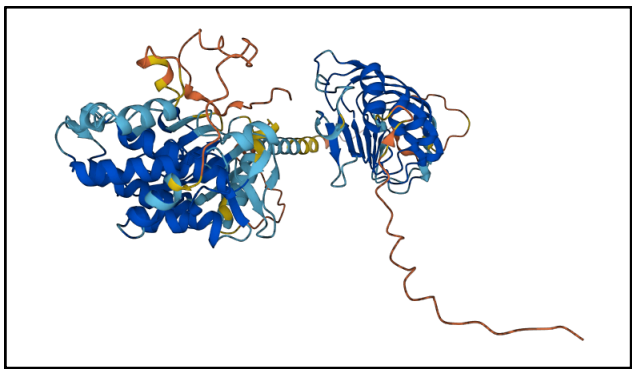
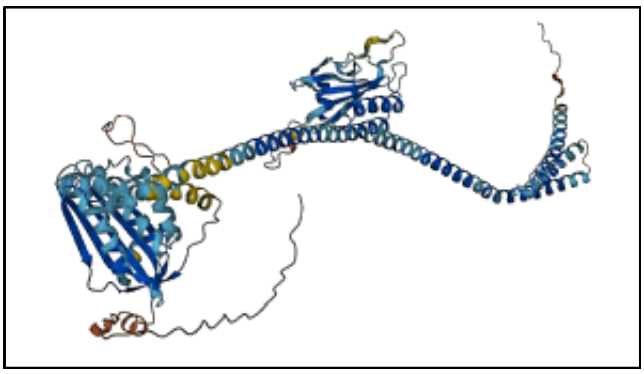


