MOLECULAR ANALYSIS OF WRKY TRANCRIPTION FACTORS IN BANANA (*Musa acuminata* cv. Berangan) PRIMED WITH PLANT GROWTH PROMOTING BACTERIA

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FACULTY OF SCIENCE
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THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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Field of Study: **BIOTECHNOLOGY (Biology and Biochemistry)**

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MOLECULAR ANALYSIS OF WRKY TRANCRIPTION FACTORS IN BANANA (*Musa acuminata* cv. Berangan) PRIMED WITH PLANT GROWTH PROMOTING BACTERIA

ABSTRACT

Saline soils pose a significant threat to food security, necessitating the exploration of solutions. In this study, halotolerant bacteria were isolated from a Malaysian mangrove forest and their effects on banana plantlets under salt stress were investigated. Among the collected isolates, three rhizobacterial strains (RB1, RB3, and RB4) and three endophytic bacterial strains (EB1, EB2, and EB3) exhibited the best performance in promoting plant growth. These strains, identified as *Bacillus* sp. and *Pseudomonas* sp., were further analyzed. Scanning electron microscopy confirmed their successful colonization of banana plantlet roots. When subjected to salt stress, the colonized plantlets, particularly those with strains EB3 and RB3, demonstrated improved growth, increased levels of chlorophyll, carotenoids, and proline, as well as reduced malondialdehyde content, reactive oxygen species generation, and electrolyte leakage. The activity of antioxidant enzymes was also enhanced in the presence of strains EB3 and RB3. These findings highlight the multifunctional plant growth-promoting activity of halotolerant *Bacillus* and *Pseudomonas* strains from the mangrove, offering potential for mitigating salt stress in bananas. Furthermore, a plant-growth promoting bacterial consortium comprising halotolerant *Bacillus* sp. and *Pseudomonas* sp. was evaluated for its priming effect on banana plants subjected to abiotic (salinity) and biotic (Foc-TR4) stresses. Primed banana plants exhibited improved growth parameters, including plant height, root length, and biomass, along with enhanced physiological characteristics such as relative water content, chlorophyll, and carotenoid contents, compared to non-primed plants under stress. Lipid peroxidation was reduced, and proline levels and antioxidant enzyme activities were increased in primed plants. In salt-stress conditions, primed plants showed elevated levels of K^+, Ca^{2+}, and Mg^{2+} ions, and reduced levels of Cl^- and Na^+. Under Foc-TR4 stress, primed plants demonstrated significant enhancements in total soluble phenolics, lignin content, and defense-related enzyme activities. Additionally, the expression analysis of stress-responsive *MaWRKY* genes revealed their upregulation in primed banana plants under salt and Foc-TR4 stresses. These findings highlight the effectiveness of the PGPB consortium in promoting banana plant growth and priming WRKY-mediated protection against abiotic and biotic stresses. In conclusion, the utilization of halotolerant *Bacillus* and *Pseudomonas* strains from mangroves as plant growth-promoting bacteria shows promise in alleviating salt stress in bananas. The priming effect of the bacterial consortium enhances plant growth, physiological parameters, ion balance, and defense mechanisms against abiotic and biotic stresses. These findings contribute to the exploration of microbial strategies for improving banana resilience and provide insights into the potential applications of halotolerant microbes in salt-stress tolerance enhancement in plants.

Keywords: Banana, Plant growth promoting bacteria (PGPB), Salinity, Fusarium, WRKY

ANALISIS MOLEKUL FAKTOR TRANKRIPSI WRKY DALAM PISANG (*Musa acuminata* cv. Berangan) DIPERBAIKI DENGAN BAKTERIA PENGGALAK PERTUMBUHAN TUMBUHAN

ABSTRAK

Tanah masin menimbulkan ancaman besar kepada keselamatan makanan, yang memerlukan penerokaan penyelesaian. Dalam kajian ini, bakteria halotolerant telah diasingkan daripada hutan bakau Malaysia dan kesannya terhadap tumbuhan pisang di bawah tekanan garam telah disiasat. Antara pencilan yang dikumpul, tiga strain rhizobakteria (RB1, RB3, dan RB4) dan tiga strain bakteria endofit (EB1, EB2, dan EB3) mempamerkan prestasi terbaik dalam menggalakkan pertumbuhan tumbuhan. Strain ini, yang dikenal pasti sebagai *Bacillus* sp. dan *Pseudomonas* sp., masing-masing, dianalisis selanjutnya. Mengimbas mikroskop elektron mengesahkan kejayaan mereka menjajah akar plantlet pisang. Apabila mengalami tekanan garam, tumbuhan yang dijajah, terutamanya yang mempunyai strain EB3 dan RB3, menunjukkan pertumbuhan yang lebih baik, peningkatan tahap klorofil, karotenoid dan prolin, serta kandungan malondialdehid yang berkurangan, penjanaan spesies oksigen reaktif dan kebocoran elektrolit. Aktiviti enzim antioksidan juga dipertingkatkan dengan kehadiran strain EB3 dan RB3. Penemuan ini menyerlahkan aktiviti penggalak pertumbuhan tumbuhan pelbagai fungsi bagi strain *Bacillus* dan *Pseudomonas* halotolerant daripada bakau, menawarkan potensi untuk mengurangkan tekanan garam dalam pisang. Tambahan pula, konsortium bakteria menggalakkan pertumbuhan tumbuhan yang terdiri daripada Bacillus sp yang bertoleransi halo. dan *Pseudomonas* sp. telah dinilai untuk kesan penyebuannya pada pokok pisang yang tertakluk kepada tegasan abiotik (kemasinan) dan biotik (Foc-TR4). Tumbuhan pisang prima mempamerkan parameter pertumbuhan yang lebih baik, termasuk ketinggian tumbuhan, panjang akar dan biojisim, bersama-sama dengan ciri fisiologi yang dipertingkatkan seperti kandungan air relatif, klorofil dan kandungan karotenoid, berbanding tumbuhan bukan prima di bawah tekanan. Pengoksidaan lipid telah dikurangkan, dan tahap prolin dan aktiviti enzim antioksidan telah meningkat dalam tumbuhan prima. Dalam keadaan tekanan garam, tumbuhan prima menunjukkan paras ion K^+, Ca^{2+},dan Mg^{2+} yang tinggi, dan paras Cl^- dan Na^+ yang berkurangan. Di bawah tegasan Foc-TR4, tumbuhan prima menunjukkan peningkatan ketara dalam jumlah fenolik larut, kandungan lignin dan aktiviti enzim berkaitan pertahanan. Di samping itu, analisis ekspresi gen *MaWRKY* yang responsif tekanan mendedahkan pengawalseliaan mereka dalam tanaman pisang prima di bawah tekanan garam dan Foc-TR4. Penemuan ini menyerlahkan keberkesanan konsortium PGPB dalam menggalakkan pertumbuhan pokok pisang dan menyediakan perlindungan pengantara WRKY terhadap tekanan abiotik dan biotik. Kesimpulannya, penggunaan strain *Bacillus* dan *Pseudomonas* halotolerant daripada bakau sebagai bakteria penggalak pertumbuhan tumbuhan menunjukkan janji dalam mengurangkan tekanan garam dalam pisang. Kesan penyebuan konsortium bakteria meningkatkan pertumbuhan tumbuhan, parameter fisiologi, keseimbangan ion, dan mekanisme pertahanan terhadap tegasan abiotik dan biotik. Penemuan ini menyumbang kepada penerokaan strategi mikrob untuk meningkatkan daya tahan pisang dan memberikan pandangan tentang potensi aplikasi mikrob halotolerant dalam peningkatan toleransi tekanan garam dalam tumbuhan.

Kata kunci: Pisang, Bakteria penggalak pertumbuhan tumbuhan (PGPB), Kemasinan, Fusarium, WRKY

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ACC	:	1-aminocyclopropane-1-carboxylic acid
ABA	:	Abscisic acid
Ar	:	Absorbance of reference
α	:	Alpha
$(NH_4)_2SO_4$	:	Ammonium sulphate
APX	:	Ascorbic peroxidase
bZIP	:	Basic Leucine Zipper Domain
BLAST	:	Basic Local Alignment Search Tool
β	:	Beta
H_3BO_3	:	Boric acid
Ca^{2+}	:	Calcium ion
CAT	:	Catalase
CEBAR	:	Centre for Research in Biotechnology for Agriculture
Cl^-	:	Chloride ion
CAS	:	chrome azurol S
CFU	:	Colony forming units
$CuSO_4.5H_2O$	:	Copper sulphate pentahydrate
CTK	:	Cytokinin
$dS\ m^{-1}$	:	decisiemens per metre
°*C*	:	degree celcius
DNA	:	Deoxyribonucleic acid
K_2HPO^4	:	Dipotassium phosphate
ECe	:	Electrical conductivity

EL : Electrolyte leakage

ET : Ethylene

EDTA : Ethylenediaminetetraacetic acid

FESEM : Field emission scanning electron microscope

$FeCl_3$: Ferric chloride

$FeSO_4$: Ferrous sulphate

GR : Glutathione peroxidase

g : Grams

HDTMA : Hexadecyl trimethyl ammonium bromide

h : Hour

HCL : Hydrogen chloride

H_2O_2 : Hydrogen peroxide

IAA : Indole-3-Acetic-Acid

JA : Jasmonic acid

LOX : Lipoxygenase

L : Litre

LB : Luria-Bertini

Mg^{2+} : Magnesium ion

$MgSO_4$: Magnesium sulphate

MDA : Malondialdehyde

$MnSO_4.H_2O$: Manganese sulphate monohydrate

mg : Microgram

µl : Microliter

µM : Micromolar

mL : Milliliter

mM : Millimolar

min	:	Minute
MEGA	:	Molecular Evolutionary Genetic Analysis
MoO_3	:	Molybdenum trioxide
MS	:	Murashige and Skoog
KH_2PO	:	Monopotassium phosphate
nm	:	Nanometer
nmol	:	Nanomoles
NCBI	:	National Center for Biotechnology
NJ	:	Neighbour-Joining algorithm
NADP	:	Nicotinamide adenine dinucleotide phosphate
NBT	:	Nitro Blue Tetrazolium
OD	:	Optical density
%	:	Percent
PSU	:	Percent siderophore units
PBS	:	Phosphate-buffered saline
PBIU	:	Plant Biotechnology Incubator Unit
+	:	Plus
PCR	:	Polymerase chain reaction
PGPB	:	Plant growth promoting bacteria
PSI	:	Phosphate solubilization index
K^+	:	Potassium ion
qPCR	:	Quantitative PCR
A260/230	:	Ratio of absorbance at wavelength 260 nm and 230 nm
A260/280	:	Ratio of absorbance at wavelength 260 nm and 280 nm

ROS	Reactive oxygen species
rpm	Revolutions per minute
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
$NaCl$	Sodium chloride
$NaOH$	Sodium hydroxide
Na^+	Sodium ion
sp.	Species
spp.	Species (plural)
x	Times
TF	Transcription factor
$Ca_3(PO_4)_2)$	Tricalcium phosphate
TBE	Tris-boric acid ethylenediaminetetraacetic acid
TCA	Tricarboxylic acid
v/v	Volume over volume
w/v	Weight over volume
$ZnSO_4.7H_2O$	Zinc sulphate

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Bananas (*Musa* spp.) are a globally important fruit crop, contributing to both food security and economic prosperity. With an annual production of over 116 million tonnes, bananas serve as a vital dietary staple in many regions (FAO, 2022). However, the productivity of banana plants is significantly affected by various abiotic and biotic stresses. Among these, soil salinity and the fungal pathogen *Fusarium oxysporum* f. sp. *cubense* tropical race 4 (Foc-TR4) pose significant challenges to banana cultivation, leading to substantial yield losses and economic repercussions (Ismaila et al., 2022; Mazumdar et al., 2019). Soil salinity, resulting from coastal flooding and human activities has become a significant global concern for agriculture. Saline soils, characterized by high levels of Ca^{2+}, Mg^{2+}, Na^{+}, Cl^{-}, HCO_3^{-} and SO_4^{2-} ions, are considered unsuitable for cultivation (Arif et al., 2020; Goswami & Suresh, 2020). Salinity disrupts multiple physiological and metabolic processes in banana plants, including nutrient uptake, ion balance, photosynthesis, respiration, and water relations (Abbas et al., 2019; Pan et al., 2021; Polash et al., 2019). Soil salinity also induces osmotic stress in plants, resulting in the generation of reactive oxygen species (ROS) and oxidative damage to cellular components (El Ghazali, 2020; Navada et al., 2020). Consequently, the growth, development and productivity of banana plants are severely affected under saline conditions. Furthermore, the devastating fungal pathogen Foc-TR4 causes Fusarium wilt disease in banana plants, leading to the decline of infected plants and ultimately the death of the crop (Ismaila et al., 2022). Fusarium wilt poses a significant threat to global banana production, as the causative pathogen is highly virulent and difficult to control. The pathogen colonizes the vascular system of banana plants, causing vascular wilting, yellowing of leaves, and premature fruit ripening (Pastuszak et al., 2021). Once established in the soil, Foc-TR4 persists for long periods, making it challenging to manage and eradicate from infected areas.

The development of resilient banana varieties that can withstand salinity and Foc-TR4 infection is crucial for ensuring sustainable banana production. Traditional breeding methods and genetic engineering approaches have been explored to enhance stress tolerance in bananas. However, the complex nature of plant stress responses, which involve both genetic and environmental factors, necessitates additional strategies to mitigate these stresses (Mazumdar et al., 2019). One promising approach is the utilization of plant growth-promoting bacteria (PGPB) to enhance plant health and stress tolerance. PGPB, such as strains of *Bacillus* spp. and *Pseudomonas* spp., have been reported to exhibit plant growth-promoting activities and improve plant performance under abiotic stresses including salinity and drought (Dixit et al., 2023; Ma et al., 2020). These bacteria can colonize the roots of plants, stimulate plant immune responses, and enhance nutrient uptake and physiological processes (Gamez et al., 2019). Moreover, some PGPB strains possess biocontrol properties, providing protection against plant pathogens (Samain et al., 2022). However, there are no reports on the application of PGPB in alleviating the detrimental effects of salinity and Foc-TR4 infection, promoting plant growth and health in banana (*Musa acuminata* cv. Berangan).

One mechanism by which PGPB enhance stress tolerance in plants is through the induction of priming, a process that involves pre-activation of plant defense mechanisms. Priming enables plants to respond more effectively to subsequent stress events, such as salinity or pathogen attack (García-Cristobal et al., 2015; Mhlongo et al., 2020). The underlying molecular mechanisms of priming and the involvement of transcription factors (TFs) in this process are still being elucidated. Members of the WRKY TF family, characterized by zinc finger domains, have been identified as key regulators in plant development and responses to abiotic and biotic stresses (Kiranmai et al., 2018; Singh et al., 2018). Several studies have demonstrated the involvement of WRKY TFs in conferring stress tolerance in various plant species (Lin et al., 2022; S. Yu et al., 2023).

However, the specific WRKY TFs involved in the regulation of gene expression during bio-priming of banana with PGPB have not been reported.

In this study, the main aim is to isolate halotolerant bacteria from the rhizosphere of mangrove plants, followed by the identification, characterization, and selection of halotolerant bacterial strains with plant growth promoting agents. The selected halotolerant PGPB (later identified as *Pseudomonas* sp. EB3 and *Bacillus* sp. RB3) are further investigated to assess their potential in priming and mitigating the detrimental effects of salt stress and Foc-TR4 infection in banana cv. Berangan. Various physiological, biochemical, and molecular changes, including the expression of WRKY genes, associated with the priming and stress treatments are examined. This research contributes to the understanding of PGPB-mediated stress mitigation mechanisms in banana and provides insights into the potential application of PGPB for enhancing banana production under challenging environmental conditions.

The specific objectives of this study were:

- To isolate halotolerant bacteria from mangrove plant rhizosphere
- To identify, characterise and select halotolerant strains of bacteria for use as plant growth promoting agents
- To investigate the potential of selected halotolerant PGPB (*Pseudomonas* sp. EB3 and *Bacillus* sp. RB3), to enhance stress tolerance in banana plants under salinity and Foc-TR4 infection
- To examine the physiological and biochemical changes in banana plants treated with the PGPB consortium, including nutrient uptake, ion balance, photosynthesis, water relations, and enzyme activities
- To investigate the molecular mechanisms involved in PGPB-mediated stress mitigation, particularly focusing on the expression of WRKY transcription factors and their regulation of gene expression during bio-priming of banana

CHAPTER 2: LITERATURE REVIEW

2.1 Banana

Bananas (*Musa* spp.) are a highly significant fruit crop cultivated and consumed extensively in tropical and subtropical regions. Between 2017 and 2022, the annual global banana production exceeded 236 million tonnes, making banana fruit a highly significant agricultural commodity (FAO, 2022). Banana are the fourth-most important food crop in developing nations following rice, wheat, and maize (Zhang et al., 2022). The majority of bananas are grown in Asia, Latin America, and Africa with India being the leading producer in 2019, followed by China, Indonesia, Brazil, and Ecuador (FAOSTAT, 2020). Banana plants belong to the Musa genus, specifically the *Musa acuminata* (A genome) and *Musa balbisiana* (B genome) species, and their cultivars are derived from crosses between these two diploid wild species. *Musa acuminata* produce sweet dessert bananas, while *Musa balbisiana* cultivars produces starchy bananas commonly known as plantains. The Cavendish banana, the most widely used commercial cultivar, accounts for approximately 47% of overall production (Vaca et al., 2020). Cavendish bananas are known for their nutritional value, containing vitamin C, vitamin A, and potassium (Pareek, 2016; Wall, 2006). In Malaysia, bananas are among the important fruits for export markets, with papaya, starfruit, and mango also playing significant roles. Malaysia was the 53rd-largest banana exporter in the world, with exports worth USD 10.3 million (Observatory of Economic Complexity, 2020; Simoes and Hidalgo (2011). However, banana production in Malaysia faced challenges, with fluctuations in output from 2017 to 2020, and pest and disease infestations affecting local production (FAOSTAT, 2022).

Banana cultivation is mostly regulated by worldwide Agri-based bioeconomy. Therefore, it is crucial to comprehend the difficulties involved in producing it and create effective plans for resolving these issues. Growing healthy and profitable varieties of bananas has garnered more interest in recent years. However, there is a significant gap in the supply and demand of healthy planting materials (Jacobsen et al., 2019; Nkengla-Asi et al., 2021). Additionally, the cultivation of bananas poses various challenges, including the threat of climate factors and pathogenic agents such as bacteria, fungi, nematodes, and viruses (Tripathi et al., 2019). To overcome these challenges and develop disease-resistant banana crops, genetic engineering strategies and traditional breeding methods such as diploid breeding (Rowe & Rosales, 1994), the 3x/2x strategy (pollination of susceptible triploids with male fertile diploids that are resistant), and the 4x/2x strategy (production of a tetraploid parent by chromosome doubling of an ancestral diploid with good agronomic trait have been employed (Menon, 2016). However, the polyploidy present in *Musa* species poses a major obstacle in banana breeding (Nansamba et al., 2020), making the development of new cultivars a time-consuming process. In Malaysia, pest and disease infestations frequently hinder local banana production which can eventually have an adverse effect on output. Farmers frequently choose well-known cultivars for cultivation, a practice known as mono-cropping, in an effort to increase profits; however, by doing so, they have reduced genetic diversity among plantations, increasing their vulnerability to pests and diseases (Ghag et al., 2015). Therefore, the development of new banana varieties and disease-free planting material using unconventional methods is crucial for preserving banana cultivation in the face of changing global climate. The next section of the literature review focuses on the major biotic (Fusarium wilt) and abiotic (salinity stress) factors that affect banana production.

2.2 Salinity Stress

Soil salinity is a significant abiotic stress factor that limits agricultural food production. Currently, over 45 million hectares of cultivated land are affected by salinity, and this number continues to increase (Velmurugan et al., 2020). Saline soil has an electrical conductivity value (ECe) greater than 4 dS m-^{1} and an exchangeable sodium content of 15% in the root zone at 25 °C (Goswami & Suresh, 2020). Salts present in saline soils include Na^+, Ca^{2+}, Mg^{2+}, Cl^-, SO_2^{4-} and HCO^{-3}, leading to a wide range of physiochemical properties in salt-affected soils (Arif et al., 2020). Salinization is caused by natural factors such as irregular rainfall patterns, weathering of rocks, high evaporation, and prolonged periods of strong winds, as well as anthropogenic behaviors including land clearing, deforestation, improper fertilizer use, subpar irrigation systems, and various agricultural practices (Corwin, 2021). Various techniques are employed for the reclamation of saline soils, including physio-chemical techniques such as leaching, drainage, and the use of soil amendments like gypsum, as well as plant-based strategies like phytoremediation and traditional breeding approaches (Fita et al., 2015; Imadi et al., 2016). However, physio-chemical techniques are not sustainable and can contribute to environmental pollution (Arora et al., 2020). Selecting salt-tolerant varieties for breeding projects from the same or closely related species with limited genetic differences often leads to the expression of undesirable traits from the donor species, limiting their suitability for saline soil reclamation (S. Jha, 2019). Additionally, the development of salt-resistant crop varieties requires substantial time and financial support.

2.2.1 Plants under salinity stress

Salt stress exerts a harmful influence on plant health, especially when high concentrations of salt are present in the root zone. This situation leads to both osmotic stress and ion toxicity, which will impact yields and crop productivity. Initially, osmotic stress inhibits plant growth which is eventually followed by ion toxicity (James et al., 2011). Osmotic stress affects various plant growth processes, such as uptake of water, germination of seeds, cell elongation, development of leaves, lateral bud formation, lateral branching, rate of photosynthesis, uptake and translocation of nutrients, carbohydrate supply to meristematic regions, and overall plant growth (Munns & Tester, 2008; Sheldon et al., 2017; Van Zelm et al., 2020). Salinity stress also causes hyperionic stress through the accumulation of Na^+ and Cl^- ions in plant tissues (Gupta & Huang, 2014). The excessive absorption of these ions disrupts ion balance, interferes with nutrient uptake, damages the photosynthetic apparatus, causes stomatal closure, reduces CO_2 availability, and negatively impacts plant physiological and biochemical processes, ultimately leading to decreased vegetative and reproductive growth (Acosta-Motos et al., 2017; Ashraf et al., 2020; Egamberdieva et al., 2019; Sharif et al., 2019). Salinity stress affects the entire plant life cycle, from seed germination to seed production, and hampers plant development at different stages (Polash et al., 2019).