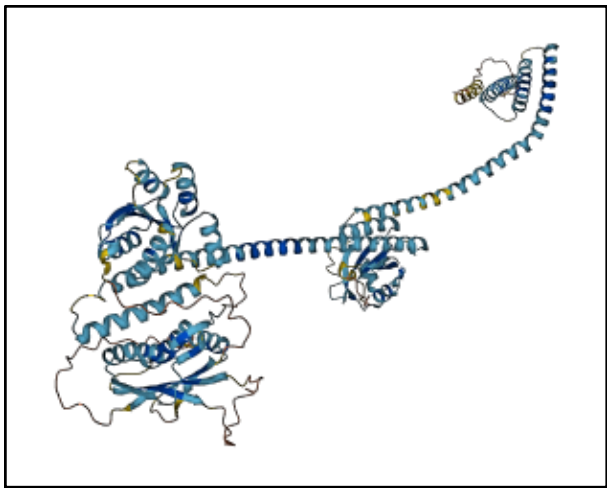

**Fig. 4.17: Protein-Protein interaction of OsMPK6 with different receptor proteins in Rice**



**BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1**

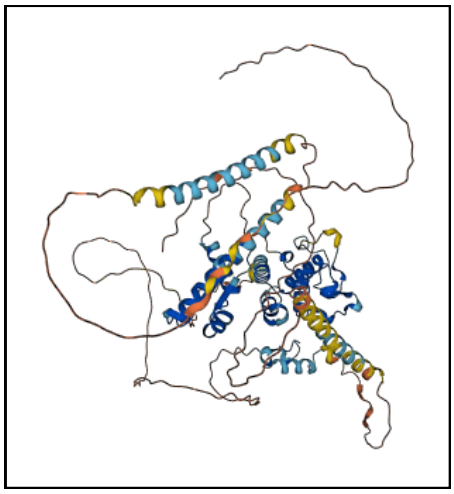


**Ethylene response sensor 1**



**Protein EIN4**

**Chitin elicitor receptor kinase 1**



**Ethylene-insensitive protein 2**

**Fig. 4.18: Three dimensional structures of the receptor protein homologs in Rice interacting with OsMPK proteins.**

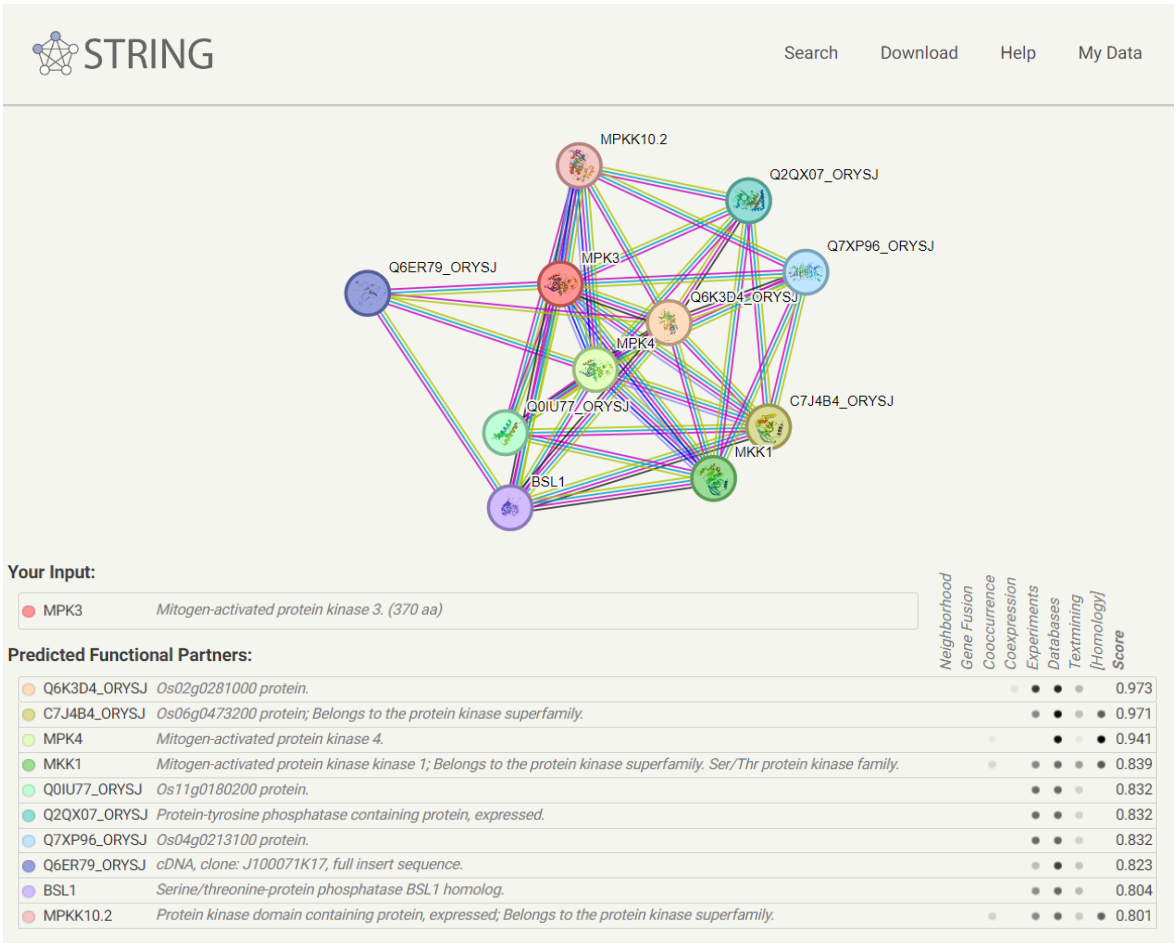



**Fig. 4.19: Protein-Protein interaction of OsMPK3 with proteins in Rice**

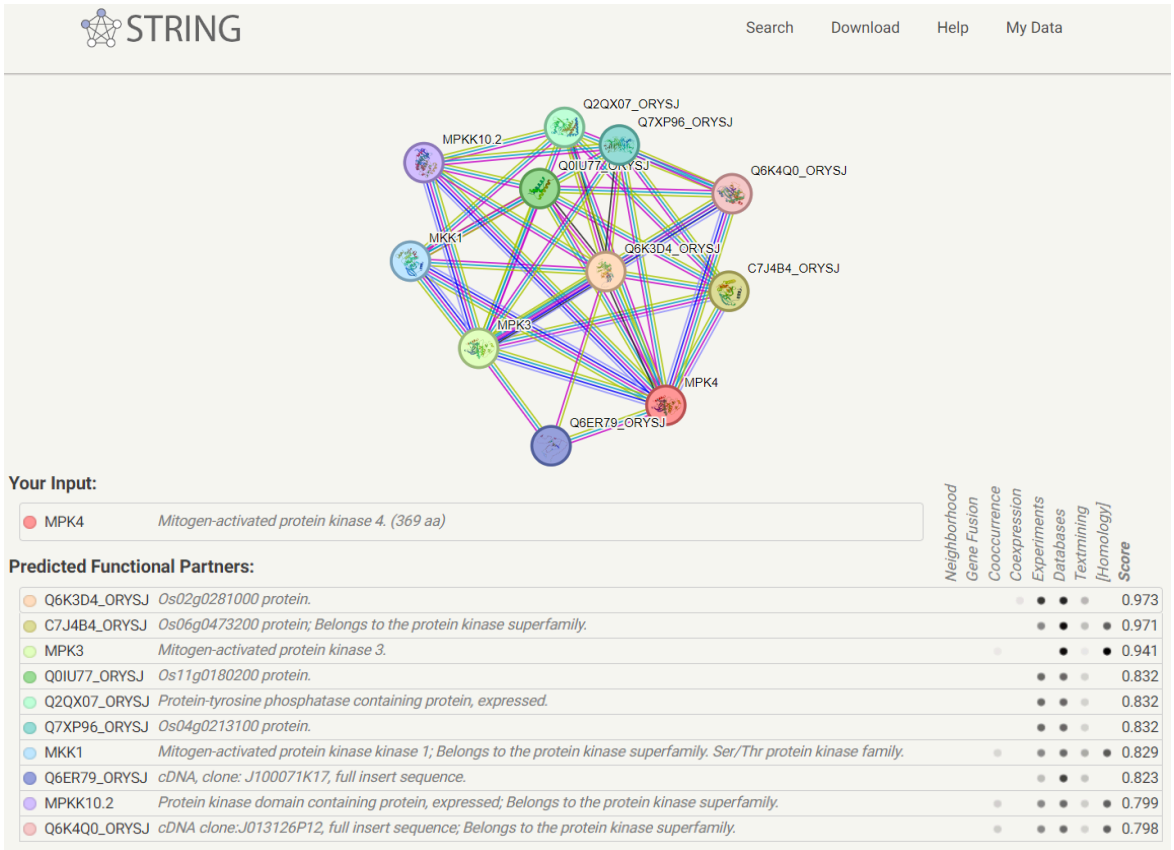



**Fig. 4.20: Protein-Protein interaction of OsMPK4 with proteins in Rice**

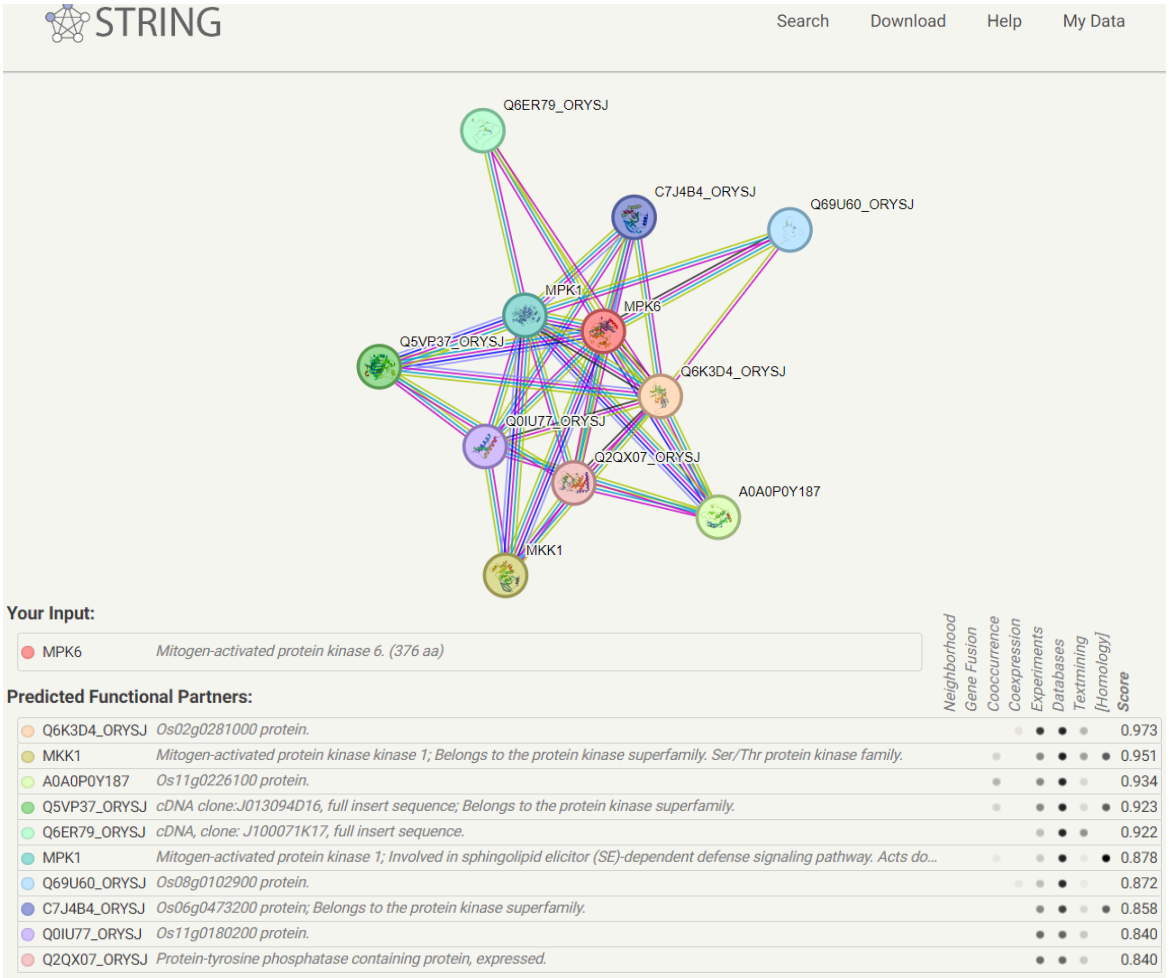



**Fig. 4.21: Protein-Protein interaction of OsMPK6 with proteins in Rice**

used for further analysis. Among all the 26 receptors identified, there was no homolog found for Toll/interleukin-1 receptor-like protein.