Banana plants, akin to many other crop species, are vulnerable to saline soils and irrigation water, leading to a substantial decrease in their growth and yield (Mazumdar et al., 2019; Santana Júnior et al., 2019). Studies have shown that bananas can survive salinity levels up to 10.2 dS m^{-1}, but higher concentrations are lethal (Mazumdar et al., 2019). While most salinity studies in bananas use NaCl treatments, which simulate salt stress, only a few studies have utilized sea salt. However, studies on aquatic macrophytes have demonstrated that the biological impacts of NaCl and sea salt are different. Aquatic macrophytes treated with sea salt exhibit greater superoxide dismutase (SOD) activity compared to those treated with NaCl, while NaCl-treated plants have higher peroxidase activity. These differences are attributed to the interaction of metals like Ca, Mg, and K with Na in sea salt, affecting water homeostasis, the activity of essential enzymes, and the expression of genes involved in antioxidative enzyme system (Rout & Shaw, 2001). Experiments that utilize sea water as a source of salinity can yield a more accurate and dependable data on the physiological effects of seawater exposure by simulating the conditions that bananas in coastal areas experience.

2.3 Fusarium Wilt

Fusarium wilt disease, caused by the fungal pathogen *Fusarium oxysporum* f. sp. *cubense*, poses a significant threat to banana production worldwide (Siamak & Zheng, 2018). Initially described in 1874 in Australia, the pathogen devastated the "Gros Michel" banana cultivar industry, which was extensively grown in monoculture farms across the Americas, Africa, and the Far East during the 1900s (Ploetz, 2015). The susceptible "Gros Michel" cultivar was subsequently replaced by resistant Cavendish group cultivars. However, around 50 years ago in the 1970s, a destructive new variant of *Fusarium oxysporum* f. sp. *cubense* known as tropical race 4 (TR4) emerged, causing the collapse of Cavendish cultivars in Australia and Southeast Asia (Dita et al., 2018). *Fusarium oxysporum* f. sp. *cubense* is categorized into races based on the cultivars they infect. The three primary races affecting bananas are: race 1, which infects cultivars Gros Michel and Lady Finger; race 2, which infects the same cultivars as race 1 as well as Bluggoe cultivars; and race 4, which infects the majority of cultivars, including Cavendish (Ploetz, 2015). Originally, it was thought that race 4 could only infect Cavendish cultivars in subtropical regions due to the cooller temperatures there (Ploetz, 2006). But in the early 1990s, Fusarium wilt began to kill Cavendish cultivars in the Southeast Asia tropical regions, leading to the discovery of a "Tropical" race 4 of *Fusarium oxysporum* f. sp. *cubense* (Foc-TR4) (Ploetz, 2015). Foc-TR4 has caused devastating losses in banana plantations across Australia, China, Malaysia, the Philippines, Indonesia, and Mozambique, spreading rapidly from the source (Sun et al., 2019). A recent field study conducted in 2019 reported that many Malaysian banana cultivars, including Berangan, Tanduk, Abu, Emas, Nangka, and Nipah, are susceptible to Foc-TR4 (Wong et al., 2019). The impact of Foc-TR4 has resulted in significant financial losses for countries such as Malaysia and Indonesia, with reported losses amounting to up to 121 and 243 million dollars, respectively (Aquino et al., 2013). The management of Fusarium wilt remains challenging due to the perennial nature of bananas and the polycyclic nature of the disease, necessitating the development of novel and alternative management techniques (Garcia-Bastidas, 2022).

2.3.1 Infection Process, Plant Pathogen Interaction and Disease Development

Foc-TR4 remains dormant in the soil until it senses or is stimulated to germinate by root exudates from the host or non-host plants or through direct contact with specific root tissues (Stover, 1962). Plant cutin layers containing dihydroxy-C16 and trihydroxy-C18 acids aid in host identification and induce cutinase activity in these fungi (Husaini et al., 2018). Within one or two days after inoculation, conidia and hyphae of Foc can be observed adhering to the root surfaces (Li et al., 2017). Penetration occurs either directly or through wounds, without the presence of real appressoria or penetration pegs (Li et al., 2011; Li et al., 2017). The hyphae swell at the point of penetration but then shrink back to their original size (Li et al., 2011). Lesions may occasionally appear at the site of infection, but they are more commonly found near the root base (Rishbeth, 1955). Subsequently, the host plant is colonized, starting with spore germination. The development of germ tube and spores is aided by root exudates. The velvet family of proteins, including VeA, VelB, and VelC, governs intense hyphal branching as the germ tube grows and comes into contact with the plant roots (López-Berges et al., 2013). With the help of several hydrolytic enzymes, the hyphae then enter the root epidermal cells before progressing to the cortex and finally reaching the xylem tissue. The transcriptional activator XlnR plays a crucial role in the breakdown of plant cell walls by activating xylanase genes such as xly3, xly4, and xyl5 (Calero-Nieto et al., 2007). The successful colonization of the xylem tissue is attributed to the activation of several key pathogenicity factors, including oxidoreductase (ORX1), secreted in xylem (SIX) regulated by *Sge1*, *FTF1*, and *FTF2* transcription factors, carbon catabolite-derepressing protein kinase (*Snf*), eukaryotic translation initiation factor 3 (*eIF-3*), pisatin demethylase 1 (*Pda1*), mitogen-activated protein kinase (*Fmk1*), mitochondrial carrier protein (*Fow1*), pectate lyase (*PelA, PelD*), pea pathogenicity peptide 1 and 2 (*Pep1, Pep2*), ras-like GTP-binding protein (*Rho1*), and transcription factor involved in pheromone response (Adhikari et al., 2020; Husaini et al., 2018). Once penetration occurs and Foc breaches the initial host barriers, the pathogen produces thicker hyphae and microconidia. Thicker hyphae then transform into chlamydospores within the intra and intercellular spaces (Li et al., 2011). As the infection progresses, Foc hinders nutrient movement and resists water flow, leading to the clogging of the water-conducting system with spores, mycelia, and other plant defense metabolites. The infection spreads further, reaching the vascular zone of the lateral roots and causing rhizome infection. Rhizome colonization is a crucial stage in disease development (Li et al., 2017). Once the rhizome is colonized, the infection spreads throughout the plant, reaching the pseudostem. This results in wilting of the foliage, splitting of the pseudostem at the base, and ultimately, plant death (Gordon, 2017). At 17 days post inoculation (dpi), a significant number of hyphae can be observed in the pseudostem, and plant death may occur by 24 dpi (Xiao et al., 2013).

2.4 Rhizosphere

The rhizosphere is a dynamic ecosystem where various microbial groups interact through interspecific and trans-kingdom communication. It refers to the small soil region in close proximity to the plant roots, where the presence of roots influences the biological and chemical characteristics of the soil (Chialva et al., 2022). The root system not only provides mechanical support and aids in nutrient and water uptake but also synthesizes, stores, and exudes a diverse range of chemicals (Walker et al., 2003). These compounds released by plant roots, known as root exudates, consist of carbohydrates, amino acids, organic acids, and other substances. Root exudates serve as chemoattractant for a wide array of metabolically active soil microbial communities (Mavrodi et al., 2021). Root exudation modifies the chemical and physical properties of the soil, thereby influencing the composition of microbial communities in the rhizosphere (Preece & Peñuelas, 2020). This process can enhance the fertility of the rhizospheric soil and indirectly stimulate the growth of specific beneficial bacteria that promote plant growth (Cordovez et al., 2021). For example, the strain *Burkholderia phytofirmans* PsJN (now known *as Paraburkholderia phytofirmans*) has been found to metabolize oxalate exuded by maize and lupine plants in the rhizosphere. This strain is known for its biocontrol properties against pathogens and its ability to stimulate plant growth (Kost et al., 2014). Recent studies suggest that plant roots can also detect bacterial quorum-sensing molecules, such as N-acyl-L-homoserine lactones (AHLs) and cyclodipeptides (CDPs), in addition to the phytohormones that regulate root growth, such as auxins and cytokinins. These bacterial and fungal compounds can influence root development and other physiological processes in plants (Ortiz-Castro & López-Bucio, 2019). The composition of these compounds in the rhizosphere depends on the physiological status of the plant, the specific plant species, and the microorganisms present (Kang et al., 2010). Thus, the rhizosphere consists of the soil influenced by root exudates, the rhizoplane (root surface) with tightly adhering soil particles, and the root itself, which is colonized by numerous microorganisms. These components interact and contribute to the complex microbial ecology and chemical dynamics in the rhizosphere (Barea et al., 2005).

2.5 Plant Growth Promoting Bacteria

The plant growth promoting bacteria (PGPB) are the bacteria residing in the root rhizoplane and are characterized by the following features; Firstly, they possess the ability to effectively colonize the root surface. This enables them to establish a presence in the root rhizoplane. Secondly, PGPB have the capacity to survive, reproduce, and compete with other microbial communities present in the rhizosphere. Lastly, these bacteria demonstrate their ability to express activities that promote and protect plant growth (Kloepper, 1994). In summary, PGPB refers to a beneficial group of bacteria that reside either freely in the rhizosphere soil or within the plant itself. These bacteria employ various mechanisms to stimulate plant growth and provide defense against both biotic and abiotic stresses (Hashem et al., 2019). Furthermore, the classification of PGPB is based on the proximity of the bacteria to the root and the nature of their association (Gray & Smith, 2005). PGPB can be categorized into two main groups: endophytic bacteria and rhizobacteria. Endophytic bacteria reside inside the cells of the root, often forming specialized nodules. Some examples of endophytic bacteria include *Azorhizobium, Bradyrhizobium, Allorhizobium, Mesorhizobium* and *Rhizobium* of the family *Rhizobiaceae*. Rhizobacteria, on the other hand, exist in the rhizosphere, on the root surface (rhizoplane), or in the intercellular spaces within the root cortex. Examples of rhizobacteria include *, Azotobacter, Bacillus, Agrobacterium, Pseudomonas*, *Burkholderia, Caulobacter, Arthrobacter Azospirillum, Chromobacterium, Erwinia, Flavobacterium, Micrococcous,* and *Serratia* (Bhattacharyya & Jha, 2012).

2.6 Mechanisms of Plant Growth Promotion by PGPB

PGPB employ diverse mechanisms to support plant growth and enhance their resilience against biotic and abiotic stress (Cao et al., 2023). These bacteria directly contribute to plant growth by facilitating the acquisition of vital resources such as nitrogen, phosphorus, and essential minerals. Moreover, they play a role in modulating the levels of plant hormones, thereby influencing growth and development. Indirectly, PGPB act as biocontrol agents, mitigating the inhibitory effects of pathogens and promoting a healthier growth environment for plants.

2.7 Direct Mechanisms

It is important to highlight that many PGPB possess multiple plant growth-promoting traits and abilities, rather than being limited to a single function. These beneficial bacteria have evolved to exhibit a wide range of mechanisms that contribute to plant growth and health.

2.7.1 Nitrogen fixation

Plant growth and productivity heavily rely on nitrogen, an essential nutrient (Ahemad & Kibret, 2014). Despite the atmosphere containing approximately 78% nitrogen, it remains unavailable to plants in its current form. Biological nitrogen fixation (BNF) plays a pivotal role in converting atmospheric N_2 into plant-usable forms. This process is facilitated by both symbiotic and non-symbiotic nitrogen-fixing bacteria (Shridhar, 2012). Symbiotic PGPB establish a symbiotic relationship with leguminous plants, enabling the fixation of atmospheric nitrogen in the soil. Examples of such bacteria include *Pantoea agglomerans*, *Azoarcus* sp., *Beijerinckia* sp., K. *pneumoniae*, and *Rhizobium* sp. (Ahemad & Kibret, 2014). On the other hand, non-symbiotic PGPB, including free-living, associative, and endophytic bacteria, fix atmospheric nitrogen without forming a symbiotic association with host plants (Glick, 2014). *Gluconoacetobacter diazotrophicus,* Cyanobacteria (*Anabaena*, *Nostoc*), *Azotobacter*, *Azospirillum*, and *Azocarus* are among the examples of non-symbiotic nitrogen-fixing bacteria (Bhattacharyya & Jha, 2012). The process of nitrogen fixation is governed by specific genes, including the *nif* gene, which activates the iron protein, donates electrons, biosynthesizes the iron molybdenum cofactor, and regulates other genes essential for the production and activation of the nitrogen-fixing enzyme (Reed et al., 2011). Treating plants with nitrogen-fixing bacteria enhances growth promotion, disease control, and soil nitrogen content (Damam et al., 2016).

2.7.2 Phosphate solubilization

Phosphorus, the second most essential nutrient for plant growth after nitrogen, holds significant importance in facilitating healthy plant development. It is essential in substantial amounts to support various metabolic processes, including macromolecular biosynthesis, photosynthesis respiration, and signal transmission (Cao et al., 2023). However, the majority of phosphorus in soil exists in forms that are precipitated, insoluble, or immobilized forms, making it inaccessible for plant uptake. As a result, the availability of soluble phosphorus forms to plants is often limited. Plants can only absorb phosphorus in two soluble forms: monobasic ($H_2PO_4^-$) and dibasic (HPO_4^{2-}) ions. This limitation has prompted the search for ecologically safe and economically viable approaches to enhance crop production in low phosphorus soils. Notably, certain bacterial strains can simultaneously solubilize and mineralize phosphate (Guang-Can et al., 2008). PGPB produce low molecular weight organic acids that facilitate the solubilization of inorganic phosphorus (Shen et al., 2021). Effective phosphate solubilizers have been identified in bacterial genera such as*, Enterobacter, Bacillus, Pseudomonas, Microbacterium Erwinia, Arthrobacter, Rhodococcus*, *Flavobacterium, Beijerinckia, Burkholderia, Rhizobium, Mesorhizobium,* and *Serratia* (Oteino et al., 2015). Besides supplying plants with accessible phosphorus, phosphate-solubilizing bacteria (PSB) offer additional benefits that contribute to plant growth and development. For example, they enhance biological nitrogen fixation and increase the availability of other essential trace elements as demonstrated by Zaidi et al. (2009). This multifaceted interaction between plants and PSB underscores the potential of harnessing beneficial microorganisms to promote sustainable agriculture and improve overall plant health and productivity.

2.7.3 Siderophore production

Iron is an essential element for the survival of nearly all forms of life. Apart from a few lactobacilli, iron is a fundamental requirement for all known microorganisms (Neilands, 1995). In aerobic environments, iron predominantly exists as Fe^{3+}, forming insoluble oxyhydroxides and hydroxides and that are typically inaccessible to microorganisms and plants (Rajkumar et al., 2010). Siderophores are low molecular weight compounds produced by microorganisms that have the ability to bind ferric ion (Fe^{3+}) from the environment, making it available for uptake by plants. Various low-molecular-weight compounds, including as sugars, organic acids, and amino acids as well as secondary compounds like flavonoids, phenols, terpenoids, and inorganic metabolites (CO_2, H_2O, etc.) present in root exudates, directly participate in the dissolution, reduction, and complexation of Fe^{3+} and provide crucial substrates for PGPB to synthesize siderophores (Gargallo-Garriga et al., 2018). The production of siderophores by PGPB enables them to serve as valuable resources for supplying plants with an adequate supply of iron.

2.7.4 Phytohormone production (Indole-3-Acetic-Acid)

Phytohormones are organic compounds that exert regulatory effects on plant growth and development at low concentrations (< 1mM) (Damam et al., 2016). Notably, approximately 80% of microorganisms present in the rhizosphere of different crops have the capability to synthesize and release phytohormones as secondary metabolites (Patten & Glick, 1996). PGPB play a significant role in promoting plant growth by producing various phytohormones, including abscisic acid (ABA), indole-3-acetic acid (IAA), ethylene (ETH), and gibberellin (GA). These phytohormones accelerate various physiological processes in plants, including cell division, elongation and differentiation, germination of seeds, seedling establishment, development, reproduction, and stress response signaling networks (Ma et al., 2020). Among these phytohormones, IAA, which is produced by numerous PGPB such as *Pseudomonas sp., Rhizobium sp., Bacillus sp*., *Enterobacter, Paenibacillus.,* etc.) plays a significant role in almost all aspect of plant growth and development. It is involved in processes such as embryo formation, induction of cell division, elongation of the stem and coleoptile, apical dominance, root induction, vascular tissue differentiation, fruit development, ethylene synthesis stimulation, adventitious root induction in cuttings, and tropic movements (Souza et al., 2017). Thus, IAA is widely recognized as an important signaling molecule in plant-microbe interactions. Its role encompasses both pathogenesis, where it influences disease development, and phytostimulation, where it promotes plant growth and development (Spaepen & Vanderleyden, 2011).

2.7.5 1-Aminocyclopropane-1-carboxylate (ACC) deaminase

Plants have the ability to respond to various stresses by modulating the levels of different hormones, which trigger the synthesis of stress-related proteins necessary for defense against adverse conditions. Ethylene is a prominent plant hormone involved in stress response modulation. While ethylene is necessary for healthy plant growth and development, excessive ethylene production, known as "stress ethylene," can have negative effects on root/shoot proliferation and overall plant growth parameters, hindering plant growth and development (Gamalero & Glick, 2015). Certain plant-associated bacteria possess the enzyme 1-aminocyclopropane-1-carboxylate deaminase (ACCD), which can mitigate the effects of stress-induced ethylene on plants. ACCD breaks down the immediate precursor of ethylene, ACC, into α-ketobutyrate and ammonia. This enzymatic activity reduces the ethylene levels in plants, thereby restoring root and shoot growth (Glick, 2014). Several microbial species, including *Pseudomonas, Enterobacter, Paenibacillus, Stenotrophomonas, Mesorhizobium, Klebsiella,* *Azotobacter, Rhizobium, Pantoea, Rhodospirillum,* and *Bacillus* have been found to produce these hormones occasionally (Singh et al., 2015).

2.8 Indirect Mechanisms of Plant Growth Promotion

PGPB promote plant growth indirectly through their role as biocontrol agents (Ma et al., 2020). The main modes of biocontrol activity exhibited by PGPB include competition for resources, niche exclusion, and the production of antifungal metabolites (Lugtenberg & Kamilova, 2009). PGPB are known to produce various antifungal metabolites that can inhibit the growth of pathogenic fungi. Examples of these metabolites include 2,4-diacetylphloroglucinol, phenazines, HCN, pyrrolnitrin, tensin, pyoluteorin, and viscosinamide (Bhattacharyya & Jha, 2012). By producing these metabolites, PGPB can suppress the growth and development of plant pathogens. Another indirect mechanism of plant growth promotion is induced systemic resistance (ISR), which occurs when specific PGPB interact with plant roots, leading to the development of resistance in plants against specific pathogenic bacteria, fungi, and viruses (Cao et al., 2023). ISR involves plant's jasmonate and ethylene signaling pathways, which stimulate the plant's defense responses against a wide range of plant diseases (Glick, 2012). Various individual components of PGPB such as 2,4-diacetylphloroglucinol, lipopolysaccharides (LPS), cyclic lipopeptides, flagella, homoserine lactones, acetoin, siderophores, and 2,3-butanediol, have been identified to contribute to ISR (Lugtenberg & Kamilova, 2009). These components play a role in signaling and communication between the PGPB and the plant, enhancing the plant's defense mechanisms. Overall, the indirect mechanisms of plant growth promotion by PGPB involve biocontrol activities, including competition for resources, production of antifungal metabolites, and the induction of systemic resistance, which collectively help protect plants from pathogens and enhance their growth and development.

2.9 Contribution of PGPB to Plant Salt Tolerance

PGPB play a vital role in enhancing plant salt tolerance by mitigating the detrimental effects of high salt concentrations. They possess beneficial traits that facilitate plant growth in saline environments through two main mechanisms: activating or modifying plant response systems when exposed to salt stress and synthesizing anti-stress molecules (Fouda et al., 2019). The mechanisms by which PGPB improve plant growth and increase resistance to salinity include enhancing nutrient uptake, influencing ion homeostasis, promoting selective ion absorption, forming biofilms, modifying root architecture, modulating the antioxidant system, regulating osmotic substances, adjusting plant hormone levels, and regulating the expression of salt-responsive genes (Bhat et al., 2020). PGPB's ability to enhance plant salt tolerance is attributed to the combined action of these mechanisms, rather than relying on a single mechanism. Table 2.1 summarizes the mechanisms and effects from recent literature on the application of PGPB in salt tolerance.

Table 2. 1: Influence of PGPB on salt tolerance

Plant	Bacterial strain	Mechanism	Effect	Reference
Maize	*Enterobacter cloacae*	IAA, Siderophore, ACC deaminase, EPS	Enhanced Osmoprotection	Ali et al. (2022)
Tomato	*Enterobacter* 64S1 and *Pseudomonas* 42P4	BNF, phosphate solubilization, enhanced proline	Enhanced Osmoprotection	Pérez-Rodriguez et al. (2022)
Tomato	*Pseudomonas oryzihabitans*	Increased APX, POD and CAT enzyme activity	Salt tolerance	Mellidou et al. (2021)

Table 2.1 continued

Plant	Bacterial strain	Mechanism	Effect	Reference
Maize	*Enterobacter cloacae*	Elevated levels of SOD, POD, APX	Increased antioxidant defense	Ali et al. (2022)
	Acinetobacter johnsonii	Decline in CAT, SOD activities	Enhanced antioxidant defense	Shabaan et al. (2022)
Pea	*Acinetobacter bereziniae, Enterobacter ludwigii,* and *Alcaligenes faecalis*	Decreased SOD and CAT	Salt tolerance	Sapre et al. (2022)
Cucumber plants,	*Burkholdera cepacia SE4, Promicromonospora sp. SE188 and Acinetobacter calcoaceticus SE370* *Planococcus* soli WZYH02	Reduced activity of CAT, POD, PPO and total polyphenol	Salt tolerance	Abbas et al. (2019); (Etesami & Beattie, 2017; Kang et al., 2014)
	Azoarcus, Azotobacter, Azospirillum, Burkholderia, Diazotrophicus, Enterobacter, Gluconacetobacter, Pantoea agglomerans, and *Pseudomonas*	BNF and IAA production	Nutrient uptake	Bhattacharyya & Jha, (2012); Damam et al. (2016)
Pea	*Pseudomonas strains*	Phosphate solubilzation	Nutrient uptake	Oteino et al. (2015)
		Siderophore production	Nutrient uptake	Scavino and Pedraza, (2013)
Radish	*Kosaconia radicincitans*	BNF, phosphorus solubilization	Nutrient uptake	Shahid et al. (2022)
Rice	*Pantoea alhagi*	EPS production	Biofilm formation	Sun et al. (2022)
Black locust	*Rahnella aquatilis* JZ-GX1	VOC production	Upregulation of *RpNHX1* expression	Li et al. (2021)
Chickpea	*Bacillus tequilensis*	EPS production	Biofilm formation	Haroon et al. (2023)
Arabidopsis	*Bacillus amyloliquefaciens*	Enhanced antioxidant	VOC production	Liu et al. (2020)

2.9.1 Osmotic Adjustment

Osmotic adjustment is a critical mechanism employed by plants growing in saline soils to counteract water loss caused by reduced osmotic pressure (Van Zelm et al., 2020). To maintain osmotic balance both inside and outside the cell, plants increase the production of low-molecular-weight compatible solutes known as osmolytes. Common osmolytes include proline, soluble sugars, glycine betaine, and ectoine (Ilangumaran & Smith, 2017; Kumawat et al., 2023). Osmotic adjustment, although energetically demanding and resulting in reduced growth, is a vital process that helps alleviate the impact of salt stress on plants (Munns & Gilliham, 2015). Proline acts as an antioxidant and stabilizes proteins, while also serving as a marker for salt stress (Cui et al., 2020). Mitogen-activated protein kinase (MAPK) and ABA signalling control the synthesis and storage of osmolytes in plants (Jogawat, 2019). Rapid activation of MAPKs occurs in response to salt stress, influencing the expression of stress-responsive genes (De Zélicourt et al., 2016). Several studies have demonstrated that PGPB inoculation significantly influences the osmoprotectant content in various plant species under salt stress. For example, *Enterobacter cloacae* PM23 enhances osmoprotectant levels in Maize (*Zea mays*) (Ali et al., 2022). Inoculation of *Pseudomonas* 42P4 and *Enterobacter* 64S1 increases proline content and alleviates salt stress symptoms in tomato (*Solanum lycopersicum*) (Pérez-Rodriguez et al., 2022).

2.9.2 Antioxidant Activity

Environmental stressors can disrupt the balance between ROS production and scavenging activity, leading to an accumulation of ROS in plants (Apel & Hirt, 2004). Salt stress, in particular, significantly increases ROS levels due to disturbances in electron transport chains and decreased water potential (Acosta-Motos et al., 2017). ROS, including $O_2^{\bullet-}$, $HO_2^{\bullet-}$, $OH^{\bullet}$, H_2O_2, $^1O_2$, ROOH, $RO^{\bullet}$ and $ROO^{\bullet}$ can react with organic molecules, resulting in detrimental effects such as DNA/RNA damage, protein oxidation, enzyme inhibition, and membrane lipid peroxidation (El Ghazali, 2020). To counteract ROS, plants have evolved antioxidant defense systems consisting of enzymatic antioxidants (e.g., such as ascorbate peroxidase (APX), superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), and glutathione reductase (GR) and non-enzymatic antioxidant compounds (e.g., phenolics, carotenoids, proline, flavonoids) that contribute to salt tolerance (Gupta & Huang, 2014).

Emerging evidence suggests that PGPB can modulate the antioxidant defense system of plants by enhancing the activity of various antioxidant enzymes under stress conditions (Abbas et al., 2019; Etesami & Beattie, 2017). However, there are contrasting reports as some studies have shown reduced levels of enzymatic antioxidants in plants inoculated with PGPB under stress, suggesting potentially reduced stress levels compared to uninoculated plants (Kang et al., 2014; Sapre et al., 2022). For example, the application of *Acinetobacter johnsonii* SUA-14 in maize led to decreased catalase and superoxide dismutase activity, as well as a reduction in malondialdehyde (MDA) content, potentially linked to the reduced uptake of Na^+ (Shabaan et al., 2022). On the other hand, other studies have demonstrated that PGPB inoculation can lead to elevated levels of antioxidants and enhanced antioxidant enzyme activity in plants under stress. For instance, the inoculation of *Enterobacter cloacae* PM23, a halotolerant bacterium, in maize resulted in elevated levels of antioxidants, including SOD, POD, APX, ascorbic acid, flavonoids, and phenolics, along with reduced H_2O_2 and MDA content. The upregulation of APX and SOD genes was associated with the higher enzyme activity, indicating improved antioxidant defense under salinity stress (Ali et al., 2022). Similarly, *Pseudomonas oryzihabitans* AXSa06 inoculation in *Solanum lycopersicum* enhanced antioxidant activity, as indicated by increased ascorbate and MDA content, and enhanced POD and CAT enzyme activity under 200 mM NaCl stress (Mellidou et al., 2021). These diverse findings collectively highlight the ability of PGPB to modulate the antioxidant defense system of plants, enhancing their capacity to scavenge ROS and mitigate oxidative damage under salt stress conditions. The activation of antioxidant enzymes and the accumulation of non-enzymatic antioxidants contribute to the improved stress tolerance observed in PGPB-inoculated plants.

2.9.3 Ion Homeostasis

Maintaining ion homeostasis, particularly with respect to sodium (Na^+) is crucial for plant growth and survival under salt stress conditions. High cytoplasmic Na^+ concentrations are toxic to both glycophytes and halophytes. The regulation of Na^+ uptake and transport are essential for maintaining high cytoplasmic K^+/Na^+ ratios, as Na^+ and K^+ compete for important metabolic processes in the cytoplasm. Numerous K^+-dependent enzymes can have their enzyme activity inhibited by Na^+, affecting cellular metabolism (Almeida et al., 2017). To achieve Na^+ control, halophytic plants employ mechanisms such as Na^+ exclusion from roots, long-distance Na^+ transport, and Na^+ compartmentalization (Munns, 2005).

PGPB, such as the endophytic strain *Bacillus megaterium* ZS-3, have been shown to induce systemic tolerance to salt stress in plants by regulating various processes, including ion homeostasis. *B. megaterium* ZS-3 restricts Na^+ accumulation, leading to increased K^+/Na^+ ratios in the presence and absence of salt. This effect is achieved by down-regulating *HKT1* and up-regulating *NHX1* and *AVP1* (a vacuolar H^+-pyrophosphatase), which pumps H^+ into vesicles, creating a H^+ gradient across the membrane. These results point to a limitation in the absorption of Na^+ by plants and the sequestration of Na^+ in plant vesicles, enabling plants to cope more effectively with salt stress (Shi et al., 2022). PGPB, such as *Pseudomonas knackmussii* MLR6, have also been shown to regulate ion homeostasis-related gene expression, including the up-regulation of *SOS1*, in *A. thaliana* plants under salt conditions (Rabhi et al., 2018). These studies highlight the role of PGPB in regulating ion homeostasis and enhancing salt stress tolerance in plants through various mechanisms, including Na^+ exclusion, Na^+ compartmentalization, and modulation of ion transporters and pumps.

2.9.4 Hormonal Modulation

Salt stress poses a significant threat to plant growth and productivity, necessitating the exploration of effective strategies for stress mitigation. Phytohormones play a crucial role in plant responses to stress, and PGPB have emerged as potential regulators of hormone dynamics. This section of the literature review examines the influence of PGPB on specific plant hormones under salt stress conditions and provides examples from relevant studies.

2.9.4.1 Ethylene Modulation under Salinity Stress Conditions

Ethylene, a gaseous phytohormone, plays a vital role in plant growth and development. However, at high concentrations, ethylene can have negative effects, including defoliation, suppression of root and stem growth, and early senescence, which can reduce crop performance (Bhattacharyya & Jha, 2012). Under normal conditions, the enzyme ACC synthetase converts ACC into ethylene. Studies have demonstrated the ability of PGPB strains to modulate ethylene accumulation in plants under salt stress. For example, three Bacillus strains (*B. subtilis* NBRI 28B, *B. safensis* NBRI 12M, and *B. subtilis* NBRI 33N) that produces ACC deaminase reduced ethylene buildup in maize under salt stress conditions, resulting in improved plant growth. The application of *B. safensis* NBRI 12M exhibited the greatest reduction in ACC-oxidase (ACO) activity, which corresponds to lower ethylene production (Forchetti et al., 2007).

2.9.4.2 ABA Modulation under Salinity Stress Conditions

ABA is a key regulator in the plant's response to abiotic stresses, including salt stress. It coordinates various physiological processes depending on its concentration. At normal levels, ABA regulates processes such as seed development and dormancy, stomatal opening, and synthesis of storage proteins and lipids (Sreenivasulu et al., 2012). However, under high concentrations induced by abiotic stress, ABA inhibits plant growth. Salt stress greatly induces ABA production, increasing the amount of ABA in the plant. To help the plant adapt and survive, this causes stomatal closure and changes in gene expression (Sah et al., 2016). Under salt stress, PGPB inoculation has been demonstrated to affect ABA levels. For example, the inoculation of and *Planococcus* soli WZYH02 and *Bacillus atrophaeus* WZYH01 in maize resulted in changes in the expression of genes associated to plant hormones and a reduction in the ABA content. The inoculated plants showed up-regulation of *ZmDREB2A* and *ZmWRKY58* genes and down-regulation of the *ZmNCED* gene, which play roles in enhancing plant salt tolerance (Hou et al., 2022). Similar findings showed that ABA buildup during salt stress was reduced in wheat (*Triticum aestivum*) that had been inoculated with *Dietzia natronolimnaea* STR1 and *Arthrobacter protophormiae* SA3 (Barnawal et al., 2017).

2.9.4.3 Cytokinin and Auxin Modulation under Salinity Stress Conditions

Cytokinins are another class of plant hormones that influence cell division, vascular cambium sensitivity and differentiation, root hair proliferation, and inhibit lateral root formation and primary root elongation (Aloni et al., 2006). Auxins, such as IAA, regulate a wide range of processes in plant development and growth (Ma et al., 2020). IAA promotes the growth of roots by increasing both their surface area and length, allowing plants to access soil nutrients more effectively (Spaepen & Vanderleyden, 2011). PGPB inoculation has been found to enhance cytokinin and auxin levels in plants exposed to salt stress. For example, citrus (*Citrus macrophylla*) inoculated with *Novosphingobium* sp or *Pseudomonas putida* KT2440 displayed increased accumulation of auxin and cytokinins. These bacterial associations resulted in a low ABA and salicylic acid (SA) content (Vives-Peris et al., 2018). *Bacillus subtilis* IB-22 inoculation in wheat increased ABA content in roots, while also producing cytokinins and raising their content, preventing stressed plants from decline in potassium concentration (Arkhipova et al., 2020).

2.9.4.4 Jasmonate Modulation under Salinity Stress Conditions

Jasmonates, particularly jasmonic acid (JA), play a crucial role in plant defense responses. PGPB inoculation has been reported to influence JA levels in plants under salt stress. *Bradyrhizobium japonicum* inoculation in Arabidopsis resulted in a rise in jasmonate (JA) levels, which led to differential gene expression compared to non-inoculated plants. The expression of abiotic-stress-related and JA genes was altered, indicating the involvement of JA in the primed state and stress response (Gomez et al., 2023).

2.9.4.5 OPDA as a Hormonal Regulator under Salinity Stress Conditions

12-oxo-phytodienoic acid (OPDA), a precursor of JA and related molecules, has emerged as a hormonal regulator involved in the activation of autonomous signaling pathways in plant responses to stress (Liu & Park, 2021). Limited research has explored the influence of PGPB on OPDA levels under salt stress. However, some studies suggest that PGPB inoculation may modulate OPDA levels. For example, Erice et al. (2017) investigated the impact of *Bacillus megaterium* application on OPDA levels in Arabidopsis. They observed a reduction in OPDA content following bacterial inoculation under normal conditions. However, no significant differences in OPDA levels were observed under saline conditions. This study highlights the potential role of PGPB in modulating OPDA levels, particularly under stress conditions.

2.9.5 Nutrient Uptake under Salinity Stress Conditions

Under saline conditions, high sodium (Na^+) concentrations induce nutritional disorders and reduce the availability of essential nutrients for plants. Nutrients such as nitrogen (N), calcium (Ca^{2+}), potassium (K^+), phosphorous (P), and iron (Fe) are adversely affected (Ben Abdallah et al., 2017). Non-symbiotic nitrogen-fixing rhizobacteria from genera like *Diazotrophicus, Gluconacetobacter, Azoarcus, Azotobacter, , Burkholderia, Azospirillum, Enterobacter,* and *Pseudomonas* promote plant growth and restore nitrogen levels in saline soil (Bhattacharyya & Jha, 2012; Damam et al., 2016). Phosphate-solubilizing bacteria, both rhizosphere-colonizing and endophytic, play a crucial role in liberating organic phosphates and solubilizing insoluble inorganic phosphate (Oteino et al., 2015). Microorganisms contribute to the release of K^+ by producing acids, protons, and chelating ions associated with potassium minerals (Parmar & Sindhu, 2013). Iron (Fe) is essential for metabolic pathways and enzymatic reactions in plants, but its bioavailability is limited due to rapid oxidation to Fe(III), which is poorly soluble (Timofeeva et al., 2022). Insoluble Fe is converted by PGPB siderophores into soluble forms available to microorganisms, promoting plant growth (Scavino & Pedraza, 2013). Zinc (Zn), an important micronutrient, is required in small concentrations and serves as a cofactor and metal activator for enzymes (Parisi & Vallee, 1969). Certain rhizosphere bacteria enhance Zn availability for plants through mechanisms such as solubilization, chelation, and release of organic acids, phytohormones and vitamins, and oxidoreductive systems (Saravanan et al., 2011). Shahid et al. (2022) observed that *Kosaconia radicincitans* KR-17 inoculation increased the contents of nitrogen, phosphorus, potassium, calcium, magnesium, zinc, iron, copper, and sodium in Radish (*Raphanus sativus* L.) under saline conditions compared to non-inoculated plants.

2.9.6 Biofilm Formation under Salinity Stress Conditions

Biofilms, consisting of nucleic acids, lipids, proteins, exopolysaccharides (EPS), and microorganisms, enable rhizosphere bacteria to attach to plant root surfaces (Danhorn & Fuqua, 2007). Due to the ability of biofilm components to act as osmoprotectors, these biofilms serves as protective layers that help plants withstand stress conditions like salinity and drought (Rojas-Solis et al., 2020). EPS, a complex mixture of polymers released by bacteria, plays a crucial role in mitigating physiological and environmental stresses, including salinity (Morcillo & Manzanera, 2021). A noteworthy study demonstrated the significance of EPS in reducing salt stress and facilitating root colonization. *Pantoea alhagi* strains, including the exopolysaccharide-deficient ΔpspD mutant and the wild-type NX-11 strain, were evaluated in hydroponic trails, for their capacity to promote salt resistance in rice. While the pspD mutant did not, the wild strain increased rice's resistance to salt. Through modifications to root exudates, the EPS produced by NX-11 facilitated rhizosphere colonization by directly influencing biofilm formation and indirectly enhancing chemotaxis and biofilm formation. Gene expression analysis revealed that EPS upregulated the *OsXTH25* gene, which is associated with lectin production (Sun et al., 2022). The inoculation of *Bacillus tequilensis* in chickpea plants also alleviated salt stress. Bacterial flocculation in *B. tequilensis* was related to the development of biofilms and EPS. The presence of proteins and carbohydrates in the biofilm matrix was confirmed by Fourier-transformed infrared spectroscopy, these components interacted with Na^+ and contributed to salinity tolerance (Haroon et al., 2023).

2.10 Contribution of PGPB to Plant Biocontrol

Biocontrol of plant diseases by PGPB refers to the use of beneficial bacteria to suppress or manage plant diseases caused by various pathogens. These bacteria can contribute to plant health and disease resistance through multiple mechanisms.

2.10.1 Phytohormone Production

Phytohormone production plays a significant role in the biological control of plant diseases, as indicated by previous studies (Mukherjee et al., 2021; Soundar Raju et al., 2020). For example, the modulation of phytohormone signaling by *Bacillus amyloliquefaciens* has been found to induce disease tolerance against *Rhizoctonia solani* in Chickpea (Srivastava & Verma, 2015). Another study by Zebelo et al. (2016) demonstrated that *Spodoptera exigua* was suppressed after cotton plants were inoculated with *Bacillus sp*. This inoculation had a significant impact on the synthesis of jasmonic acid. In a study conducted by Karimi et al. (2012), it was observed that the inoculation of chickpea with *B. subtilis*, an IAA producer, resulted in enhanced plant development and exerted resistance against *F. oxysporum f. sp ciceris*. Furthermore, ethylene signals the onset of systemic resistance, which is mediated by rhizobacteria in defense against pathogens. Dixit et al. (2018) found the manipulation of ethylene levels in plants through the inoculation of ACC deaminase producing *Paenibacillus lentimorbus*, infected by *S. rolfsii,* resulted in effective control of southern blight disease. This control was attributed to the plant-beneficial bacteria's regulation of the ethylene pathway and the activities of antioxidant enzymes.

2.10.2 Lytic enzymes

Microbial enzymes including pectinases, glucanases, cellulases, lipases, chitinases, and proteases, have gained attention for their potential in controlling phytopathogens, earning them the name "cell-wall-degrading enzymes" (Fadiji & Babalola, 2020). These enzymes also play a significant role in the nutrient cycle of ecosystems by breaking down organic materials. By targeting the structural components of fungal cell walls, these enzymes inhibit spore germination and germ-tube elongation, thereby impeding fungal growth (Pandey et al., 2019). In another study, endophytic bacteria were isolated from horseradish (*Armoracia rusticana*) and evaluated for their lytic enzyme activity (Egamberdieva et al., 2020). These bacterial strains were found to inhibit plant diseases caused by *Rhizoctonia solani* and *Fusarium culmorum, F. solani*. Similarly, Muniroh et al. (2019) discovered that the plant-beneficial bacterium *Pseudomonas aeruginosa* effectively prevented basal stem rot in oil palm induced by *G. boninense*. This bacterium produced hydrolytic enzymes, such as cellulase, β-1,3-glucanase, and chitinase which contributed to the disease control. These findings highlight the potential of microbial enzymes as effective tools in controlling phytopathogens and the role of specific enzymes in degrading the cell walls of fungi.

2.10.3 Antifungal Compounds

PGPB possessing biocontrol capabilities are known to produce secondary metabolites, including antifungal and antibacterial compounds, which aid in inhibiting phytopathogens (Fadiji & Babalola, 2020). Numerous studies have reported the capacity of endophytic bacteria and fungi to produce antifungals, which are linked to the development of systemic resistance in plants (Jacob et al., 2020; Xu et al., 2019). Microbial antifungal compounds have emerged as key players in plant defense mechanisms and the biological management of developing plant diseases (Bolívar-Anillo et al., 2020; De Silva et al., 2019; Haidar et al., 2016). Bacillus, Aspergillus, Penicillium, Trichoderma, and Streptomyces species are among the well-known antibiotic-producing endophytes (De Silva et al., 2019). For example, *Streptomyces* sp. has been found to produce trimethyl sulfide and dimethyl sulfide, which contribute to the reduction of tomato bacterial wilt and red pepper leaf spot (Le et al., 2022). *Bacillus* sp., isolated from soybean and exhibiting biocontrol abilities against *Phytophthora sojae*, has been found to produce two types of antifungal compounds (Zhao et al., 2018). Additionally, iturin A synthesized by *Bacillus* sp. CY22 has been identified as an inhibitory agent against *Rhizoctonia solani*, which is the pathogen responsible for balloon flower root rot (Cho et al., 2003). Microbial antifungal compounds hold promise as potential substitutes to conventional fungicides in agriculture, mitigating the harmful effects on the environment and public health. These compounds can effectively limit the development and dessimination of fungal pathogens, providing protection against various plant diseases.

2.10.4 Siderophore Production

PGPB have the ability to produce volatile compounds that directly inhibit the development of pathogens (Xia et al., 2022). Siderophore secretion by PGPB plays a role in enhancing plant growth by enabling plants to compete with iron and providing protection against pathogenic organisms (Ryu et al., 2003). Iron is essential for the growth and survival of harmful microorganisms, including fungi and bacteria. Beneficial microorganisms can produce siderophores that compete with pathogens for iron, thereby restricting their ability to proliferate and survive. This mechanism aids in defending plants against various infections that rely on iron (Egamberdieva et al., 2023). The use of siderophore-producing endophytes as biocontrol agents holds promise in addressing plant health. For example, *Bacillus subtilis* CAS15, a strain known for its siderophore production, was found to significantly prevent Fusarium wilt and also promote the growth of pepper plants (Yu et al., 2011). Another study by Lecomte et al. (2016) demonstrated that *Pseudomonas* sp. are capable of regulating *F. oxysporum* f. sp. *dianthi* through mechanisms such as heightened competition for resources and ecological niches.

2.10.5 Induce Systemic Resistance (ISR)

In the realm of sustainable agriculture, induced resistance has gained prominence as a promising strategy to combat plant diseases (Fadiji & Babalola, 2020). PGPB employ two primary mechanisms, induced systemic resistance (ISR) and systemic acquired resistance (SAR), to protect host plants against diseases (Gao et al., 2021; Ullah et al., 2019). ISR enhances plant resistance to pathogens by activating pathogen-related proteins, polyphenols, phytoalexins, and signal transduction pathways mediated by salicylic acid (SA)/jasmonate (JA) or ethylene (ET) (Kloepper & Ryu, 2006; Romera et al., 2019). Pathogenesis-related (PR) proteins, including enzymes such as chitinases and β-1,3-glucanases, play a crucial role in mitigating the effects of plant infections and improving defense against them, particularly under biotic stresses (Gao et al., 2010). These enzymes contribute to strengthen cell wall defenses against infections and cell death as well as to lyse invasive fungal cells. For example, in pepper plants, *P. polymyxa* induces ISR, defending them against *Xanthomonas axonopodis* pv. *Vesicatoria*, a the bacterial spot pathogen and reducing disease severity (Phi et al., 2010). *Bacillus velezensis* inoculation in Arabidopsis, expressing the senescence-promoting gene *phytoalexin deficient4* (PAD4), led to decreased proliferation of the green peach aphid *Myzus persicae* (Rashid et al., 2017). *Burkholderia gladioli* strain E39CS3 significantly elevated endogenous JA levels and upregulated the expression of JA-regulated plant defense genes, resulting in a notable reduction in corm rot caused by *F. oxysporum* (Ahmad et al., 2022). In a study by Y. Jha (2019), it was observed that in response to *P. pseudoalcaligenes* pathogen inoculation, rice plants demonstrated upregulation of PR proteins, including catalase and β-1,3-glucanase. Moreover, the acyl-homoserine lactones (AHL) produced by beneficial bacteria such as *P. putida* and *Serratia liquefaciens* elicited systemic resistance in tomatoes against *Alternaria alternata* (Schuhegger et al., 2006). *Bacillus* sp. was found to upregulate the expression of *PR1a, PR2a,* and *PR3* genes, leading to the production of chitinases and glucanases that inhibit the proliferation of *S. rolfsii* (Sahu et al., 2019). Similarly, Gupta et al. (2022) demonstrated that *Pseudomonas aeruginosa* induced ISR and stimulated enhanced total phenolic content and antioxidant enzyme activity in pea plants infected with *Fusarium oxysporum* f. sp. *pisi*. Overall, ISR plays a significant role in plant’s defense mechanism by priming the plant, triggering systemic signaling, activating defense genes, and interacting with other defense pathways. As a result, ISR significantly enhances the plant's resistance against infections, leading to enhanced overall health and growth.