The conserved domains within these sequences was found using NCBI Conserved Domain Database (https://www.ncbi.nlm.nih.gov/cdd/) (Fig. 4.14). The major domain present and conserved in most of the sequences is the leucine-rich domain and the protein kinase domain, which suggests that they may be involved in a sequential phosphorylation mechanism such as MAPK cascade (Table 4.7). "ProtPARAM" (https://web.expasy.org/protparam/) tool by Expasy was used to find the chemical composition and iso-electric point of the proteins (Table 4.7).

The three dimensional structure of the homologs in *Oryza sativa* was predicted using "AlphaFold" database (https://alphafold.ebi.ac.uk/). The structure of most proteins showed prominent leucine-rich domains which are constitutive of a receptor protein (Fig. 4.18). The protein-protein interaction was studied using "STRING" database (https://string-db.org/). Among the receptor proteins that were identified it was found that MPK3 interacts with Protein EIN4, Ethylene receptor 1, Chitin elicitor receptor kinase 1, Chitin elicitor-binding protein and BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 (Fig. 4.15); MPK4 interacts with Protein EIN4, Ethylene receptor 1, Chitin elicitor-binding protein and BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 (Fig. 4.16) and MPK6 interacts with Protein EIN4, Ethylene receptor 1, Ethylene-insensitive protein 2, Chitin elicitor receptor kinase 1 and BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 (Fig. 4.17). When checked for interaction with MAPK proteins MPK3 is found to interact with MPK4, MKK1 and MPKK10.2 (Fig. 4.19); MPK4 interacts with MPK3, MKK1 and MPKK10.2 (Fig. 4.20); MPK6 interacts with MKK1 and MPK1 (Fig. 4.21). Uncharacterized protein kinase superfamily proteins interacting with MPK3 is