2.10.6 Antioxidant Enzymes

Antioxidant enzymes play a crucial role in plant defense by scavenging harmful ROS generated during diverse stress conditions, including pathogen attacks (X. Wang et al., 2023). The antioxidant system in plants can also be stimulated by microbes associated with plants (Cavalcanti et al., 2020). For example, Sebestyen et al. (2022) discovered that the production of antioxidants and iron-binding metabolites by *Hypocrea atroviride* and *Bacillus subtilis* inhibit the growth of the fungal pathogen *Eutypa lata* that causes grapevine trunk diseases. Moreover, Sahu et al. (2019) showed that by promoting lipid peroxidation and the synthesis of ascorbate oxidase, peroxidase, and polyphenol oxidase, *Bacillus* sp. induces ISR and reinforces the cell wall.

2.10.7 Competition for Nutrients and Niches

Soil and rhizospheres are an intricate system characterized by the presence of abundant carbon, nutrients, oxygen, and diverse range of microbes. Within these environments, various microbes, including both beneficial bacteria and pathogenic fungi, coexist and engage in competitive interactions for nutrients and niches (Compant et al., 2005; Lugtenberg et al., 2001). All organisms depend on nutrients for growth and reproduction, and in natural ecosystems, competition for these resources can be intense (Köhl et al., 2019). The microbial community already present in the target environment must be considered when introducing a biocontrol agent as it must effectively compete with other microorganisms for nutrients and space (Srebot et al., 2021). If the biocontrol agent fails to establish itself or maintain a sufficient population, its ability to provide effective pest control may be compromised. Studies have shown that limiting mineral elements, nutrients and space can inhibit the fungal pathogens spore germination and the development of infection on host tissue (Fokkema et al., 1983). Biocontrol bacteria play a critical role in root systems by actively colonizing and occupying niches while consuming nutrient sources from root exudates. This process enables them to compete with pathogens for essential resources required for their growth and proliferation (Eisendle et al., 2004; van Dijk & Nelson, 2000). Effective colonization of the root system by these bacteria also serves as a delivery system for biologically active metabolites, such as cell-wall-degrading enzymes, hydrogen cyanide (HCN), and antifungal compounds, which exert negative effect on the physiology of fungal pathogens (Chin-A-Woeng et al., 1998). For example, *Pseudomonas extremorientalis* strain TSAU20 exhibited improved root colonization and a 10% reduction in cucumber root rot caused by *Fusarium solani*. Although this strain does not produce antifungal compounds or HCN, cellulase, lipase, or glucanase, its main biocontrol mechanism was competition for nutrients and niches (Egamberdieva et al., 2011). Similarly, *Pseudomonas fluorescens* strain PCL1751 demonstrated effective colonization of rhizosphere and shown efficacy in reducing tomato root and foot rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Kamilova et al., 2005). Pseudomonas have been seen to colonize the rhizosphere and interact with plants by motility and chemotaxis towards root exudates (de Weert & Bloemberg, 2006). In another study, *Pseudomonas syringae*-induced disease symptoms in Arabidopsis were inhibited by *Sphingomonas* strains, demonstrating the critical role that bacterial competition for a carbon supply plays in preventing pathogen development (Innerebner et al., 2011). The ability to effectively colonize and form biofilms is considered crucial traits exhibited by biocontrol bacteria, enabling them to establish and maintain their presence in the rhizosphere ecosystem. Research has shown that *Stenotrophomonas maltophilia* and *Pseudomonas fluorescens* occupy similar niches as the pathogen *Pseudomonas syringae* on the phyllosphere of beans and can suppress diseases caused by plant pathogens (Ji & Wilson, 2002). Therefore, for biocontrol to be effective, it is crucial to understand the niche requirements of both the biocontrol agent (BCA) and the target pest (Situ et al., 2023). A key element in determining a BCA's success is competition for nutrients and niches. It is critical to take into account the current microbial community as well as the niche preferences of the target pest and the BCA when creating a biocontrol plan or product. Table 2.2 provides a summary of the findings on the application of PGPB in biocontrol.

Table 2. 2: **Influence of PGPB on biocontrol**

Plant	Bacterial strain	Mechanism	Effect	Reference
Chickpea	*Bacillus amyloliquefaciens*	Phytohormone signaling	Disease resistance against *Rhizoctonia solani*	Srivastava and Verma (2015)
Cotton	*Bacillus sp*.	Increased synthesis of jasmonic acid,	Biocontrol against beet armyworm *Spodoptera exigua*.	Zebelo et al. (2016)
Chickpea	*B. subtilis*, *umsongensis* KRT21, *Brevibacterium frigoritolerans* KLT2 and *Pantoea agglomerans* KLT4	IAA production	Biocontrol against *F. oxysporum* f. sp *ciceris*	Karimi et al. (2012)
Oil palm	*Pseudomonas aeruginosa*	Production of CHI, GLU and cellulase	Biocontrol against Stem root *G. boninense*.	Muniroh et al. (2019)
Tomato	*Paenibacillus lentimorbus*	ACC deaminase production	Biocontrol against *S. rolfsii*	Dixit et al. (2018)
Tomato	*Streptomyces* sp.	Production of dimethyl sulfide and trimethyl sulfide		Le et al. (2022)
Soybean	*Bacillus* sp.,	Antifungal production	Biocontrol against *Phytophthora sojae*,	Zhao et al. 2018)
Balloon flower	*Bacillus* sp. CY22	Production of iturin A	Biocontrol against *Rhizoctonia solani*	Cho et al. (2003)
Pepper	*Bacillus subtilis* CAS15	Siderophore production	Control against Fusarium wilt	Yu et al. (2011)

Table 2.2 continued

Plant	Bacterial strain	Mechanism	Effect	Reference
Pepper	*P. polymyxa*	Induction of ISR	*Resistance* against *Xanthomonas axonopodis* pv. *vesicatoria*	Phi et al. (2010)
Arabidopsis	*Bacillus velezensis*	Expressing of phytoalexin *deficient4*	Control against *Myzus persicae*	Rashid et al. (2017).
Saffron	*Burkholderia gladioli*	Elevated endogenous JA levels and defense genes	Biocontrol against *F. oxysporum*	Ahmad et al. (2022).
Rice	*P. pseudoalcaligenes*	Induction of GLU and CAT	Control against *Pyricularia grisea*	Y. Jha (2019).
Tomato	*Serratia liquefaciens,* *P. putida*	Production of acyl-homoserine lactones (AHL)	Resistance against *Alternaria alternata*	Schuhegger et al. (2006)
Tomato	*Bacillus* sp.	Upregulation of *PR1a, PR2a,* and *PR3* expression	Resistance against *S. rolfsii*	Sahu et al. (2019)
Pea	*Pseudomonas aeruginosa*	Induction of ISR	Resistance against *Fusarium oxysporum* f.sp. *pisi*	Gupta et al. (2022)
Grapevine	*Bacillus subtilis* and *Hypocrea atroviride*	Induction of ISR	Control against *Eutypa lata*	Sebestyen et al. (2022)
Tomato	*Pseudomonas fluorescens*	Colonization and competition for niche	Resistance against *Fusarium oxysporum* f. sp. *radicis-lycopersici*	Kamilova et al. (2005)

Table 2.2 continued

Plant	Bacterial strain	Mechanism	Effect	Reference
Cucumber	*Pseudomonas extremorientalis*	Enhanced root colonization and competition for niche	Resistance against *Fusarium solani*	Egamberdieva et al. (2011)
Arabidopsis	*Sphingomonas*	Competition for carbon source	Resistance against *Pseudomonas syringae*	Innerebner et al. (2011)
Beans	*Pseudomonas fluorescens* and *Stenotrophomonas maltophilia*	Competition for niche	Resistance against *Pseudomonas syringae*	Ji and Wilson (2002)

2.11 Transcription factors

Transcription factors (TFs) play a critical role in regulating gene expression and are key components of plant signaling pathways. They are involved in plant growth, development, and stress responses by binding to specific DNA sequences in the promoter regions of target genes. TFs act as master regulators that coordinate various biological processes and enable plants to adapt to changing environmental conditions (Joshi et al., 2016; Shahzad et al., 2021). TFs can activate or suppress gene activity based on their binding to DNA-binding sites. They possess activator or repressor domains that modulate gene expression once they bind to specific DNA sequences. TFs often have additional domains that interact with other proteins, such as other TFs or signaling molecules, increasing their functional diversity (Phillips & Hoopes, 2008). Posttranslational modifications, such as phosphorylation, can also regulate TF activity, as observed in the MAPK cascade system (Guan et al., 2014). TFs are not only regulated by internal cues but also respond to various external stimuli. They play a crucial role in stress management, metabolism, and plant growth. For instance, TFs like *WRKY* and *AP2* have been implicated in the regulation of plant-specific metabolism and stress tolerance (K.

Kumar et al., 2016; Mishra et al., 2013). The plant genome encodes a significant number of TFs, with approximately 5% of the Arabidopsis genome consisting of TF gene (Arabidopsis Genome Project; (Riechmann & Ratcliffe, 2000). Different categories of TFs have been identified in plants, including AP2/ERF (APETALA2/ethylene-responsive factor), bHLH (basic helix-loop-helix), bZIP (basic leucine zipper), MYB (myeloblastosis related), NAC (no apical meristem), and WRKY TFs. These TFs exhibit diverse functions and regulate various molecular events in plant biology (Kilian et al., 2012; Udvardi et al., 2007). Understanding the functional diversity of TFs is essential for unraveling the molecular mechanisms underlying plant biology. Table 2.3 presents an overview of TFs associated with abiotic stress-related, while Table 2.4 an overview of TFs associated with biotic stress-related both found in different plant species. By studying the roles and interactions of these TFs, researchers gain insights into the molecular processes that govern plant growth, development, and stress responses. The availability of genome-scale metabolic pathway databases for numerous plant genomes aids in understanding the functional implications of TFs across different plant species (Hawkins et al., 2021). In the subsequent section, the focus will be on the WRKY TF family, which has been extensively studied for its involvement in plant responses to biotic and abiotic stresses.

Table 2. 3: Transcription factors involved in abiotic stress in plants

Transcription factor	Crop species	Functional validation	Function in Stress response	Reference
MaWRKY18	Banana	Overexpression	Drought, salt and cold	Tak et al., 2021
TaWRKY1-2D	Arabidopsis	Overexpression	Drought	Y. Yu et al., 2023.
SbWRKY45	Arabidopsis	Overexpression	Drought	BAILLO et al. (2023)
SbWRKY30	Sorghum	Overexpression	Drought	Yang et al., 2020

Table 2.3 continued

Transcription factor	Crop species	Functional validation	Function in Stress response	Reference
SlWRKY81	Tomato	Overexpression	Drought	Ahammed et al., 2020.
OsWRKY45	Rice	Overexpression	Drought	Qiu & Yu, (2009).
TaWRKY146	Arabidopsis	Overexpression	Salinity and Drought	Ma et al., 2017
FtWRKY46	Arabidopsis	Overexpression	Salt	Lv et al., (2020)
AtWRKY46	Arabidopsis		Salinity and Drought	Ding et al., (2014).
CiWRKY75-1 and *CiWRKY40-4*	*Arabidopsis*		Drought	Wan et al. (2018)
PgWRKY	Pearl Millet		Salinity and Drought	Chanwala et al. (2020)
MuWRKY3	Horse gram		Drought	Kiranmai et al., 2018
AtWRKY29	Arabidopsis	Overexpression	Heat	D. Li et al., 2017; Y. Li et al., 2017
CaWRKY40	Tobacco	Overexpression	Heat	DANG et al., 2013
CaWRKY6	Pepper	Overexpression	Heat	Cai et al., (2015).

Table 2.4 Transcription factors involved in biotic stress in plants

Transcription factor	Crop species	Functional validation	Function in Stress response	Reference
OsWRKY62 and OsWRKY67	Rice	Overexpression	*Xanthomonas oryzae pv. oryzae (Xoo)* and *Magnaporthe oryzae*	Vo et al. (2018)
CsWRKY2 and *CsWRKY14*	Sweet Orange	Overexpression	*P. digitatum*	Xi et al. (2023)
AtWRKY38 and At*WRKY62*	Arabidopsis	Overexpression	*Pseudomonas syringae*	Kim et al. (2008)
ShWRKY81	Tomato	Overexpression	*O. neolycopersici*	H. Wang et al. (2023)
GhWRKY41	Cotton and Arabidopsis	Overexpression	*V. dahliae*	(Xiao et al. (2023)
OsWRKY31	Rice	Overexpression	*Magnaporthe oryzae*	S. Wang et al. (2023)
VqWRKY56 and *VqbZIPC22*	Grapevive	Overexpression	*Erysiphe necator*	Y. Wang et al. (2023)
OscWRKY1	Arabidopsis	Overexpression	*Pseudomonas syringae pv. tomato Pst DC3000*	Joshi et al. (2022)
JrWRKY21	Walnut	Overexpression	*C. gloeosporioide*	Zhou et al. (2022)
CsWRKY23	Citrus	Overexpression	*Penicillium digitatum*	W. Wang et al. (2023)
GhWRKY70	Arabidopsis	Overexpression	*Verticillium dahliae*	Zhang et al. (2023)
TaWRKY49 and TaWRKY62	Wheat		*Puccinia striiformis f. sp. tritici*	N. Wang et al. (2016)
CaWRKY40b	Pepper		*Ralstonia solanacearum*	Ifnan Khan et al. (2018)

2.12 WRKY Transcription factors

The WRKY gene family, which is one of the largest transcription factor gene families, plays a crucial role in various plant activities from germination to senescence (Goyal et al., 2023). These transcription factors are named WRKY due to the presence of a highly conserved sequence WRKYGQK at their N-terminus (Jiang et al., 2017). Additionally, a conserved zinc finger motif (C-X4-5-C-X22-23-H-X-H or C-X5-8-C-X25-28-H-X1-2-C) is found at the C-terminus of the WRKY motif. Through binding with the W-box DNA sequence (C/T) TGAC(T/C) in target gene promoters, WRKY proteins regulate gene expression (H. Li et al., 2020; X. Li et al., 2020). It is important to highlight the considerable diversity in both the structure and function of WRKY TFs. This variability may be seen in the diverse numbers of WRKY genes and proteins found in distinct plant genomes, as well as in the variations in intron counts and nucleotide sequences found across various plant evolutionary levels. Due to their participation in numerous plant processes, such as growth, development, and responses to biotic and abiotic stressors, WRKY TFs have drawn considerable interest. Additionally, they are essential for effector-triggered immunity (ETI) and microbe- or pathogen-associated molecular pattern-triggered immunity (MTI or PTI) in plants (Tang et al., 2023; Xi et al., 2023; Y. Yu et al., 2023). These TFs can function as activators or repressors, forming a network that regulates various cytoplasmic and nuclear functions and mediates signaling between organelles and the nucleus (Bakshi & Oelmüller, 2014).

2.12.1 Structural characteristics and classification of functional domains

The different binding capacities of WRKY TFs could be attributed to presence of different numbers of DNA-binding domains (DBDs) and zinc-finger-like motifs despite possessing a highly conserved W-box. (Wani et al., 2021). WRKY genes are categorized into three primary groups. Group I and II contain two and one WRKY domains, respectively, with a single C2-H2 zinc finger motif shared by both groups (C–X4–5–C–X22–23–H–X1–H). Group III WRKY genes have a single WRKY domain but show variation in C2-HC zinc finger motif patterns (C–X7–C–X23–H–X1–C). Furthermore, Group II WRKY genes are further classified into five subgroups based on primary amino acid sequences (IIa, IIb, IIc, IId, and IIe) (Eulgem et al., 2000). Pre-WRKY structures (Pro-WRKY) are thought to have evolved from a single domain, according to studies on WRKY structures, with Group I members possibly arising from gene duplication and Group IIc members potentially resulting from the loss of the N-terminal WRKY domain. Group III represents the youngest and least diverse group, while Group IIc may have branched out to give rise to additional subgroups within Group II (Song & Gao, 2014; J. Wu et al., 2017). Strong evidence suggests that group II and III WRKY members should have evolved from group I based on sequence similarities between the WRKY domains of those members and the C-terminal WRKY domain of group I WRKY TFs (Chen et al., 2019). Despite the WRKY domain's high degree of conservation, sequence variations have been observed within it. For example, variations such as WRKYGRK in banana and populus, FWRKYGQK in populus, and WRKYGEK in banana and rice. Banana also exhibits variations like WRKYGHK and WRKYGNK. WRKY TFs have been extensively studied in various monocot crops, and their distribution within chromosomes and genomes is not uniform (Goel et al., 2016; Chanwala et al., 2020; Xu et al., 2016; Zhang et al., 2017).

WRKY TFs are known to bind to specific cis-elements called W box motifs in the promoters of target genes, either activating or inhibiting gene transcription (C. X. Li et al., 2020; H. Li et al., 2020; X. Li et al., 2020). The W-box comprises a degenerated/core TGAC motif, which serves as the central sequence recognized by WRKY factors. Additionally, the W-box contains a conserved GAC core, which is essential for WRKY factor binding. The W-box is further characterized by flanking thymine (T) and pyrimidine (C/T) residues that contribute to the overall interaction between WRKY factors and the cis-element (Chen et al., 2019; Jiang et al., 2017). When WRKY TFs bind to other WRKY TFs or their own promoters, auto- or cross-regulation is frequently seen, suggesting the presence of functioning W-box components (Liu et al., 2021). The hydrophobic W-box region's main groove is bound by WRKY-DBDs through α-helices or β-sheets, allowing for specific interactions between the DNA and protein (Deeba et al., 2017). The WRKY45-DBD in OsWRKY45 can form a homodimer by switching β4–β5 strands, providing flexibility (Cheng et al., 2019; Xu et al., 2018). Key residues such as Tyr, Trp, and Lys in the WRKYGQK sequence, as well as zinc finger structures, are crucial for DNA binding, as evidenced by inhibitor and mutational studies (Chen et al., 2019; Ciolkowski et al., 2008). The WRKY domain's structure can be altered by changes in the amino acid heptad, enabling coordinated and precise regulation of target genes by WRKY TFs (Chen et al., 2019).

Different plant species exhibit variations in the distribution of WRKY TF groups. For example, Arabidopsis and rice have a higher abundance of group I WRKY proteins compared to strawberry. Poplar, on the other hand, has 50% of its WRKY TFs belonging to group I. Mulberry consists solely of group I members in the group Ia sub-group, while a distinctive motif of GGDFDDNEPEAKR-WKGE is found in the C-terminal regions of all group Ia members and one member of the group IIb family (Baranwal et al., 2016). Tea also displays variations, such as the replacement of glutamine with lysine in the protein sequence (Wu et al., 2016). WRKY III may have originated through duplication events during the divergence of monocots and dicots according to studies focusing on woody dicots, such as Populus (Wang et al., 2015). The structural complexity of WRKY genes is shown by the low diversity between monocots and dicots in relation to lower plant groups and the presence of spliced domains with R-type or V-type introns (Liang et al., 2017). In wheat and strawberries, the number and type of introns vary, with V-type introns exclusive to type IIa and IIb groups. (Ning et al., 2017; Zhou et al., 2016). Dicots like pepper have leucine zipper structures in Group IIa members, which act as elicitors for WRKY TF binding to the W-box (Cormack et al., 2002). The WRKY domain sequence and Zn-finger motifs differ between Group IIe and Group III members in tomato (Huang et al., 2012). Gene duplications, losses, and positive or neutral selection have also shaped the evolution of WRKY TFs (Zhou et al., 2016; Tang et al., 2013). The presence of multiple duplicated blocks in the WRKY family provides evidence for duplications that have occurred during evolution and genome expansion (Xu et al., 2016; Zhang et al., 2017).

2.13 WRKY TF and Plant Stress Response

2.13.1 Role in Biotic Stress

Plants have developed mechanisms in response to a variety of biotic stresses that they experience during their life cycle, such as pathogen attacks (Jha et al., 2020; Sun et al., 2015). Numerous studies have been conducted on the significance of WRKY TFs as regulators of the plant immunological response to various biotic stressors (Cui et al., 2019; Singh et al., 2017). The binding of WRKY TFs to the W-box regulates the transcriptional activation or suppression of defense genes, thus influencing the plant's immune response. Peng et al. (2018) reviewed PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI), the two lines of plant immune response, which are involved in recognizing molecular patterns of the pathogen (PAMPs) and detecting toxins/effectors through pattern-recognition receptors (PRRs) and plant resistance (R) proteins, respectively. WRKYs have been shown to play crucial roles in in both PTI and ETI by directly or indirectly interacting PAMPs/effector proteins or by their regulation through mitogen-activated protein kinases (MAPKs) (Phukan et al., 2016). It has been shown that PTI and ETI in rice are mediated by the WRKY transcription factor

OsWRKY62 (Peng et al., 2008). Overexpression of one of its splice variants, *OsWRKY62.1* in transgenic rice plants resulted in impaired PTI and ETI responses, accompanied by the suppression of defense-related genes, upon infection with bacterial blight. In contrast, *OsWRKY67* acts as a positive regulator of PTI and ETI in rice against *Xanthomonas oryzae* pv. *oryzae* (Xoo) and *Magnaporthe oryzae* (Vo et al., 2018).

Xi et al. (2023) reported that Sweet Orange *CsWRKY2* and *CsWRKY14* were significantly expressed in response to *P. digitatum* infection. Similarly, Kim et al. (2008) found that Arabidopsis *AtWRKY38* and *AtWRKY62* regulate resistance to *Pseudomonas syringae* through the SA-dependent pathway, affecting the expression of *Pathogenesis-Related1* (PR1) genes. (H. Wang et al., 2023) demonstrated how *ShWRKY81* regulates tomato's resistance against *O. neolycopersici* through the SA-dependent expression of PR genes. Xiao et al. (2023), showed that knocking down *GhWRKY41* makes cotton more susceptible to the fungus, while overexpressing it promotes resistance to *V. dahliae* in transgenic cotton and Arabidopsis. Additionally, *GhWRKY41* physically interacts with itself and directly stimulates its own transcription. Similarly, S. Wang et al. (2023) reported that the rice TF gene *OsWRKY31* is an essential part of an MPK signaling pathway involved in rice's resistance to plant diseases, particularly against *Magnaporthe oryzae*, causing rice blast.

In grapevine, *VqWRKY56* and *VqbZIPC22* interacts to increase proanthocyanidin biosynthesis and enhance resistance to powdery mildew (Y. Wang et al., 2023). Similarly, Joshi et al. (2022) showed that *OscWRKY1* positively regulates genes in the phenylpropanoid pathway, altering the level of rosmarinic acid and enhancing Arabidopsis' resistance to bacterial pathogens. Zhou et al. (2022) demonstrated that the overexpression of *JrWRKY21* favors walnut resistance to *C. gloeosporioides* and indirectly induces the expression of PR gene *JrPR5L* via the *WRKY21-PTI5L* protein complex. In citrus, the overexpression of *CsWRKY23* enhanced the resistance to

Penicillium digitatum through the SA pathway, leading to ROS accumulation and thickening of cell walls (W. Wang et al., 2023). In transgenic Arabidopsis, overexpression of *GhWRKY70* displayed better growth characteristics, greater lignin content, increased antioxidant enzyme activities, and elevated levels of jasmonic acid, resulting in resistance to *Verticillium dahliae* (Zhang et al., 2023). In wheat, specific WRKY genes have been identified as key players in conferring resistance to stripe rust (*Puccinia striiformis* f. sp. *tritici*) under high-temperature conditions. Among these genes, *TaWRKY4*9 and *TaWRKY62* have been found to be differentially expressed and associated with seedling-plant resistance to stripe rust. Interestingly, the silencing of *TaWRKY49* has been shown to enhance resistance to stripe rust, indicating its negative regulatory role in the defense response against the pathogen. On the other hand, the silencing of *TaWRKY62* results in decreased resistance to stripe rust, suggesting its positive regulatory role in plant defense (Wang et al., 2016). In pepper, the *CaWRKY40b* gene has been identified as a negative regulator controlling a group of genes linked to immunity during infection with the pathogen *Ralstonia solanacearum.* Silencing of *CaWRKY40b* using VIGS and overexpression of a dominant repressor form of *CaWRKY40b* known as *CaWRKY40b-SRDX* resulted in the upregulation of positive regulators and the downregulation of negative regulators associated with immunity (Ifnan Khan et al., 2018). Moreover, increased *CaWRKY40* transcript levels during *R. solanacearum* infection stimulate ET, JA, and SA-mediated pathways.

In banana, the genes *MaWRKY23, MaWRKY4, MaWRKY25,* and *MaWRKY28* were specifically expressed during the early stages of corm development at 1, 7, and 14 days after infection with Foc-TR4. These genes exhibited differential expression between the resistant Pahang and susceptible Brazilian banana cultivars, suggesting their potential involvement in the plant-pathogen interaction pathway (Zhang et al., 2019). Additionally, in *Musa acuminata* and *Musa balbisiana*, the genes *MaWRKY145, MaWRKY45,* *MaWRKY89, MaWRKY24,* and *MaWRKY65,* were highly expressed in banana roots at 3, 27, and 51 hours after Foc-TR4 infection, indicating their potential role in the response to the pathogen (Goel et al., 2016).

2.13.2 Role in Abiotic stress

The role of WRKY transcription factors (TFs) in regulating plants' response to abiotic stress has gained substantial support from a growing body of research. With the pressing need to increase agricultural yields to feed a growing population amidst escalating challenges of drought and salinity, understanding the mechanisms underlying stress tolerance is paramount (S. Jha, 2019). Recent studies have shed light on the contributions of specific WRKY TFs in conferring drought resistance in transgenic Arabidopsis. For example, *TaWRKY1-2D* was found to enhance drought tolerance by posttranslational regulation of *TaDHN3* (Y. Yu et al., 2023). Furthermore, under drought stress conditions, transgenic Arabidopsis expressing *TaWRKY1-2D* displayed significantly elevated expression of stress response and antioxidant system genes, including *AtRD29A*, *AtP5CS1*, *AtPOD1*, *AtCAT1*, and *AtSOD* (Cu/Zn) (Cu/Zn) (Y. Yu et al., 2023). In another study, overexpression of *SbWRKY45* in transgenic Arabidopsis enhanced root development rates and germination rates under drought stress, suggesting its potential role in sorghum's response to abiotic stress, particularly drought (BAILLO et al., 2023). Similarly, in sorghum, *SbWRKY30* was found to regulate the drought stress response gene *SbRD19* through its binding to W-box elements in the *SbRD19* promoter, thereby improving drought tolerance by protecting plant cells from ROS damage (Yang et al., 2020). In tomato, *SlWRKY81* played a crucial role in enhancing drought tolerance by preventing the accumulation of H_2O_2 and acting as a negative regulator of stomatal closure (Ahammed et al., 2020).

Increased resilience in transgenic Arabidopsis under salinity stress was caused by the overexpression of *PbWRKY40*, which resulted in increased resilience, manifested by improved germination rate, root development, chlorophyll content, and total organic acid content, while exhibiting reduced electrolyte leakage, Na^+/K^+ ratio, and MDA content (Lin et al., 2022). Similarly, heterologous expression of *DcWRKY12* in yam (*D. composita*) enhanced salt and osmotic stress tolerance by modulating antioxidant enzyme activity, osmoregulatory substance content, ion homeostasis, and the expression of salt stress-responsive genes (S. Yu et al., 2023). Furthermore, *WRKY46* in Arabidopsis was found to play a critical role in regulating lateral root development during osmotic/salt stress, involving intricate interactions with ABA signaling and auxin homeostasis (Ding et al., 2015). Additionally, *IbWRKY47* in sweet potato (*Ipomoea batatas*) positively regulated stress resistance-related genes, enhancing salt stress tolerance (Qin et al., 2020). In Moso bamboo (*Phyllostachys edulis*), *PeWRKY83* expression levels varied significantly under abiotic stressors, and transgenic Arabidopsis overexpressing *PeWRKY83* displayed improved tolerance to salt stress (M. Wu et al., 2017). Similarly, *DgWRKY5* in Chrysanthemum showed increased expression in response to various stressors, and its overexpression enhanced salt tolerance in transgenic plants (Liang et al., 2017). On the other hand, overexpression of *PcWRKY33* (*Polygonum cuspidatum*) and *CmWRKY17* (Chrysanthemum) in Arabidopsis increased salt sensitivity (Bao et al., 2018; J. b. Li et al., 2015).

These studies collectively highlight the diverse roles of WRKY transcription factors in modulating salt stress responses and tolerance in different plant species. The findings underscore the complexity of WRKY-mediated regulatory networks and the importance of species-specific responses in the context of salinity stress. Further research is needed to elucidate the underlying molecular mechanisms and explore the potential application of WRKY genes in improving salt tolerance in crop plants.

CHAPTER 3: MATERIALS AND METHODS

3.1 Equipment and Commercial Kits

3.1.1 Equipment

Equipment used in this study is listed below (in alphabetical order):

1. Autoclave sterilizer (Hirayama, Japan)
2. Agilent RNA 6000 Nano LabChip Kit and an Agilent 2100 Bioanalyzer (Agilent Technologies, United States).
3. Centrifuge 5430R (Eppendorf, Germany)
4. DocuCentre-IV 2060 scanner (Fuji Xerox, Japan)
5. EC meter (Themo Scientific, United States)
6. Field Emission Scanning Electron Microscope FEI Quanta FEG 650 (FEI, USA)
7. Gel documentation system (Cambridge Scientific, United States)
8. Heat block SBH1300 (Cole Parmer, United States)
9. Incubator (Memmert, Germany)
10. Inductively coupled plasma mass spectrometry 7500 Single Turbo System (Agilent Technologies, United States)
11. Laboratory refrigerator SJD226M (Sharp, Japan)
12. Liquid nitrogen Tank (Taylor Wharton, United States)
13. Micropipettes (Eppendorf, Germany)
14. Microscope CX23 (Olympus, Japan)
15. MilliQ Integral Water Purification System (Elga, United States)
16. Mini-rotator SCF-5 (DAIHAN Scientific, South Korea)
17. Nanophotometer Pearl (Implen, Germany)
18. Oven UNB400 (Memmert, Germany)
19. pH meter (Sartorius, Germany)

20. QuantStudio^{TM} 12k Flex system (Applied Biosystems, USA)

21. Shaking incubator LM-575D (Yihder, Taiwan)

22. Sputtering Hummer II (Tectonics, Springfield, United States)

23. Stereomicroscope TL3000 (Leica Microsystems, Wetzlar, Germany)

24. Thermocycler (Esco, Singapore)

25. Ultra Low Temperature Freezer (New Brunswick, USA)

26. Water bath (Being Instrument, China)

3.1.2 Commercial Kit

Commercial kits used in this study are listed below (in alphabetical order):

1. SYBR® Green Master Mix (Vanzyme, China)

2. Transcript® One-Step gDNA remover and cDNA synthesis Supermix (TransGen Biotech, China).

3.2 Oligonucleotide Primers

Oligonucleotide primers used in this study were ordered from Integrated DNA Technologies Pte. Ltd., Singapore and listed in Table 3.1.

Table 3. 1: Oligonucleotide primers used in this study.

Primers	Sequence (5' – 3')	Length	Function	Locus ID
8F	AGA GTT TGA TCC TGG CTC AG	21	Amplification of 16S rRNA gene	Khan et al. 2016
1510R	GGC TAC CTT GTT ACG A	16	Amplification of 16S rRNA gene	Khan et al. 2016
MaWRKY4F	GGA TAG GGC AGG CGA TTT ATT	21	qPCR	*GSMUA_Achr10G06060_001*
MaWRKY4R	ATT AGG GTC GCA GAT CAG	18	qPCR	*GSMUA_Achr10G06060_001*
MaWRKY23F	GAC GGT GCC CTT GTC TTA TT	20	qPCR	*GSMUA_Achr6G22160_001*
MaWRKY23R	GAC TCT AGC CTA TGA AGC TGT G	22	qPCR	*GSMUA_Achr6G22160_001*
MaWRKY24F	TTC AGT GGT ATC AGC CAA GTC	21	qPCR	GSMUA_Achr8G01790_001
MaWRKY24R	GAC TTC AAC CTT TCC AAG CTT AC	23	qPCR	GSMUA_Achr8G01790_001

Table 3.1 continued

Primers	Sequence (5' – 3')	Length	Function	Locus ID
MaWRKY25F	CCT AAT GGA CAC CTC CTC ATA C	22	qPCR	GSMUA_Achr6G32720_001
MaWRKY25R	CAT CAT GGA CCA CAC CAA ATC	21	qPCR	GSMUA_Achr6G32720_001
MaWRKY28F	CAG AAG ATG GCG AAG GGA AA	20	qPCR	*GSMUA_Achr3G31130*
MaWRKY28R	CAC ACC GCT GAA CTT GTT TG	20	qPCR	GSMUA_Achr3G31130
MaWRKY45F	CGA AGA GAG GAA CGA GGA ATT T	22	qPCR	*GSMUA_Achr4G20600*
MaWRKY45R	GGC CTA ACC ATG GAC TTC AC	20	qPCR	*GSMUA_Achr4G20600*
MaWRKY65F	GAG AAG CAT GTC AGG GAA GAT G	22	qPCR	*GSMUA_Achr6G07620*
MaWRKY65R	CGG ACT TGG AAG GAG CTA ATC	21	qPCR	*GSMUA_Achr6G07620*

Table 3.1 continued

Primers	Sequence (5' – 3')	Length	Function	Locus ID
MaWRKY89F	GTG CAG AGA AGT GCA GAA GA	20	qPCR	*GSMUA_Achr7G19340*
MaWRKY89R	CTG CTC CCT GTG AAA GAT GT	20	qPCR	*GSMUA_Achr7G19340*
MaWRKY145F	GGA GAC GCA AAG AAC GAG AT	20	qPCR	*GSMUA_AchrUn_randomG21800*
MaWRKY145R	TCG AGT TTG GAA GGC ATA GC	23	qPCR	GSMUA_Achr8G01790_001

3.3 Sample Collection

Soil samples from the rhizosphere and plant roots were acquired using a sterile scoop and subsequently deposited into sterile plastic containers from three locations within the mangrove forest on Carey Island, Selangor. Each container was properly labeled with information including the sampling location and date. These sites are situated on Peninsular Malaysia western coast (2°86′05″N, 101°36′58″E). To conduct the visit to the area and collect samples, formal permission was secured in advance from both the local district office in Selangor, Malaysia and the Forest Department of Peninsular Malaysia. The identification of mangrove tree species at each sampling site was conducted by observing the morphology of propagules, leaves, and flowers with reference to online resources provided by Wetlands International (https://indonesia.wetlands.org). A Botanist expert from University of Malaya, Institute of Biological Sciences further validated the identification (Fig. 3.1). In order to evaluate the salinity levels, soil samples were extracted from the rhizosphere of mangrove plants at three designated sites. The analysis encompassed measurements of electrical conductivity (EC) and soil pH, carried out using an electrical conductivity meter and a pH meter, respectively. The mangrove tree species identified at the sampling sites were *Rhizophora apiculata* (site 1), *Avicennia alba* (site 2), and *Sonneratia alba* (site 3). All collected samples were carefully transferred into sterile plastic containers, labeled, and transported to the Molecular Biology Facility at the Centre for Research in Biotechnology for Agriculture (CEBAR) at University of Malaya. Upon arrival, the samples were stored at 4°C until further experiment.

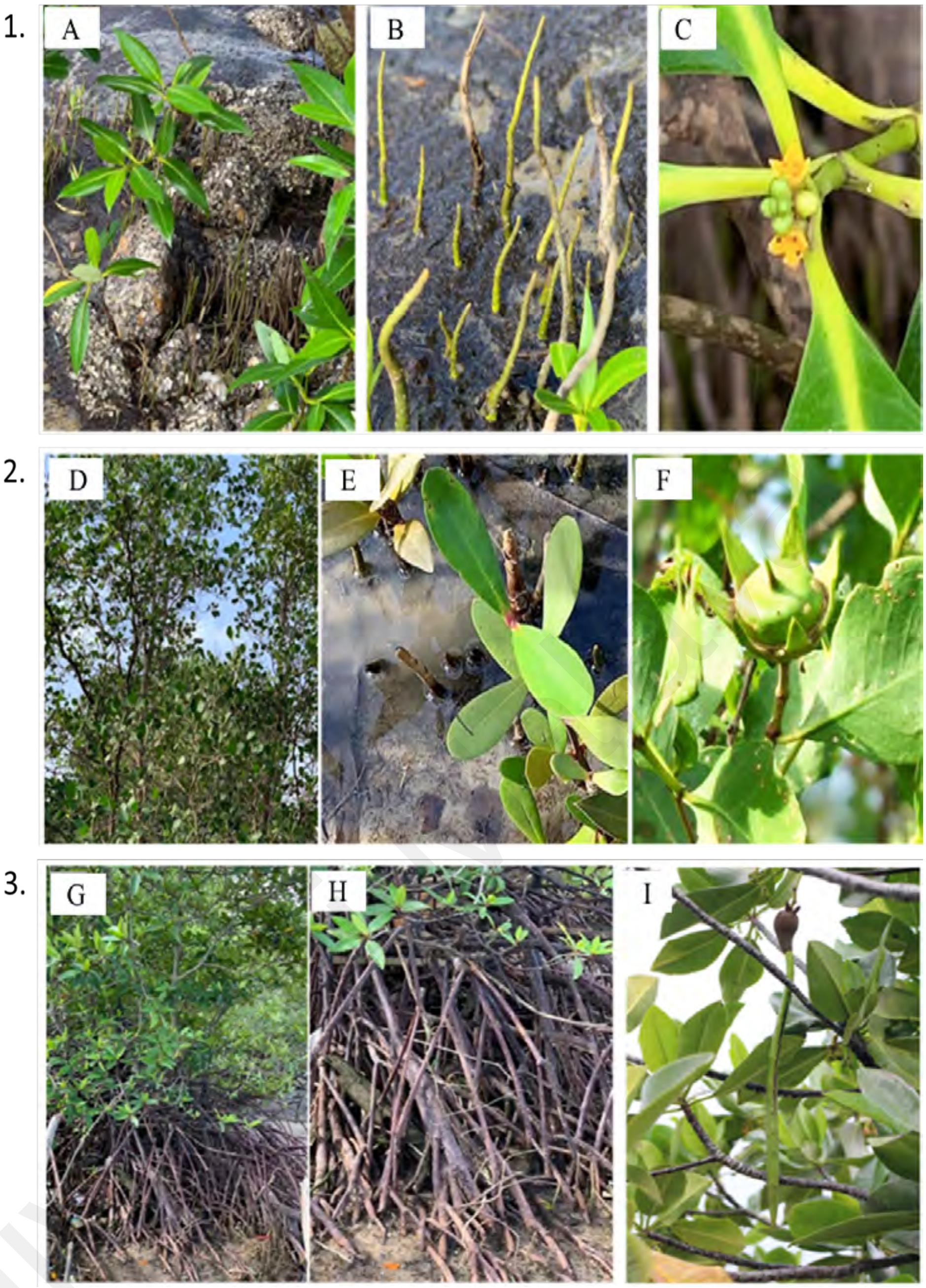


Figure 3. 1: Mangrove plants at Carey Island, Selangor: 1. *Avicennia alba* (A) leaves, (B) roots, (C) flowers; 2. *Sonneratia alba* (D) tree, (E) plant with root, (F) fruit; 3. *Rhizophora apiculata* (G) plant, (H) roots (I) propagules

3.4 Isolation of bacteria

3.4.1 Rhizobacteria Isolation

All culture media used in this study and media composition are listed in Appendix A. Rhizobacteria were isolated from the rhizosphere soil samples following a modified protocol based on Komaresofla et al. (2019). One gram of rhizosphere soil was suspended in 100 ml phosphate-buffered saline (PBS). The soil suspension was then serially diluted in PBS, with 10-fold dilutions up to 10^{-9}. Each dilution (100 µl) was plated on Luria Bertani (LB) agar medium for 24 h at 28°C. Morphologically distinct colonies were isolated and obtained as pure cultures using the traditional streak plate technique. Colony morphology, including pigmentation, shape, size, appearance, elevation, margin, texture, and opacity, was recorded for each isolate. Gram staining and microscopic observation at 1000x magnification were performed to assess the cellular morphology. A 30% (v/v) glycerol stock was prepared for long-term storage of all rhizobacterial isolates at -80°C.

3.4.2 Endophytic Bacteria Isolation

The isolation of endophytic bacteria from mangrove plant roots were conducted following the methodology described by Sun et al. (2008). The roots were washed to remove adhered soil and subjected to surface sterilization using a series of treatments: 3 min with 70% ethanol, 20 sec with 35% hydrogen peroxide, and another 30 sec with 70% ethanol. The roots were then washed five times with sterile water for 2 min each time. To check for the presence of any colonies, the final washed solution (200 µl) was spread on LBA plates and incubated for 120 h at 28°C. Surface-sterilized roots (1 g) were macerated in 10 ml PBS using a sterilized mortar and pestle, and 10-fold serial dilutions were prepared up to 10^{-9}. Aliquots of 100 µl from each dilution were plated on LBA agar. Morphologically distinct colonies were isolated on LBA plates, and pure cultures were obtained for each isolate. Gram staining and microscopic observation were performed to examine the cellular morphology. Colony morphology, including shape, appearance, size, elevation, pigmentation, texture, opacity, and margin, was recorded for each isolate. To maintain pure culture, a 30% (v/v) glycerol stocks was prepared and kept at -80°C.

3.5 Salt Tolerance Assay

To evaluate the salt tolerance of the endophytic and rhizobacterial isolates, a salt tolerance test was conducted utilizing aquarium sea salt (calcium, magnesium and bicarbonates) (Red Sea, China) as a surrogate to replicate the salinity levels found in seawater. The experimental procedure followed a modified protocol based on Komaresofla et al. (2019). Each endophytic and rhizobacterial isolate was subjected to individual assessment of salt tolerance. Agar medium (LB) was prepared and treated with aquarium sea salt in quantities ranging from 50, 100, 150, 300, 350 mM to 400 mM sea salt. The plates were incubated for a period of 24 h at 28 ± 2°C to allow for bacterial growth and development. The growth of the bacterial isolates on the agar plates was examined to determine their tolerance to varying salinity levels. The ability of the isolates to grow and form colonies on the agar plates indicated their tolerance to the respective salt concentrations.

3.6 In Vitro Assessment of Bacteria for Plant Growth-Promoting Traits

The bacterial isolates that demonstrated growth on LB agar supplemented with 400 mM aquarium sea salt concentration were selected for further evaluation of their plant growth-promoting traits. The following traits were assessed: siderophore, ACC deaminase activity, nitrogen fixation, phosphorus solubilization, and IAA production.

3.6.1 Nitrogen Fixation Activity

To assess the nitrogen fixation activity of the bacterial isolates, the method described by Jensen (1954) was followed. Pure cultures of the bacterial isolates were spot-inoculated onto nitrogen-free Jensen's medium. The plates were then incubated at 28°C for 72 h. Bacterial isolates that showed visible growth on the nitrogen-free medium were considered capable of fixing atmospheric nitrogen.

3.6.2 Phosphate Solubilizing Activity

On Pikovskaya's (PVK) agar medium, pure cultures of the bacterial isolates were spot-inoculated and incubated for 120 h at $28 \pm 2°C$ (Paul & Sinha, 2017). The formation of a clear halo zone surrounding the bacterial colonies was measured to calculate the phosphate solubilization index (PSI). The PSI is calculated as the ratio of the total diameter (colony + halo zone) to the colony diameter that was determined as:

$$\text{Phosphate solubilization index (PSI)} = \frac{\text{Colony diameter+halozone diameter}}{\text{Colony diameter}} \qquad (3.1)$$

Subsequently, colorimetric technique was used to quantitatively analyze the solubilization of phosphate. Pure cultures of the bacterial isolates were inoculated into PVK broth with 1 ml 0.5 g $Ca_3(PO_4)_2$ as the insoluble phosphate source. The cultures were incubated for 4 days at 28°C under 150 rpm on a shaker. After incubation, the bacterial cultures were centrifuged for 30 min at 15,000 rpm and 4 °C. Filter-sterilized supernatant (1 ml) was combined with 45 ml of distilled water, 10 mL of chloromolibdic acid, and 1 ml of the supernatant. A spectrophotometer (Implen, Germany) was used to measure the solution's absorbance at 587 nm after it had been incubated in the dark for 30 min. Standard solution was prepared by dissolving 0.2195 g of KH_2PO_4 in 1L water to get 50 µg mL^{-1} solution. Further dilutions (2, 4, 8, 12, 20, 30 µg mL^{-1}) were made to get standard curve of KH_2PO_4.The standard KH_2PO_4 curve was used to calculate the amount of soluble phosphate.

Strains that exhibited nitrogen fixing and phosphate solubilization activities were selected for analysis of the remaining growth-promoting traits.

3.6.3 IAA Production

The assessment of IAA production by the bacterial isolates was conducted following the methodology outlined by Gordon and Weber (1951). The bacterial isolates were first incubated in 10 mL of LB broth for 24 h at 28 °C at 120 rpm. Once transferred, 1 mL of the culture was incubated for 48 hours at 28 °C and 120 rpm in Tryptic soy broth (TSB) medium with 1 mL of tryptophan (5 mM) added as a supplement. After specific time intervals (12, 24, and 48 hours), the amount of IAA produced was measured. The bacterial cultures were centrifuged at 10,000 rpm at 4 °C for 10 min, and 1 mL of the supernatant was combined with 2 mL of Salkowski's reagent to measure the generation of IAA. The mixture was vortexed and incubated in the dark for 30 min at 28 ± 2°C. Pink color emergence indicated IAA production. A spectrophotometer (Implen, Germany) was used to measure the solution's absorbance at 530 nm. IAA was quantified as $\mu g\ mL^{-1}$ and its concentration of IAA was determined from a standard curve (Yousef, 2018).