**Table 4.8: Various kinase domain containing proteins interacting with the receptor homologs**

| Sr. No. | Receptor Protein | Interacting kinase proteins |
|---|---|---|
| **1** | BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 | Q7XPI1_ORYSJ and Q0DZD2_ORYSJ |
| **2** | Chitin elicitor receptor kinase 1 | Osl_32002, Osl_21625, Osl_21981, Osl_08557 and Osl_02814 |
| **3** | Chitin elicitor-binding protein | CERK1, RLCK185, CERK, XA21, FLS2 and MPK5 |
| **4** | Ethylene response sensor 1 | Osl_07476 and Osl_17347 |
| **5** | Ethylene-insensitive protein 2 | ---------- |
| **6** | G-type lectin S-receptor-like serine/threonine-protein kinase | A0A0E0FWD3 |
| **7** | Leucine-rich repeat receptor-like protein kinase PEPR1 | ---------- |
| **8** | Leucine-rich repeat receptor-like protein kinase PEPR2 | ---------- |
| **9** | Leucine-rich repeat receptor-like protein kinase TDR | A0A0E0I7R, A0A0E0H1M8 and A0A0E0HMX5 |
| **10** | LRR receptor kinase SERK2 | BR1, Q7XPI1_ORYSJ and Q0DZD2_ORYSJ |
| **11** | LRR receptor-like serine/threonine-protein kinase EFR | ---------- |
| **12** | LRR receptor-like serine/threonine-protein kinase FLS2 | Osl_10836, Osl_13190, Osl_09091 and BAK1 |
| **13** | LysM domain receptor-like kinase 3 | ---------- |
| **14** | LysM domain receptor-like kinase 4 | A0A0E0FWD3 and A0A0E0HTZ3 |
| **15** | MDIS1-interacting receptor like kinase 2 | Osl_17982, Osl_34730, Osl_22227, Osl_16145 and Osl_09868 |

| | | |
|---|---|---|
| **16** | Nodulation receptor kinase | ---------- |
| **17** | Probable serine/threonine-protein kinase PBL2 | Osl_27334, SAPK2, Osl_11876, Osl_07615, Osl_13649 and Osl_18368 |
| **18** | Probable serine/threonine-protein kinase PBL3 | Osl_27334, SAPK2, Osl_11876, Osl_07615, Osl_13649 and Osl_18368 |
| **19** | Protein BRASSINOSTEROID INSENSITIVE 1 | BAK1, BSK3, Q10S67_ORYSJ, BSK1-2, SERK2, Q8S7N5_ORYSJ and SERK3 |
| **20** | Protein EIN4 | ---------- |
| **21** | Protein LYK5 | A0A0E0FWD3 and A0A0E0HTZ3 |
| **22** | Receptor like protein 23 | Osl_32002, Osl_06757, Osl_21981, Osl_08557, Osl_02814 |
| **23** | Senescence-induced receptor-like serine/threonine-protein kinase | Osl_09663 and Osl_19502 |
| **24** | Serine/threonine-protein kinase BIK1 | Osl_03528, Osl_21981, Osl_02814 and Osl_18856 |
| **25** | Serine/threonine-protein kinase RIPK | Q8RUE8_ORYSJ |

C7J4B4_ORYSJ; MPK4 are C7J4B4_ORYSJ and Q6K4Q0_ORYSJ and MPK6 are C7J4B4_ORYSJ and Q5VP37_ORYSJ. C7J4B4_ORYSJ and Q5VP37_ORYSJ are mitogen-activated protein kinase kinase proteins (Table 4.8). The receptor protein sequences were individually screened for their interaction with other kinase proteins and 61 interactions were identified.