3.6.4 Siderophore Production

Following the protocol outlined by Schwyn and Neilands (1987), the production of siderophore from selected bacterial isolates was determined. Pure cultures for the bacterial isolates were spot-inoculated on Chrome-azurol S (CAS) agar plates, and uninoculated plates served as negative controls. After 5 days of 28°C incubation, an orange halo zone formed surrounding the bacterial colonies on the plates.

The bacterial isolates were cultured at 28°C for 48 h and 120 rpm for the quantitative evaluation of siderophore production. Following a 10-min centrifugation of the bacterial cultures at 10,000 rpm, 1 mL of the supernatant was mixed with 1 mL of CAS reagent. A spectrophotometer (Implen, Germany) was used to measure the mixture's absorbance at 630 nm after it had been incubated for 20 min. The percentage of siderophore units (PSU) produced by the bacterial isolates was calculated using the formula (Payne, 1993):

$$\%\ \text{of siderophore units} = \frac{\text{Ar}-\text{As}}{\text{Ar}} \text{ x } 100 \tag{3.2}$$

Ar represent the absorbance of the reference (CAS solution and uninoculated broth), and As for the sample (CAS solution and cell-free supernatant).

3.6.5 ACC Deaminase Activity

To evaluate the ACC deaminase activity of the bacterial isolates, a standard protocol based on Dworkin and Foster (1958) was followed. Pure cultures of the bacterial isolates were spot inoculated on Dworkin and Foster (DF) agar medium. As a viability control, plates with DF medium supplemented with an alternate nitrogen source, such as $(NH_4)_2SO_4$ were used. The plates were then incubated at 28°C for 72 h. Bacterial isolates that showed visible growth on plates supplemented with ACC as the sole nitrogen source were considered capable of using ACC for growth. The quantitative estimation of ACC deaminase activity was determined by measuring the production of α-ketobutyrate and ammonia resulting from the enzymatic cleavage of ACC. This was accomplished using the standard protocol described by Penrose and Glick (2003). The following steps were followed: The bacterial isolates were incubated in 5 mL of TSB medium at 28°C for 24 h at 120 rpm. The bacterial cultures were then centrifuged for 5 min at 8000 rpm to separate the cell pellet. The cell pellet was suspended in 2 mL of DF medium with 3 mM

ACC after being washed twice in 1 mL of 0.1 M Tris-HCl (pH 7.5). The suspension was cultured in an incubated at 28°C for 48 h and 120 rpm. Following the incubation period, the bacteria were separated by centrifugation for 10 min at 3000 rpm and were then resuspended in 500 µL of 0.1 M Tris-HCl (pH 8.5). To facilitate cell lysis, toluene 5% (v/v) was added to the suspension, followed by vertexing and homogenization for 30 sec. A microcentrifuge tube containing 50 µL of lyzed cell suspension and 5 µL of 0.3 mM ACC was incubated at 28°C for 30 min. A negative control without ACC and a blank containing 50 µL of 0.1 M Tris-HCl (pH 8.5) with 5 µL of 0.3 M ACC were also prepared. After incubation, the samples were mixed with 500 µL of 0.56 N HCl, and the cell debris was separated by centrifugation for 5 min at 12,000 rpm. A 500 µL aliquot of the supernatant was transferred into a glass test tube and mixed with 400 µL of 0.56 N HCl and 150 µL of DNF solution. The mixture was incubated at 28°C for 30 min, and then 1 mL of 2N NaOH was added. The absorbance of the mixture was measured at 540 nm. The quantity of α-ketobutyrate was estimated by comparing it with a standard curve of α-ketobutyrate.

3.7 16S rRNA Gene Amplification and Analysis

To determine the identity of bacterial isolates that exhibited nitrogen fixation and phosphate solubilization activities, the 16S rRNA gene analysis was performed. The CTAB method described by Russell and Sambrook (2001) was used to extract genomic DNA from the bacterial isolates. The extracted genomic DNA served as the template for PCR amplification of the 16S rRNA gene. Universal primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1510R (5'-GGCTACCTTGTTACGA-3') were used for the PCR amplification. PCR amplification was performed in 25 μL reaction containing 1X buffer, 1.5 mM $MgCl_2$, 0.12 mM of dNTPs mixture, 0.3 μM of each primer, 1 U Taq polymerase, and 10 ng of template. The thermal cycling program included an initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 0.5 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min, concluding with a final extension step at 72°C for 10 min. The PCR reaction was carried out in a Thermocycler (Esco, Singapore). Subsequently, the PCR products were subjected to gel electrophoresis on a 1% agarose gel for separation. To purify the PCR products, the GFXtm PCR DNA and Gel Band Purification Kit from Amersham Pharmacia Biotech, Inc. (NJ, USA) was employed. The purified PCR products were then sent to Apical Scientific Sdn Bhd in Selangor, Malaysia, for commercial Sanger sequencing. The obtained raw sequence data from Sanger sequencing were subjected to analysis and assembled to form 16S rRNA gene sequences using the Molecular Evolutionary Genetic Analysis (MEGA) software version 7.0 (S. Kumar et al., 2016). Ambiguous nucleotides were trimmed, and the forward and reverse strands were assembled at the overlapping regions. The assembled 16S rRNA gene sequences were compared against the NCBI and EzBioCloud databases using BLAST (Basic Local Alignment Search Tool) to determine closely related neighbor taxa for each bacterial isolate. The 16S rRNA gene sequences of these neighbor taxa were obtained and aligned using ClustalW (Larkin et al., 2007). The aligned 16S rRNA gene sequences were used to construct phylogenetic trees using the neighbor-joining (NJ) method in the MEGA7 program (Saitou & Nei, 1987). The robustness of the phylogenetic inference was estimated by performing 1000 bootstrap replicates (Felsenstein, 1985). The 16S rRNA gene sequences of the selected bacterial strains were submitted to GenBank (http://www.ncbi.nlm.nih.gov), and the accession numbers were listed in Table 4.2.

3.8 In Vivo Root Colonization of Banana Plantlets By PGPB

3.8.1 Selection of Rhizobacteria and Endophytic Bacteria for Root Colonization

For the banana root colonization study, bacterial strains RB1, RB3, and RB4, as well as endophytic strains EB1, EB2, and EB3, were selected. The selection criteria for these strains were their demonstrated high salt tolerance, reaching up to 400 mM, as well as their possession of plant growth-promoting traits. After isolating bacterial colonies, purification was performed using streak plates to obtain individual isolates (Komaresofla et al. (2019). These isolates were then subjected to various tests, including morphology assessment, gram staining, salt tolerance, and 16S rRNA gene sequencing. To distinguish between different strains, isolates showing identical results across all the tests were considered to belong to the same strain. On the other hand, isolates displaying differences in any of these tests were regarded as different strains. Thus, RB1, RB3, and RB4, as well as EB1, EB2, and EB3, were identified as distinct bacterial strains. It is important to note that the taxonomy of bacteria in the isolation process progresses from isolates to strains, species, genus, family, order, class, phylum, and kingdom. Therefore, in this study, the selected strains RB1, RB3, RB4, EB1, EB2, and EB3 represent different bacterial populations with unique traits and characteristics.

3.8.2 Inoculum Preparation

To prepare the inoculum, six selected PGPB strains were utilized, including three rhizobacteria (RB1, RB3, and RB4) and three endophytic bacteria (EB1, EB2, and EB3). Overnight cultures of these strains were prepared in LB broth at 28°C at 125 rpm. A spectrophotometer (Implen, Germany) was used to measure the optical density (OD_{600}) at the growth phase of the bacterial cultures. Centrifugation for 5 min at 7,000 rpm was used to separate the bacterial cells. The resulting cell pellets were washed twice with PBS solution. The pellets were resuspended in a liquid medium known as ½ Murashige and

Skoog (MS) at (pH 5.8) to obtain a concentration of 1 x 10^6 colony-forming units (CFU) per mL, as described by Mia et al. (2010).

3.9 Plant Treatments to Study Colonization of Banana Roots

Clonally propagated banana plantlets (*Musa acuminata* cv. Berangan) were obtained from the Plant Biotechnology Incubator Unit (PBIU) at the University of Malaya. Plantlets of similar heights, approximately ranging between 10 and 10.5 cm, were carefully chosen for the root colonization and salt stress experiments. To allow for acclimatization and ensure uniform growth conditions, the plantlets were placed in clear plastic cups with a diameter of 10 cm. These cups contained a half-strength MS (Murashige and Skoog) broth solution at a pH of 5.8. (Fig. 3.2). The acclimation was conducted in a growth room at 25°C, 70% relative humidity (RH), and a 16-hour light/8-hour dark photoperiod provided by fluorescent white light (40 W). A photoperiod of less than 12h is associated with slowing the growth of banana (Turner et al., 2007) the longer photoperiod used here was due to standard settings for other plant experiments in the room. Following the acclimation period, the hydroponic banana plantlets were treated by adding 1 mL of freshly prepared bacterial suspension solution. The inoculated plants were compared against control plants grown in non-inoculated medium. All treatments were conducted in triplicate.

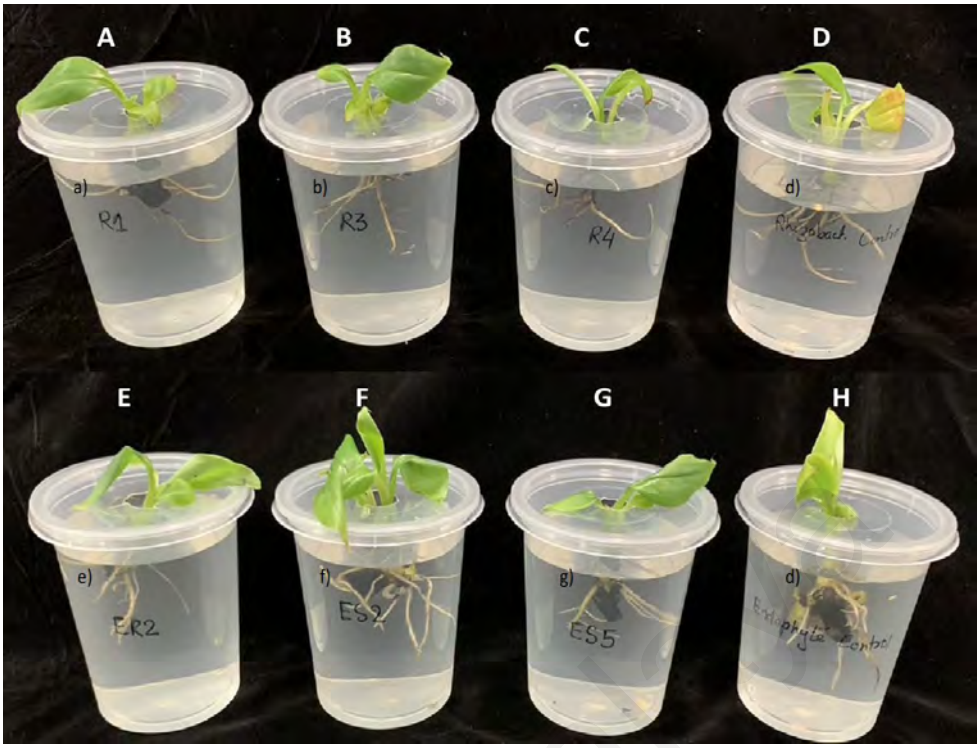


Figure 3. 2: Experimental setup for root colonization study of banana plantlets with (a) RB1, (b) RB3, (c) RB4, (d) Control, (e) EB1, (f) EB2, (g) EB3, (h) Control

3.10 Microbial Root Colonization Analysis by Field Emission Scanning Electron Microscopy (FESEM)

At 72 hours post-bacterial inoculation, samples were obtained from distinct sections of the banana roots, including the root cap, elongation zone, and root hairs. These root sections were carefully rinsed with distilled water to remove any residual medium. Using a sterile razor blade, individual root sections were precisely excised and affixed onto a brass stub using a 2.5% glutaraldehyde adhesive. The samples were then dehydrated using a graduated ethanol series. Subsequently, they were washed with acetone following the protocol described by Gamez et al. (2019). To facilitate imaging, a thin layer of gold coating was applied to the root specimens using a sputtering Hummer II (Tectonics, Springfield, VA). The field emission scanning electron microscopy

(FESEM) analysis was performed at the Infra Analytical Laboratory located at the University of Malaya.

3.11 Hydroponic Salt Stress Treatments of Banana Plantlets

To simulate seawater conditions and investigate the effects of the six selected PGPB isolates on banana plantlets under salt stress, aquarium salt (Red Sea, China) was utilized. These study involved three rhizobacteria (RB1, RB3 and RB4) and three endophytic bacteria (EB1, EB2 and EB3). The methodology, with slight modifications based on Patel et al. (2019), involved diluting the aquarium sea salt to create a 90 mM salt solution equivalent to NaCl concentration. The banana plantlets were divided into four treatment groups: (i) non-inoculated banana plantlets as non-stressed controls (C), (ii) non-inoculated banana plantlets subjected to salt stress (CS), (iii) PGPB-inoculated plantlets without salt stress (PGPB), and (iv) PGPB-inoculated plantlets under salt stress (PGPBS). The experimental setup followed a completely randomized design with ten replicates, consisting of ten plants per treatment group. The plants were harvested for morpho-physiological studies after 7 days of treatment, and selected plantlet materials were quickly frozen in liquid nitrogen and kept at -80°C for future biochemical investigations.

3.12 Consortium Selection

3.12.1 Microorganisms Used and Culture Condition

The Gram-positive strain *Bacillus* sp. RB3 (MZ881955) and the Gram-negative strain *Pseudomonas* sp. EB3 (MZ881952) were isolated from the root of the mangrove plant species *Sonneratia alba*. To prepare the bacterial consortium, each bacterial strain was individually grown in 150 ml of LB broth for 24 h at 28 °C and 125 rpm. After incubation, the bacterial cultures were centrifuged at 6000 rpm for 6 min to collect the cell pellets. The cell pellets were then washed with a saline solution and suspended in distilled water containing 2% glycerol. The concentration of the bacterial suspension was adjusted to 10^8 CFU/ml. The individual cultures were combined in a 1:1 ml ratio to create the consortium, which was then stored at 4 °C until further use. These strains were selected based on their ability to fix nitrogen, solubilize phosphate, produce siderophores, possess ACC-deaminase activity, and alleviate salt stress in a preliminary hydroponic study conducted on *Musa acuminata* cv. Berangan. For routine maintenance, both *Bacillus* sp. RB3 and *Pseudomonas* sp. EB3 were cultured in LB medium at 28°C. Long-term preservation was achieved by storing them in 30% (v/v) glycerol stocks under -80°C.

For the *Fusarium oxysporum* f. sp. *cubense* tropical race 4 isolate (Foc-TR4) strain C1HIR_9889, a spore suspension was prepared prior to plant infection. A volume of 500 ml of Potato Dextrose Broth (PDB) was used to incubate the Foc-TR4 culture for 5 days at 28 °C and 125 rpm. After the incubation period, the spore suspension was separated from the culture using sterile cheesecloth. The spores were then resuspended in PDB to achieve a concentration of 10^6 conidia per mL, following the procedure outlined by Li et al. (2017). This strain was routinely maintained in potato dextrose medium at 28°C and preserved long term 30% (w/v) glycerol stocks under -80°C.

3.13 In Vitro Assessment of Antagonistic Activity of Bacterial Strains

To assess the antagonistic activity of *Bacillus* sp. RB3 and *Pseudomonas* sp. EB3 against Foc-TR4, a dual culture method was used on potato dextrose agar (PDA) plates. The procedure followed the method described by Skidmore and Dickinson (1976). Control plates without bacterial inoculation were also prepared for comparison. The PDA plates were inoculated with Foc-TR4 and the bacterial strains, ensuring a suitable distance between the fungal mycelium and bacterial colonies. The plates were then incubated for 7 days under optimal conditions. During this period, the growth of the fungal mycelium and the bacterial colonies were monitored. After incubation, the diameter of the fungal mycelium (FG) growing on the antagonistic plates and the diameter of the fungal mycelium (CG) growing on the control plates were measured. Using these measurements, the percentage inhibition of fungal colony growth was calculated using 68ollowingg formula:

$$\% \text{ inhibition} = \left[1 - \frac{\text{FG}}{\text{CG}}\right] \times 100 \qquad (3.3)$$

Where FG; diameter (mm) of fungal mycelium growing on antagonistic plates and CG; diameter (mm) of fungal mycelium growing on control plates.

3.14 Greenhouse Experiments

3.14.1 Plant Materials

One-month-old clonally propagated banana (*Musa acuminata* cv. Berangan) plantlets were procured from Horus Green Sdn Bhd and maintained at the Plant Biotech Facility, University of Malaya, Malaysia. The plantlets were acclimatized in autoclaved soil for one week, and plantlets of similar height (10-10.5 cm) were selected for the subsequent experiments.

3.14.1.1 Experimental Design for Salt Stress Treatments

To simulate salt stress, aquarium sea salt was used to prepare a solution with a concentration of 100 mM (equivalent to NaCl). The experimental setup included four treatment conditions, each with ten replicates: (i) non-inoculated and non-salt-stressed control. (ii) Non-inoculated and salt-stressed with 100 mM solution. (iii) Inoculated with the PGPB consortium (EB3+RB3) and non-salt stressed. (iv) Inoculated with the PGPB consortium (EB3+RB3) and salt-stressed with 100 mM solution. Inoculation with the bacterial consortium was done by adding 50 ml of the PGPB consortium (at a concentration of 10^8 CFU/ml) near the plant stem in each pot. One week after inoculation, salt treatment was performed on alternate days for two weeks by watering the pots with 100 ml of the 100 mM sea salt solution. Non-salt-treated samples were watered with an equivalent amount of distilled water. Subsequently, the plants were allowed to recover for two weeks by watering them with 50 ml of distilled water every alternate day. After the two-week recovery period, the plants were harvested. The pots were arranged in a completely randomized design, and the harvested samples were either used immediately or rapidly frozen in liquid nitrogen and stored at -80 °C.

3.14.1.2 Experimental Design for Foc-TR4 Treatments

To investigate the ability of the PGPB consortium (EB3+RB3) to promote resistance against Foc-TR4 stress, four treatment conditions were implemented, each with ten replicates: (i) Non-inoculated (no PGPB and no Foc-TR4). (ii) Foc-TR4 inoculated only. (iii) EB3+RB3 inoculated only. (iv) EB3+RB3 with Foc-TR4 inoculated. Inoculation of the PGPB consortium (treatments iii and iv) involved adding 50 ml of the consortium (at a concentration of 10^8 CFU/ml) to each pot. For treatments i and ii, plants of the same size and age were grown under the same conditions for one week. After one week, plants with uniform height were selected, and their roots were cut to a standardized length of 10 cm. Subsequently, the roots were soaked either in 50 ml of (10^6 conidia per mL) Foc-TR4 suspension for 30 min (Foc-TR4 treatment groups ii and iv) or in 50 ml distilled water for 30 min (non-Foc-TR4 treatment groups i and iii). Plant harvest was performed at 4 and 6 weeks after the pathogen challenge. The pots were arranged in a completely randomized design, and the harvested samples were either used immediately or rapidly frozen in liquid nitrogen and stored at -80 °C.

3.14.2 Disease Severity Assessment

The disease severity of the plants was assessed at 4 and 6 weeks after inoculation using a method described by W. Li et al. (2015). The banana roots and stalks were dissected at cross-sections to examine both internal and external symptoms of each plant, which were used to assign scores for determining the disease index values. The scoring criteria for external symptoms were as follows: 0: Healthy plant showing no wilting or yellowing. 1: Plant with yellowing of the lower leaves. 2: Plant with yellowing of the upper leaves. 3: Plant with yellowing of most of the leaves. 4: Plant with severe wilting or dead plant. The internal symptoms were scored based on the extent of necrosis or lesion on the rhizome using the following scale: 0: No symptoms. 1: 1-10% lesion area in the rhizome. 2: 11-30% lesion area in the rhizome. 3: 31-50% lesion area in the rhizome. 4: More than 50% lesion area in the rhizome. The disease index values were calculated using the following formula:

$$\text{Disease index (\%)} = \left[\Sigma \frac{(\text{grade} \times \text{no of plants in the grade})}{(4 \times \text{total no of plants assessed})}\right] \times 100 \qquad (3.4)$$

3.15 Analysis of Plants

A schematic representation of the experimental flow is show in the results (Fig. 4.9 and Fig. 4.20)

3.15.1 Assessment of plant growth parameters

The plant height, number of leaves, root length and weight were recorded for each plant after harvesting.

3.15.2 Determination of Photosynthetic Pigments

To determine the photosynthetic pigment content in banana leaves, the following method, proposed by Inskeep and Bloom (1985) was followed. First, 200 mg of banana leaves were homogenized in 2 mL of pre-chilled 100% N, N-dimethylformamide (DMF) in the dark for 2 h.

The solution was then centrifuged for 10 min at 13,000 rpm. The supernatant was collected and thrice diluted. A spectrophotometer (Implen, Germany) was used to record absorbance of the diluted supernatant at three different wavelengths: 663 nm, 645 nm, and 480 nm. The Chl a, Chl b and carotenoid contents were estimated using the following formulae:

$$\text{Chl a} = 12.25\text{A}_{663.2} - 2.78\text{A}_{646.8}\ (\text{mg mL}^{-1}\ \text{solution}) \qquad (3.5)$$

$$\text{Chl b} = 21.50\text{A}_{646.8} - 5.10\text{A}_{663.2}\ (\text{mg mL}^{-1}\ \text{solution}) \qquad (3.6)$$

$$\text{Total carotenoids} = 1000\text{A}_{470} - 1.82\text{Ca} - 85.02\ \text{Cb}\ (\text{mg mL}^{-1}\ \text{solution}) \qquad (3.7)$$

$$\text{Total carotenoids} = \frac{1000\text{A}_{470} - 1.82\text{Ca} - 85.02\text{Cb}\ (\text{mg mL}^{-1}\text{solution})}{198} \qquad (3.8)$$

A represents absorbance measured in 1.00 cm cuvettes and Chl = mg L^{-1}.

3.15.2 Cell Death Determination

The protocol outlined by Qin et al. (2017) was employed to assess cell death in the banana leaves using Evan's blue stain. Banana leaves were stained with a 0.2% (w/v) Evan's blue stain for 16 h at 25°C. Following staining, the leaves were properly rinsed with distilled water to eliminate any remaining pigment after staining. Chlorophyll interference was eliminated by subjecting the leaves to a bleaching solution composed of methanol, acetic acid, and glycerol in a ratio of 3:2:1. After blotting the leaves dry, digital scanning was performed with a DocuCentre-IV 2060 scanner (Fuji Xerox, Japan) to capture the stained regions.