## 4.2. Discussion

In the present study, the previously isolated salt tolerant bacterial strains (*Pseudomonas multiresinivorans* ABSK11 and *Bacillus subtilis* ABSK186) from the salt pans of Ribandar, Goa were screened for their plant growth promoting attributes in salt sensitive rice variety IR64 in the presence of salt stress in *in vitro*. It has been shown that various plant growth attributes including plant height, root length, dry weight, proline content, antioxidant enzyme activity is reduced in non-inoculated salt treated plants as compared to bacterial inoculated plants (Fig. 4.12). There is effective enhancement in plant growth attributes in inoculated plants as compared to non-inoculated control (Zhang et al., 2018; Ansari et al., 2019).

Plants are known to show higher accumulation of proline as an adaptation to salt stress (Omari, 2021). In ABSK11 inoculated salt treated plants proline content was higher than that in the salt treated control plant after 4 days of salt treatment. In the same plant after 6 days the proline content increased to give the highest peak among all the considered treatments. After which the proline content was seen to decrease on the $10^{th}$ day of salt treatment. In ABSK186 inoculated salt treated plant the proline content remained consistent with the salt treated control plant. There was a significant increase in proline content observed on the $10^{th}$ day of inoculation, suggesting that the proline accumulation activity of ABSK186 is delayed than that of ABSK11. The difference in

performance in the two different bacterial strains is similar to the findings of Sahu et al. (2020). Δ1-pyrroline-5-carboxylate synthetase is a proline synthesis enzyme encoded by *P5CS* gene which has been shown to be upregulated when MKK2 (a MAPKK) is overexpressed and the downstream targets of which are MPK3/6 (Teige at al., 2004, Verma et al., 2019). This can be the possible cause for the increase levels of proline in the inoculated plants. The upstream of MKK2 may be some receptor which interacts with the bacterial signalling components and starts a pathway that provides increased level of the osmoprotectant.

ROS accumulation is widely reported in plants under salt stress (Kesawat et al., 2023). $H_2O_2$ has been is one of these species which is known to reacts with DNA, oxidizes proteins and forms HO• and $O_2^-$•, it further reacts with proteins by attacking methionine and cysteine residues and also with heme proteins. Plants have shown increase in the scavenging capacity of ROS by production of CAT, SOD and POD with bacterial treated plants than untreated plants under salinity stress (Habib et al., 2016; Shobana et al., 2020). In ABSK11 inoculated salt treated plants the ROS levels are observed to decrease after 7 days of salt treatment in relation to un-inoculated plants. Here further decrease is observed after 14 days of salt treatment. A peculiar trend is observed in ABSK186 inoculated plant without salt where the ROS content is seen to rise after bacterial treatment. The same is observed when these plants are treated with salt where ROS concentration reaches a peak. But significant decrease in ROS is observed after 14 days of salt treatment and bacterial inoculation. These findings can be correlated research showing that $H_2O_2$ is crucial for plants' defense mechanisms under biotic stress. $H_2O_2$ is well established for impeding the growth and survival of plant pathogens, which further limit infection transmission (Sahu et al., 2022). The rice plant will have sensed ABSK186 as a pathogenic bacterial strain and thus the accumulation

of $H_2O_2$. There are two phases in ROS production: the first phase is the rapid, transient, nonspecific ROS production, and the second phase occurs later, where the concentration of ROS is much higher than the first (Abdul et al., 2020). This was observed in the $H_2O_2$ estimation where a sudden increase was seen and then decreased, possible due to activation of salt tolerant responses like SERF1, SIT1 or any other genes involved. Rice Salt Intolerance 1 (SIT1) is reported to be upstream of MPK3/6 and is a negative regulator of salt stress (Li et al., 2014). The expression of SALT- RESPONSIVE ERF1 (SERF1), a rice ERF transcription factor, is specifically induced in roots upon salt and hydrogen peroxide ($H_2O_2$) treatment. It plays a positive role in salinity tolerance (Schmidt et al., 2013). OsMPK3 is known to have promoting effects on rice by modulate the ROS accumulation in the plant body (Zhang et al., 2018). Along with OsMPK3, OsMPK4, which is activated by salt induced OsMPKK1, is also known to positively regulate salt stress (Wang et al., 2014).

It has been reported that salt stress-induced nutrient and osmotic imbalance in plants which leads to reduced photosynthesis and stunted growth (Khan et al., 2016). High salinity causes a reduction in chlorophyll content due to suppression of specific enzymes involved in the synthesis of chlorophyll and subsequent reduction in biomass and plant height (Jaleel et al., 2008). It has been reported by Zhang et al. (2018) that the metabolism of bacteria inoculated salt treated plants showed significantly lower weakening than the un-inoculated salt treated plants. In the present study it is observed that the shoot-root length of the salt treated control plant decreased in comparison to the inoculated salt treated plants. The shoot of ABSK11 showed significant increase whereas the root showed lesser enhancement in growth. In ABSK186 inoculated and salt treated plants the enhancement of shoot length was meager but there was significant enhancement in the root length. The increase in root length can be a result of IAA

production by the bacterial strains (Etesami et al., 2014; Bartakke, 2018). It has been observed that MPK3/6 are involved in the root elongation mechanism in response to exogenous auxins (Huang et al., 2019; Jonwal et al., 2023). In addition to this OsMPK6 is known to regulate biomass production and help to increase height in rice (Liu et al., 2015). The increase in shoots is possibly associated with the modulation of various phytohormones which are responsible for advanced growth in response to bacterial inoculation (Zamanzadeh-Nasrabadi et al., 2023; Oubaha et al., 2024).

The MAPK cascade function as a relay of signalling by phosphorylation of the various MAPKs and subsequent activation of multiple downstream substrates such as transcription factors, protein kinases, other enzymes, and structural proteins, leading to the activation of cellular responses (Zhang & Zhang, 2022). There are 75 MAPKKKs identified in rice *in silico* (Rao et al., 2010). These MAPKKK are known to interact downstream with MAPKKs (Zagodzik, 2018). The signal is carried forward by MAPK which then activates certain transcription factors which in turn produce a response.

The gene expression analysis has brought to light the involvement of the MAPK cascade in alleviating the salt stress in plants. The upregulation of the *MPK3* and *MPK6* suggests that these genes are highly expressed in inoculated plants in comparison to un-inoculated plants. OsMPK3 and OsMPK4 are identified as the strongest downstream target of OsMKK1 (Wang et al., 2014) and involved in salt tolerance. A study on the OsMKKK10-OsMKK4-OsMPK6 cascade and the OsMPK6 interactors, revealed its role salt tolerance was upregulated when rice was exposed to the stress (Huang et al., 2009; Guo et al., 2020). The allevation of salt stress through accumulation of proline can be attributed to the *P5CS* gene which has been shown to be upregulated when MKK2 (a MAPKK) is overexpressed and the downstream targets of which are MPK3/6 (Teige at al., 2004, Verma et al., 2019).