3.15.3 Determination of Lipid Peroxidation

Lipid peroxidation levels were assessed following the method outlined by Draper and Hadley (1990). Banana leaves (200 mg) were homogenized in 2 mL of a 0.1% (w/v) trichloroacetic acid (TCA) solution. The homogenate was then centrifuged at 4°C and 13,000 rpm for 15 min. Then, 2 mL of a 0.5% (w/v) thiobarbituric acid (TBA) solution made in 20% (w/v) TCA was added to 500 µL of the supernatant. After 30 minutes of incubation at 95°C in a water bath, the mixture was immediately cooled on ice to terminate the reaction. A spectrophotometer (Implen, Germany) was used to measure the absorbance of the resultant supernatant at 450 nm, 532 nm, and 600 nm after centrifugation for 5 min at 10,000 rpm. The concentration of malondialdehyde (MDA), an indicator of lipid peroxidation, was determined using a molar extinction coefficient of 155 mM^{-1} cm^{-1}.

3.15.4 Determination of Proline Content

The protocol outlined by Bates et al. (1973) was followed to determine the proline content of banana leaves. A total of 200 mg of banana leaves were homogenized in 2 mL of 3% sulfosalicylic acid. A Sulfosalicylic acid was used to bring the homogenate's volume to 10 mL after centrifugation for 15 min at 4 °C and 13,000 rpm. For the reaction, 2 mL of the homogenate was combined with 2 mL of glacial acetic acid and 2 mL of ninhydrin reagent, and the mixture was incubated in a water bath at 100 °C for 30 min. After cooling to room temperature, 6 mL of toluene was added to the reaction mixture and vortexed. A spectrophotometer (Implen, Germany) was used to measure the solution's absorbance at 570 nm.

3.15.5 Determination of Ion Content

The concentration of Potassium (K^+), Sodium (Na^+), Calcium (Ca^{2+}), Chloride (Cl^-), and Magnesium (Mg^+) in banana leaves were analyzed using the methodology described by Zou et al. (2015). Banana leaves weighing 200 mg were rinsed with distilled water and dried for 48 h at 65°C. The dried tissue was then crushed into a powder and added to test tubes containing 1 ml of HNO_3. The mixture was incubated in a water bath at 95°C for 4 h. After cooling to room temperature, 1 ml of H_2O_2 was added to each tube, and the mixture was boiled for 10 min at 100°C. The final volume of the sample was adjusted to 10 ml with distilled water. Inductively coupled plasma mass spectrometry (ICP-MS 7500 Single Turbo System) used to examine the ion content of the sample.

3.15.6 Determination of Electrolyte Leakage

The methodology outlined by Lata et al. (2011) was followed to determine the electrolyte leakage. Banana leaves weighing 200 mg were rinsed with distilled water to remove any surface-adhered electrolytes. The initial electrical conductivity of the leaves (L1) was measured after incubating the leaf samples in closed tubes containing 10 ml of distilled water at 25 °C for 2 h. The samples were then subjected to a 20-min incubation in a water bath at 95 °C to release all the electrolytes. After cooling the samples back to 25 °C, the final electrical conductivity (L2) was measured. The electrolyte leakage (EL) was calculated using the formula:

$$\text{Electrolyte leakage (\%)} = \left(\frac{\text{L1}}{\text{L2}}\right) \times 100 \qquad (3.9)$$

L1 represent the leaves initial conductivity prior to incubation and L2 denotes thier final conductivity following incubation.

3.15.7 Reactive Oxygen Species and Scavenging Enzyme Activity Analysis

For enzyme extraction, the procedure was carried out according to methods described by (Fung et al., 2019) with modifications. 200 mg of banana leaves were homogenized in a mixture containing 1% polyvinylpyrrolidone (PVP), 100 mM ethylenediaminetetraacetic acid (EDTA), and 100 mM phosphate buffer (pH 7). The homogenate was then centrifuged at 12,000 rpm for 15 min at 4°C to separate the cellular debris and obtain the supernatant containing the extracted enzymes. The collected supernatant was used for the determination of enzyme activities involved in scavenging ROS or other relevant enzymatic assays.

3.15.7.1 Hydrogen peroxide (H_2O_2)

To determine the level of H_2O_2, the methodology outlined by Sagisaka (1976) was employed. Banana leaves (200 mg) were homogenized in 5 mL of 5% TCA. After that, the homogenate was centrifuged for 10 min at 13,000 rpm. 20 mL of potassium thiocyanate, 40 mL of ferrous ammonium sulfate, and 40 mL of 50% TCA were added to the resultant supernatant. A spectrophotometer (Implen, Germany) was used to measure the reaction mixture's absorbance at 480 nm.

3.15.7.2 Catalase (CAT)

The Aebi (1984) procedure was used to determine the level of CAT activity. A 3 mL reaction mixture containing 3.2 mM H_2O_2 in 50 mM phosphate buffer (pH 7.0) was added to 100 μL of the enzyme extract. The decomposition of H_2O_2 was monitored by measuring the decrease in absorbance at 240 nm over a period of one min. Using an extinction coefficient of 36 M^{-1} cm^{-1}, the CAT activity is expressed as millimoles of H_2O_2 decomposed per minute per milligram of protein.

3.15.7.3 Superoxide Dismutase (SOD)

The procedure outlined by Gupta et al. (1993) was employed to assess SOD activity. A 3 mL reaction mixture comprising 63 μM NBT, 50 mM phosphate buffer (pH 7.8), 13 μM methionine, and 1.3 μM riboflavin was added to 100 μL of the enzyme extract in total. The increase in absorbance at 560 nm, brought on by the photoreduction of NBT into formazan blue, was measured following a10 min exposure of the reaction mixture to 30 V of white, fluorescent light. The SOD activity was calculated as the amount of enzyme necessary to inhibit NBT photoreduction by 50%, which is equal to one unit of SOD.

3.15.7.4 Ascorbate Peroxidase (APX)

For the determination of ascorbate peroxidase (APX) activity, the methodology outlined by Nakano and Asada (1981) was employed. A total of 100 μL of the enzyme extract was added to a 3 mL reaction mixture consisting of 1 mM H_2O_2, 50 mM phosphate buffer (pH 7.5), and 0.8 mM ascorbate. The oxidation of ascorbate was monitored by measuring the decrease in absorbance at 290 nm over a period of one min. Using an extinction coefficient of 2.8 M^{-1} cm^{-1}, the APX activity was calculated as micromoles of ascorbate oxidized per minute per milligram of protein.

3.15.7.5 Glutathione Reductase (GR)

To determine the activity of GR, the method described by Smith et al. (1989)was followed. A total of 100 μL of the enzyme extract was added to a 1 mL reaction mixture containing 1 mM oxidized glutathione and 2 mM NADPH. Over a one-minute period, the decrease in absorbance at 340 nm was measured. GR activity was calculated based on the rate of NADPH oxidation and expressed as micromoles per minute per milligram of protein.

3.15.7.6 Peroxidase (POX)

For the assessment of POX activity, the procedure outlined by Kar and Mishra (1976) was utilized. A total of 100 μL of the enzyme extract was added to a 3 mL reaction mixture consisting of 100 mM phosphate buffer, 0.50% (w/w) H_2O_2, and 5% pyrogallol solution. The change in absorbance at 420 nm was recorded over a period of one min. Using the extinction coefficient of 2.47 mM^{-1} cm^{-1}, POX activity was calculated based on the oxidation of pyrogallol and expressed as micromoles of purpurogallin produced per minute per milligram of protein.

3.15.8 Defense Related Enzymes Analysis

For the extraction of defense-related enzymes, the procedure described by (Fortunato et al., 2015) was followed. A solution containing 100 mM phosphate buffer at pH 6.8, 2% (w/v) polyvinylpyrrolidone (PVP), 1 mM EDTA, and 1 mM phenylmethylsulphonyl fluoride (PMSF) was used to homogenize 200 mg of leaves. The homogenate was then centrifuged at 4°C for 15 min at 12,000 rpm. The resulting supernatant was collected and used for the determination of the activities of several defense-related enzymes.

3.15.8.1 β-1,3-glucanase (GLU)

The GLU activity was determined following the methodology outlined by Lever (1972). A reaction mixture comprising 50 mM sodium acetate buffer (pH 5.0), 1 mg/ml laminarin, was added to a 100 µL enzyme extract. After that, the reaction mixture was incubated for 1 h at 45°C in a Thermomixer (Eppendorf, Germany). Following the incubation, 1.5 mL of dinitro salicylic acid (DNS) was added to 500 L of the reaction mixture, which was then heated for 15 minutes at 100°C. The combination was then submerged in an ice bath until it reached a temperature of 25°C. To quantify the amount of reducing sugars released, A spectrophotometer (Implen, Germany) was used to record the absorbance of the solution at 540 nm. Using glucose (Sigma-Aldrich, Brazil) as a standard, a calibration curve was created and used to calculate the β-1,3-glucanase activity. The initial incubation stage was skipped for the control samples, but otherwise the procedures were the same (Miller, 1959). The GLU activity was determined in Units/Milligram i.e. U/mg.

3.15.8.1 Chitinase (CHI)

Harman et al. (1993) methodology was followed to calculate the CHI activity. A 1900 mL reaction mixture comprising 50 mM sodium acetate buffer (pH 5.0), 0.1 mM p-nitrophenyl-b-D-N-N'-diacetylchitobiose, was added to a 100 µL of enzyme extract. The process was halted by adding 500 µL of 0.2 M sodium carbonate following two hours of incubation at 37°C. For the control samples, 500 µL of 0.2 M sodium carbonate was added in place of the enzyme extract. At 410 nm, the CHI product's wavelength, the CHI activity was determined using an extinction coefficient of 70 mM/cm.

3.15.8.3 Phenol Ammonia Lyase (PAL)

The procedure outlined by Guo et al. (2007) was followed to measure the PAL activity. To 900 µL solution comprising 20 mM L-phenylalanine and 40 mM sodium borate buffer (pH 8.8), a 100 µL of crude enzyme extract was added. For one h, the reaction mixture was incubated at 30°C. The enzyme extract was not used in the control samples, instead borate buffer was applied. The derivatives of trans-cinnamic acid were detected at 290 nm after adding 50 µL of 6 N HCl to terminate the reaction. The extinction coefficient used in the calculation of PAL activity was 10 M/cm.

3.15.8.4 Polyphenol Oxidase (PPO)

The determination of PPO activity was conducted following the method outlined by Kar and Mishra (1976). A total of 100 µL of enzyme extract was added to 900 µL of a reaction mixture comprised of 20 mM pyrogallol and 25 mM potassium phosphate buffer (pH 6.8). The absorbance was measured for 1 minute at 25°C and 420 nm as soon as the reaction started. The extinction coefficient used to compute the PPO activity was 2.47 mM/cm.

3.15.8.5 Lipoxygenase (LOX)

The LOX activity was determined following the protocol outlined by Axerold et al. (1981). A total of 100 µL of enzyme extract was added to 900 µL of a reaction mixture consisting of 10 mM sodium linoleate substrate and 50 mM sodium phosphate buffer (pH 6.8). The decrease in absorbance at 340 nm was recorded over a period of one min following the reaction mixture's 4-min incubation at 25 °C. The extinction coefficient used to compute the LOX activity was 25,000 M/min. The protein concentration, determined using the Bradford (1976) method, was used to express the enzyme activity.

3.15.9 Determination of lignin-thioglycolic acid (LTGA) and total soluble phenolics (TSP)

To ascertain the TSP and LTGA contents in the plant samples subjected to Foc-TR4 treatment, the methodology outlined by Stein et al. (2019) was followed. A1 ml 80% (v/v) methanol solution was used to homogenize 200 mg banana leaf tissue. For 2 h, the crude extract was shaken at 25°C and 300 rpm. The mixture was then centrifuged for 30 min at 14,000 rpm. The methanolic extract was collected for TSP determination, and the pellet was kept at 20°C for LTGA derivative determination.

3.15.9.1 Total Soluble Phenolics (TSP) Determination

The methanolic extract was combined with 750 µL of the 0.2 M Folin-Ciocalteu phenol reagent to a volume of 1 mL. The mixture was incubated for 5 min at 25°C. Then, 0.1 M sodium carbonate (Na_2CO_3) was added and maintained at 25°C for 10 min. The solution was then given 1 ml of deionized water, and it was allowed to sit for 1 hour at 25°C. Based on a calibration curve with catechol as the standard, the TSP content was determined. At 725 nm, the absorbance was measured.

3.15.9.2 Lignin-Thioglycolic Acid (LTGA) Derivatives Determination

The centrifugation-produced pellet was again suspended in 1.5 ml of distilled water. The suspension underwent homogenization and a 15-min centrifugation at 12,000 rpm. The pellet was dried at 65°C for 12 h after the supernatant was removed. The concentration of LTGA derivatives was calculated using the alcohol-insoluble dry residue. The LTGA derivatives supernatant's absorbance was calculated at 280 nm. Using lignin, alkali, and 2-hydroxypropyl ether as standards, a calibration curve was used to measure the concentration.

3.16 Analysis of Cis-regulatory Elements

The Phytozome database (https://phytozome-next.jgi.doe.gov/) provided the promoter sequences for the *MaWRKY* genes. The 2000 bp regions upstream (5') of each gene's AUG start codon were the sequences that were recovered. The resulting promoter sequences were analyzed to determine the identity and abundance of cis-regulatory elements (CREs). PlantCARE, a web-based program accessible at http://bioinformatics.psb.ugent.be/webtools/plantcare/html/, was used to conduct the analysis (Lescot et al., 2002).

3.17 RNA Extraction

Using Khairul-Anuar et al. (2019)'s CTAB method, RNA was isolated from the root tissues of banana plants. Banana roots weighing 200 mg were collected. Plasticware and pipette tips were autoclaved at 121°C and for 45 min. All chemicals used were of molecular biology grade. Distilled water treated with 0.01% DEPC (Diethyl pyrocarbonate) was used for soaking plasticware and preparing reagents. Spray RNase Destroyer (Favorgen, Taiwan) was applied on the workbench to prevent RNase contaminationUsing liquid nitrogen and a mortar and pestle that had already been pre-chilled, the tissues were ground into a fine powder. The powdered material was transferred to a 2 ml centrifuge tube. A total of 1500 µl of freshly prepared, pre-warmed modified CTAB extraction buffer was added to the tube. The mixture was vortexed and incubated at 65 °C for 30 min. After incubation, the mixture was allowed to cool at room temperature for 2 min. The mixture was then centrifuged at 7000 rpm for 5 min. The supernatant was mixed with an equal volume of phenol, chloroform, and isoamyl alcohol (P: C: I) (125:24:1, pH 4.5), vortexed for 3 min, and centrifuged at 17,500 rpm for 15 min at 4 °C. The aqueous fraction was carefully collected without disturbing the interface layer containing waste material. The collected aqueous fraction was transferred to a new microcentrifuge tube, and an equal volume of chloroform and isoamyl alcohol (C: I) was added. The tube was gently mixed, centrifuged at 17,500 rpm for 15 min at 4 °C, and the aqueous fraction was collected. To the collected aqueous fraction, an equal volume of 8 M LiCl was added and mixed. The tubes were incubated overnight at 20 °C. After incubation, the tubes were centrifuged at 17,500 rpm for 15 min at 4 °C. The pellet was washed twice with 70% cold ethanol and air dried. The cleaned RNA pellet was resuspended in 25 µL of RNase-free water.

3.17.1 Quantification and Quality Assessment Of RNA

The purity and concentration of RNA samples were measured using a Nanophotometer Perl® (Implen, Germany) at 230 nm, 260 nm, and 280 nm absorbance. The quality and integrity of the extracted RNA were evaluated using denaturing agarose gel electrophoresis. A 1% denaturing gel was prepared by melting agarose in MOPS gel buffer (200 mM 3-(N-morpholino) propane sulfonic acid, 50 mM sodium acetate, and 10 mM EDTA, pH 7). The cooled agarose gel was mixed with 1 ml of 37% formaldehyde and 1 ml of ethidium bromide (0.5 g/ml). The gel was run at 90 V for approximately 45 min. The bands were visualized and captured using a Gel documentation system (Cambridge Scientific, US). The quality of the extracted RNA was further assessed using an Agilent RNA 6000 Nano LabChip Kit and an Agilent 2100 Bioanalyzer (Agilent Technologies).

3.17.2 cDNA Synthesis and Quantitative Real-Time PCR Analysis

Transcript® One-Step gDNA remover and cDNA synthesis Supermix (TransGen Biotech, China) were used to perform the cDNA synthesis. The QuantStudio™ 12k Flex system from Applied Biosystems, USA was used to perform qRT-PCR in order to ascertain the expression of a specific *MaWRKY* TF in banana roots. PrimeQuest IDT was used to build the primers described in Table 3.1 (https://sg.idtdna.com/PrimerQuest/Home/Index). Each reaction mixture contained 10 µl of SYBR® Green Master Mix (Vanzyme, China), 2 µl of cDNA (5 ng/µl), 7 µl of nuclease-free water, and 0.5 µl of each 10 M primer. The relative expression of each *WRKY* gene was calculated by normalizing it to the expression level of the banana tubulin gene. Non-inoculated, non-treated banana roots (without PGPB, without Foc-TR4 and not treated with salt) were used as calibrator for all comparisons. The qRT-PCR reactions involved an initial denaturation at 95°C for 5 min, followed by 40 cycles of annealing at 60°C for 20 s, and extension at 95°C for 15 s. Subsequently, the dissociation curves were obtained at 95°C for 15 s, 60°C for 1 min followed by 95°C for 15 s. The relative expression of *MaWRKY* TF was determined using $2^{-\Delta\Delta CT}$ method described by Livak and Schmittgen (2001).

3.18 Validation of *MaWRKY* Orthologs

The banana genome hub (https://banana-genome-hub.southgreen.fr/) provided the nucleotide sequences for the *MaWRKY* genes. These sequences were then subjected to a BLAST query against the plant transcription factor database (http://planttfdb.gaolab.org/) in order to confirm that the ortholog that was discussed in the discussion section and the chosen stress-responsive MaWRKY genes were highly similar.

3.19 Statistical analysis

Using the SPSS software, statistical analysis was performed on all experimental data. To ascertain the statistical significance between the treatments, a one-way analysis of variance (ANOVA) was performed on the data, followed by a Tukey post hoc test.

CHAPTER 4: RESULTS

4.1 Rhizosphere Soil Characteristics

The rhizosphere soil collected from different zones of the mangrove exhibited differences in several characteristics (Table. 4.1). Notably, the electrical conductivity (ECe) values differed among the sites. The soil adjacent to *Rhizophora apiculata* (red mangrove) at site one exhibited an ECe value of 14.46 dS/m, while the soil adjacent to *Avicennia alba* (black mangrove) at site two had an ECe value of 8.9 dS/m. The soil adjacent to *Sonneratia alba* (white mangrove) at site three showed an ECe value of 11.81 dS/m. In terms of organic carbon content, variations were observed among the sites. The soil adjacent to *Rhizophora apiculata* at site one had the lowest organic carbon content, measuring 40.74%. In contrast, the soil adjacent to *Avicennia alba* at site two exhibited a higher organic carbon content of 47.06%. The soil adjacent to *Sonneratia alba* at site three demonstrated the highest organic carbon content, measuring 49.55%. Regarding nutrient content, the highest nitrogen content was observed at site two (0.61%), followed by site three (0.45%), and site one (0.33%).

Table 4. 1: Collection sites description, soil characteristics and plant species of mangrove used in the study

Location characteristics						Soil characteristics			
Location	Collection Date	Latitude	Longitude	Soil Texture	EC (dS/m)	pH	N (%)	Total organic carbon (%)	Plant species
Carey Island	03/01/2020	2°86′05″N	101°36′58″ E	Sandy Clay loam	14.46	6.54	0.33	40.74	*Rhizophora apiculata*
Carey Island	03/01/2020	2°86′05″N	101°36′58″ E	Clay loam	8.9	7.51	0.61	47.06	*Avicennia alba*
Carey Island	03/01/2020	2°86′05″N	101°36′58″ E	Clay loam	11.81	7.4	0.45	49.55	*Sonneratia alba*

Table 4. 2: 16S rRNA gene analysis of selected plant growth promoting bacteria with reference to EzBioCloud

Mangrove site	Isolate	Sequence length (bp)	GenBank accession number of isolates	Top match in EzBioCloud database	GenBank accession number of top matches	Similarity (%)
Sonneratia alba	RB1	1489	MZ881953	*Bacillus velezensis* CR-502	AY603658	100
Sonneratia alba	RB2	1489	MZ881954	*Bacillus siamensis* KCTC13613	AJVF01000043	99.93
Sonneratia alba	RB3	1486	MZ881955	*Bacillus altitudinis* 41KF2b	ASJC01000029	100.00
Sonneratia alba	RB4	1489	MZ881956	*Bacillus tequilensis* KCTC13622	AYTO01000043	99.93
Avicennia alba	RB5	1474	MZ881957	*Bacillus albus* N35-10-2	MAOE01000087	100.00
Rhizophora apiculata	EB1	1474	MZ881950	*Pseudomonas luteola* NBRC 103146	BDAE01000066	99.93
Sonneratia alba	EB2	1487	MZ881951	*Bacillus siamensis* KCTC13613	AJVF01000043	99.86
Avicennia alba	EB3	1473	MZ881952	*Pseudomonas plecoglossicida* NBRC103162	BBIV01000080	99.86

4.2 Isolation and Salt Tolerance Efficiency of Rhizobacteria and Endophytic Bacteria

A total of 406 rhizobacterial isolates were isolated on LB medium. Among these isolates, 213 demonstrated the ability to grow on at least one of the media formulations supplemented with a range of aquarium salt concentrations (50-400 mM) (Appendix B). As the salt concentration increased, there was a decline in the number of viable bacteria, with only 88 isolates exhibiting growth at a concentration of 400 mM. Of the 29 isolates of endophytic bacteria, 24 exhibited growth on LB medium supplemented with 50 mM aquarium salt. As the salt concentration increased, there was a decrease in the number of bacteria able to grow, with only 16 isolates growing at a concentration of 400 mM aquarium salt (Appendix C, D). The shape, morphology, and Gram staining characteristics of the halotolerant rhizobacteria are presented in Appendix E, while those of the endophytic bacteria are displayed in Appendix F.

4.3 Screening of Halotolerant Bacteria

To evaluate the plant growth promoting traits of the halotolerant bacterial isolates, a total of 88 rhizobacterial and 16 endophytic bacterial isolates were subjected to screening for primary activities, namely phosphate solubilization and nitrogen fixation. Nitrogen fixation is important for providing plants with accessible nitrogen, while phosphate solubilization aids in the availability of phosphorus for various plant processes. All 88 halotolerant rhizobacterial isolates, as well as all 16 endophytic bacterial isolates, exhibited both nitrogen fixation and phosphate solubilization activities. The two isolates with the highest phosphate solubilizing activity were EB1 (169 µg ml^{-1}) and RB1 (115 µg ml^{-1}) (Fig. 4.1B).