***Results and Discussion***

The *in silico* analysis of the various receptors and MAPK proteins have yielded certain kinase type proteins that have a kinase domain and can possibly be present downstream of the receptors and upstream of MAPKs. Homologs to all the receptors from *Arabidposis* were found in rice. The identified kinases which interacts with these receptors may function as the upstream targets of MAPKKKs, MAPKKs or MAPKs. OsMPK3, OsMPK4 and OsMPK6 are observed to interact with some of the receptors, giving clue of the involvement of these receptors in signal transduction for salt tolerance. Some of the strong interactors with OsMPK3 were Q6K3D4_ORYSJ, C7J4B4_ORYSJ, M2K1_ORYSJ (MKK1) and Q8H7S4_ORYSJ (MPKK10.2). These having a conserved kinase domain suggesting that they may probably be the upstream transducers of the MAPK signalling cascade (MPKKs) involved in alleviating salt stress. Further, some of the strong interactors with OsMPK3 were Q6K3D4_ORYSJ, M2K1_ORYSJ (MKK1), A0A0P0Y187_ORYSJ, Q5VP37_ORYSJ and C7J4B4_ORYSJ. OsMPK3 was found to interact only with MKK1, thus suggesting that as per the current knowledge MKK1 is the probable upstream protein of both the OsMPK3 and OsMPK6, whereas MPKK10.2 is the upstream protein of OsMPK3 only. MKK1 is known to participate in defense responses to the bacterial elicitors (Mészáros et al., 2006). OsMPK6 is known to be negative regulator of bacterial infection (Yuan et al., 2007). Suppression of host innate immunity appears to be required for the establishment of symbiosis, thus, it will be suppressing the defense response to the PGPB. The various other kinase type proteins (Table 4.8) are can possibly be upstream or downstream targets of these MAPKs. Various genes that are involved in salt tress were supposed to be studied and their primers were also prepared, but due constrain time and unavailability proper equipments these studies could not be done.

# CHAPTER 5

# CONCLUSION

# 5. Conclusion

Plant Growth Promoting activity and role in salt stress alleviation of two halotolerant bacterial strains from the salt-pans of Ribandar, Goa was investigated on a salt sensitive rice varieties IR64. It was found that there was an increase in the shoot-root length, dry weight and proline content in inoculated plant samples compared to un-inoculated samples. ROS species which are known to accumulate as a result of high salt concentration are also lowered in inoculated samples. Quantitative real-time analysis was done, on *Pseudomonas multiresinivorans* ABSK11 inoculated plants because it showed persistent plant growth promoting activity, to check for the regulation of MAPK cascade. *MPK3* and *MPK6* showed enhanced expression in inoculated salt-stressed plants. *In silico* analysis on the various plant receptors was done to ascertain their role in signal transduction through the MAPK cascade and it led to the identification of various MAPK proteins and other kinase domain containing proteins that interacts with the receptors. It has been observed that MKK1 and MPKK10.2 the upstream regulators of MPK3 and MPK6. This regulated the proline content, ROS content and biomass changes in the plant. Comparing the biochemical, morphological and gene expression analysis, it can be concluded that *Pseudomonas multiresinivorans* ABSK11 is involved in alleviation of salt stress in rice through the regulation of MAPK cascade.

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# APPENDIX

*Appendix*

# APPENDIX I

Chapter 3: 3.1. Composition of Zobell Marine Broth

| **Ingredients** | **Gms / Litre** |
|---|---|
| Peptone | 5.000 |
| Yeast extract | 1.000 |
| Ferric citrate | 0.100 |
| Sodium chloride | 19.450 |
| Magnesium chloride | 8.800 |
| Sodium sulphate | 3.240 |
| Calcium chloride | 1.800 |
| Potassium chloride | 0.550 |
| Sodium bicarbonate | 0.160 |
| Potassium bromide | 0.080 |
| Strontium chloride | 0.034 |
| Boric acid | 0.022 |
| Sodium silicate | 0.004 |
| Ammonium nitrate | 0.0016 |
| Disodium phosphate | 0.008 |
| Sodium fluorate | 0.0024 |
| Final pH (at 25°C) | 7.6±0.2 |

3.2.3.a. Composition of Acid Ninhydrin

| **Chemical** | **Amount** |
|---|---|
| Ninhydrin | 1.25 g |
| GAA | 30 mL |
| 6 M orthophosphoric acid | 20 mL |

Mix and warm till ninhydrin is dissolved and clear solution obtained. Prepare fresh.

## *Appendix*

3.2.5 Primers

| Gene | Forward Primer | Reverse Primer |
|---|---|---|
| *MPK3* | ATCAGCAGCTTGGACC | AGTCGCAGATCTTGAGG |
| *MPK4* | CGAGGTCTCCTCCAAGTACG | GCGAAGCAGCTTGATTTCTC |
| *MPK6* | AGGTCACCGCCAAGTACAAG | AGCAGCTTGATCTCCCTGAG |