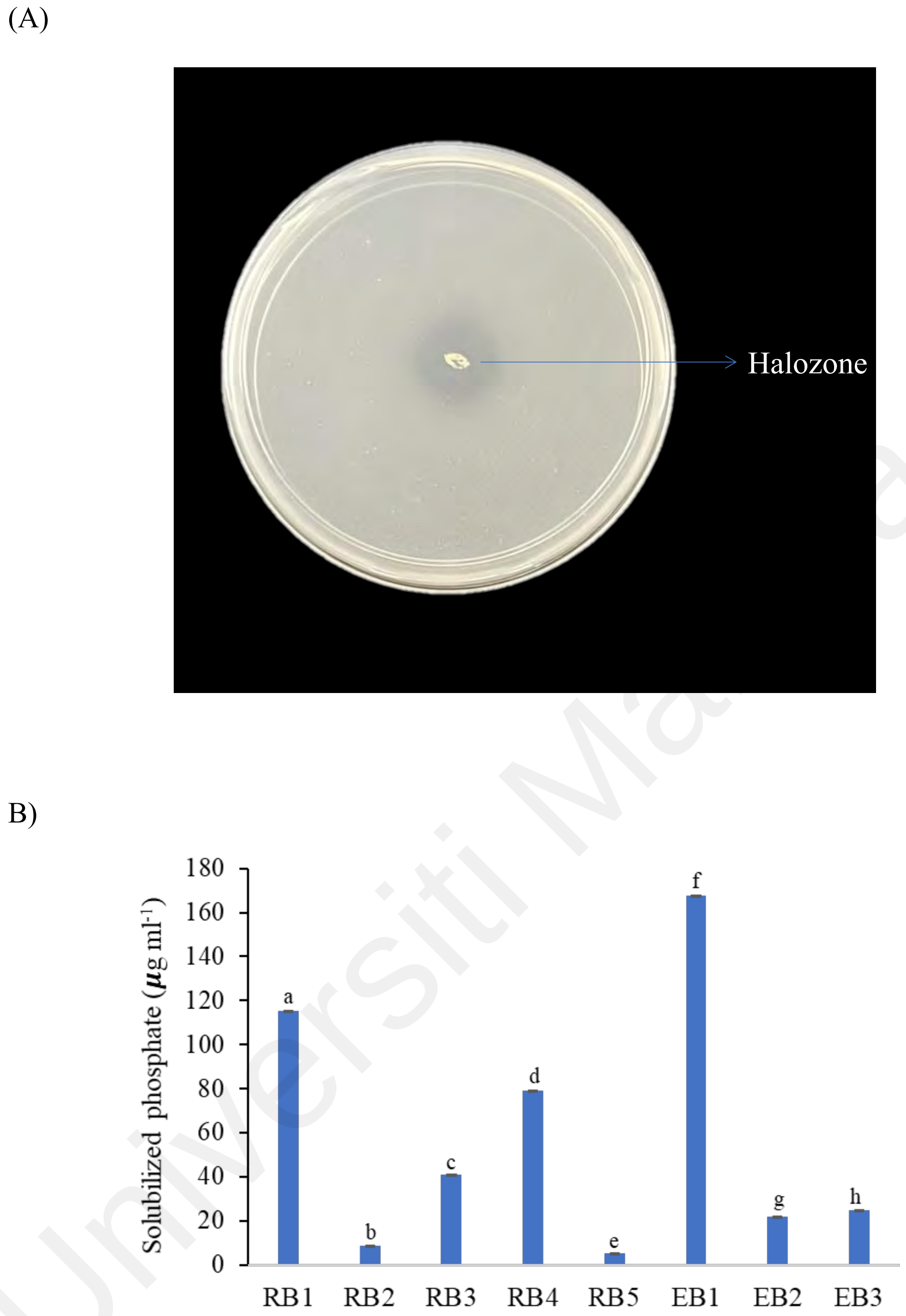


Fig. 4.1: Phosphate solubilizing activity of bacterial isolates. (A) Phosphate solubilization activity of *Bacillus* sp. RB1 on Pikovskaya's agar plate (B) Quantitative estimation of phosphate solubilization. Each panel displays data from the selected eight bacterial isolates (RB1-RB5 and EB1-EB3). Error bars indicate the standard error (n = 10). Different letters indicate a significant difference at ($p < 0.05$) using one-way ANOVA and Tukey post-hoc tests.

4.4 Molecular Identification of Halotolerant Bacteria

Further analysis involved partial 16S rRNA gene sequencing and phylogenetic analysis. Examination of the sequence similarity showed numerous isolates with 16S rRNA gene sequences that were type strains and highly similar (>99%) or identical to the known species in the EzBioCloud database (Appendix F). These results led to the selection of three endophytic bacterial strains (EB1-EB3) and five rhizobacteria strains (RB1-RB5) for additional examination. The RB isolates' 16S rRNA gene sequences shared considerable similarity (99.93% to 100%) with a number of *Bacillus* species. *Pseudomo*nas spp. was found to make up the majority (21 out of 24) of the endophytic bacterial isolates (Appendix G). Strain EB1 display a 99.93% sequence similarity in its 16S rRNA gene with *Pseudomonas luteola* NBRC 103146 and similarly, EB3 share 99.86% sequence similarity with *Pseudomonas plecoglossicida* NBRC103162 (Table 4.2). Notably, strain EB2 exhibit 99.93% sequence similarity to the rhizobacterium *Bacillus siamensis* KCTC13613, while being identified as an endophytic bacterium from root tissue.

Phylogenetic trees constructed with the neighbor joining method were used to show the link between the strains and type members of their respective genera (Fig 4.2, 4.3, and 4.5). In these trees, all strains, except for EB3, showed a high degree of relatedness and formed clusters with organisms that were closest to them. The strain EB3 displayed a 99.86% similarity in its 16S rRNA gene sequence with *P. plecoglossicida* NBRC103162, indicating a close relationship with them. However, its position in the phylogenetic tree was closer to *P. alloputida* Kh7 (Fig. 4.4). Following the phylogenetic analysis, all eight strains underwent were subjected to further evaluation for their plant growth promoting traits and response to salt stress.

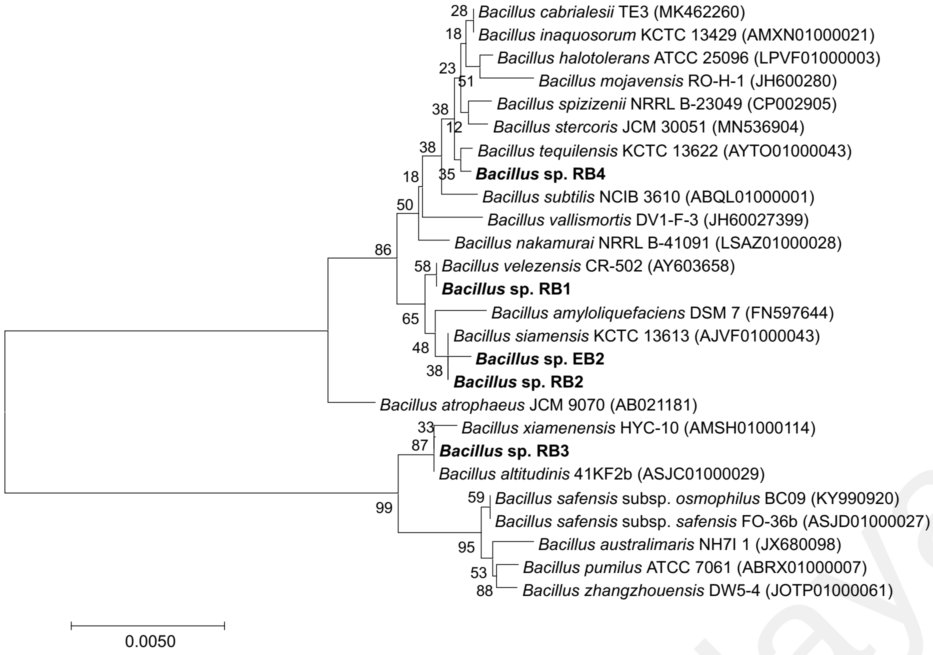


Fig. 4.2: Phylogenetic Tree of *Bacillus* isolates. Neighbor-joining tree showing the phylogenetic relationship of plant growth promoting bacterial strains EB2, RB1, RB2, RB3, RB4 and their closest relatives in the genus Bacillus based on their 16S rRNA gene sequences. The number displayed at nodes are bootstrap values, based on 1000 replications.

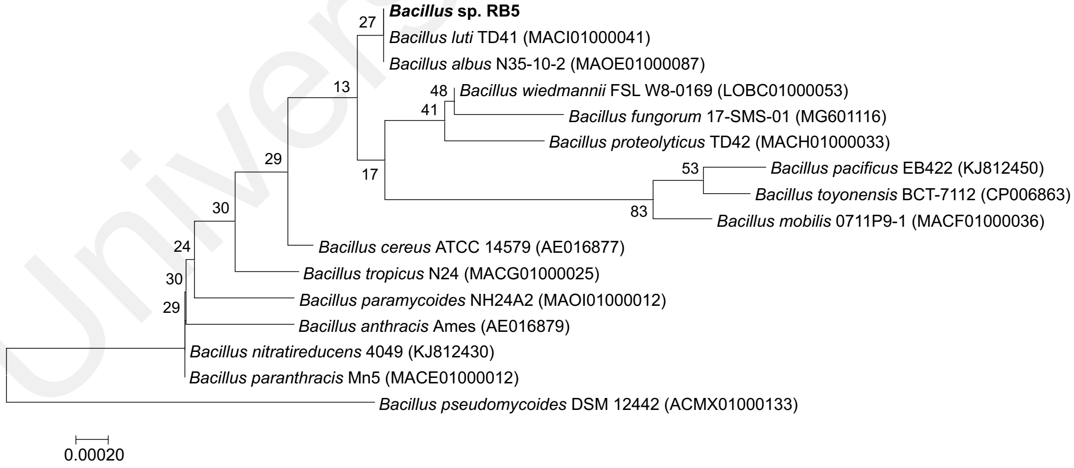


Fig. 4.3: Phylogenetic Tree of bacterial isolate RB5. Neighbor-joining tree showing the phylogenetic relationship of plant growth promoting bacterial strains RB5 and its closest relatives in the genus Bacillus based on their 16S rRNA gene sequences. The number displayed at nodes are bootstrap values, based on 1000 replications.

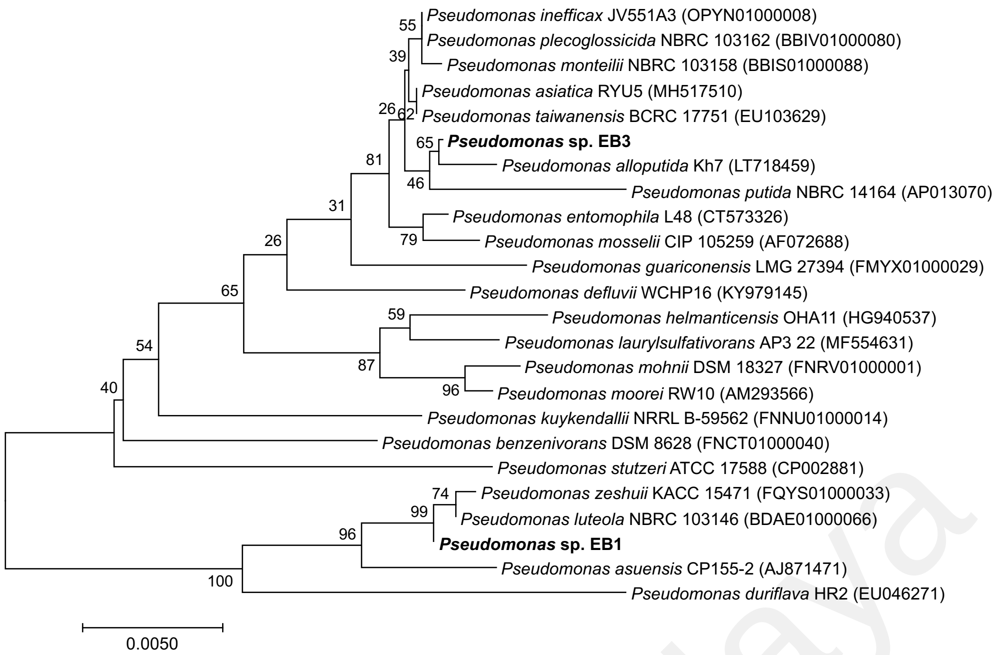


Fig. 4.4: Phylogenetic Tree of *Pseudomonas* isolates. Neighbor-joining tree showing the phylogenetic relationship of plant growth promoting bacterial strains EB1, EB3 and their closest relatives in the genus Pseudomonas based on their 16S rRNA gene sequences. The number displayed at nodes are bootstrap values, based on 1000 replications.

4.5 Plant Growth Promoting Traits of Bacterial Isolates

In this study, the selected bacterial strains, five rhizobacteria strains (RB1-RB5) and three endophytic bacteria strains (EB1-EB3), are collectively termed as plant growth promoting bacteria (PGPB) since they showed characteristics that promote plant growth. These characteristics included the ability to produce siderophores, IAA, and ACC deaminase activity.

4.5.1 IAA Production

The production of IAA, a phytohormone, is an important characteristic exhibited by PGPB in the rhizosphere. IAA plays a crucial role in enhancing root length and surface area, particularly under salt stress conditions, as it helps improve nutrient availability and regulate ion influx (Ma et al., 2020). In this study, quantitative analysis of the eight selected strains revealed an increase in IAA production over time. Among the strains, RB1 and RB3 exhibited the highest IAA production, with concentrations ranging from 7.4 to 48.3 µg ml^{-1} and 6.69 to 30.13 µg ml^{-1}, respectively, over a 48-hour period (Fig. 4.5).

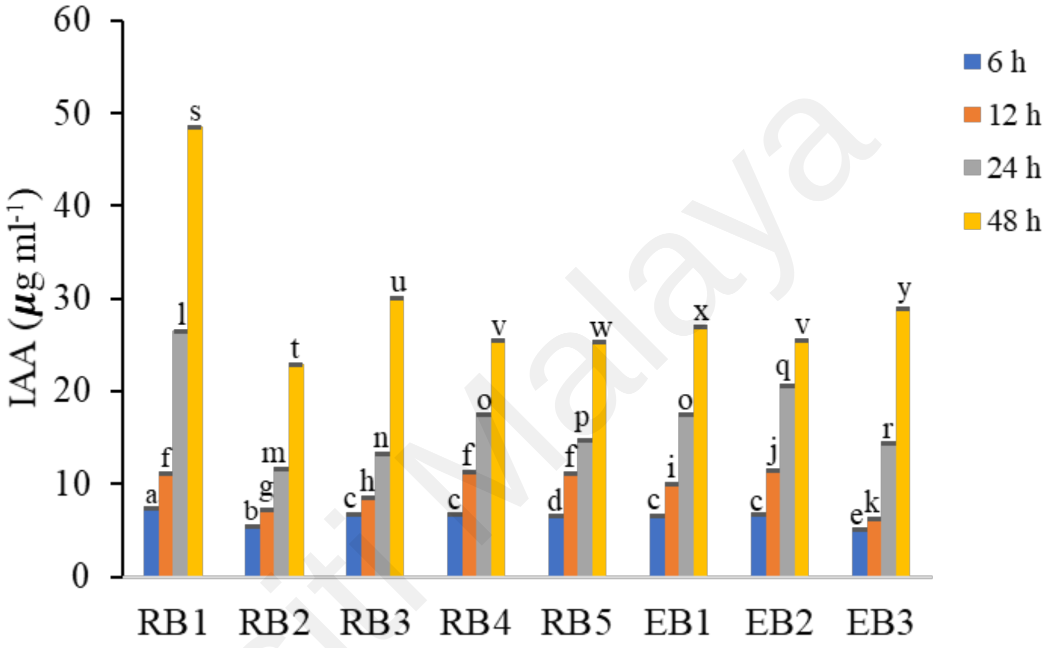


Fig. 4.5: Quantification estimation of IAA production at 6, 12, 24 and 48 h of incubation exhibited by bacterial isolates. Each panel displays data from the selected eight bacterial isolates (RB1-RB5 and EB1-EB3). Error bars indicate the standard error (n = 10). Different letters indicate a significant difference at ($p < 0.05$) using one-way ANOVA and Tukey post-hoc tests.

4.5.2 Siderophore Production

Iron (Fe) is an essential element for various metabolic activities in plants, but its bioavailability as ferric iron (Fe^{3+}) is limited. PGPB address this challenge by producing and secreting siderophores, which are low molecular weight compounds that chelate ferric ion from the environment, making it available for plant uptake (Ryu et al., 2003).

In this study, all eight selected strains demonstrated siderophore production. Among them, RB4 (50.93%) and RB5 (50.21%) exhibited the highest siderophore production, with the RB strains typically displaying greater % values compared to the EB strains (Fig. 4.6B).

(A)

(B)

Fig. 4.6: Siderophore activity exhibited by bacterial isolates. (A) Siderophore production of *Pseudomonas* sp. EB3 on CAS-LB plate, (B) Quantification estimation of siderophore production. Each panel displays data from the selected eight bacterial isolates (RB1-RB5 and EB1-EB3). Error bars indicate the standard error (n = 10). Different letters indicate a significant difference at ($p < 0.05$) using one-way ANOVA and Tukey post-hoc tests.

4.5.3 ACC Deaminase Activity

Increased ethylene content in plant roots under saline conditions can inhibit growth. ACC deaminase is an enzyme produced by PGPB that converts ACC, a substrate for ethylene biosynthesis, into α-ketobutyrate and ammonia, effectively reducing ACC levels and mitigating the negative effects of ethylene (Glick, 2014). In this study, the ACC deaminase activity ranged from 1318 to 3477 nmol α-ketobutyrate protein h^{-1} in all eight of the tested strains. In comparison to the EB strains, the majority of the RB strains had greater activity. The two strains with the highest ACC deaminase activity were strain RB2 (3902 nmol α-ketobutyrate protein h^{-1}) and strain RB4 (3561nmol α-ketobutyrate protein h^{-1}) (Fig. 4.7).

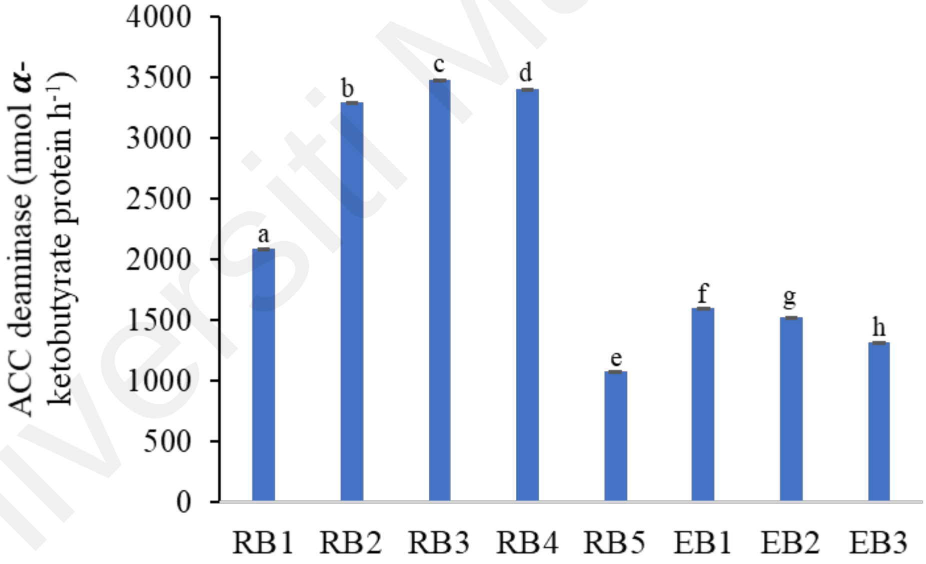


Fig. 4.7: Quantitative estimation of ACC deaminase activity exhibited by the bacterial isolates. Each panel displays data from the selected eight bacterial isolates (RB1-RB5 and EB1-EB3). Error bars indicate the standard error (n = 10). Different letters indicate a significant difference at ($p < 0.05$) using one-way ANOVA and Tukey post-hoc tests.

Six strains (RB1, RB3, RB4, EB1, EB2, and EB3) were chosen for further studies based on plant growth promoting traits analysis.

4.6 Root Colonization of Banana Plantlet with Plant Growth Promoting Bacteria

Banana roots were examined using a field emission scanning electron microscope (FESEM) 72 hours after being inoculated with one of the rhizobacterial strains RB1, RB3, and RB4 (Fig. 4.8 A-C) or one of the endophytic bacterial strains EB1, EB2, and EB3 (Fig. 4.8 D-F). All of the selected PGPB strains were visible on the root cap and root elongation zone in the FESEM pictures showing that these bacterial strains successfully colonized the root surface. The non-inoculated plant root, in comparison, exhibited no bacterial colonization (Fig. 4.8G).

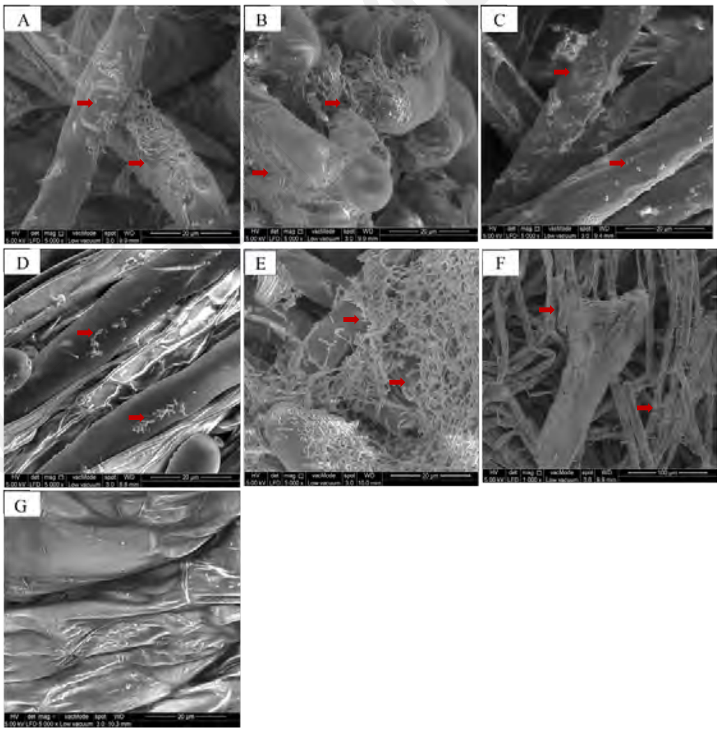
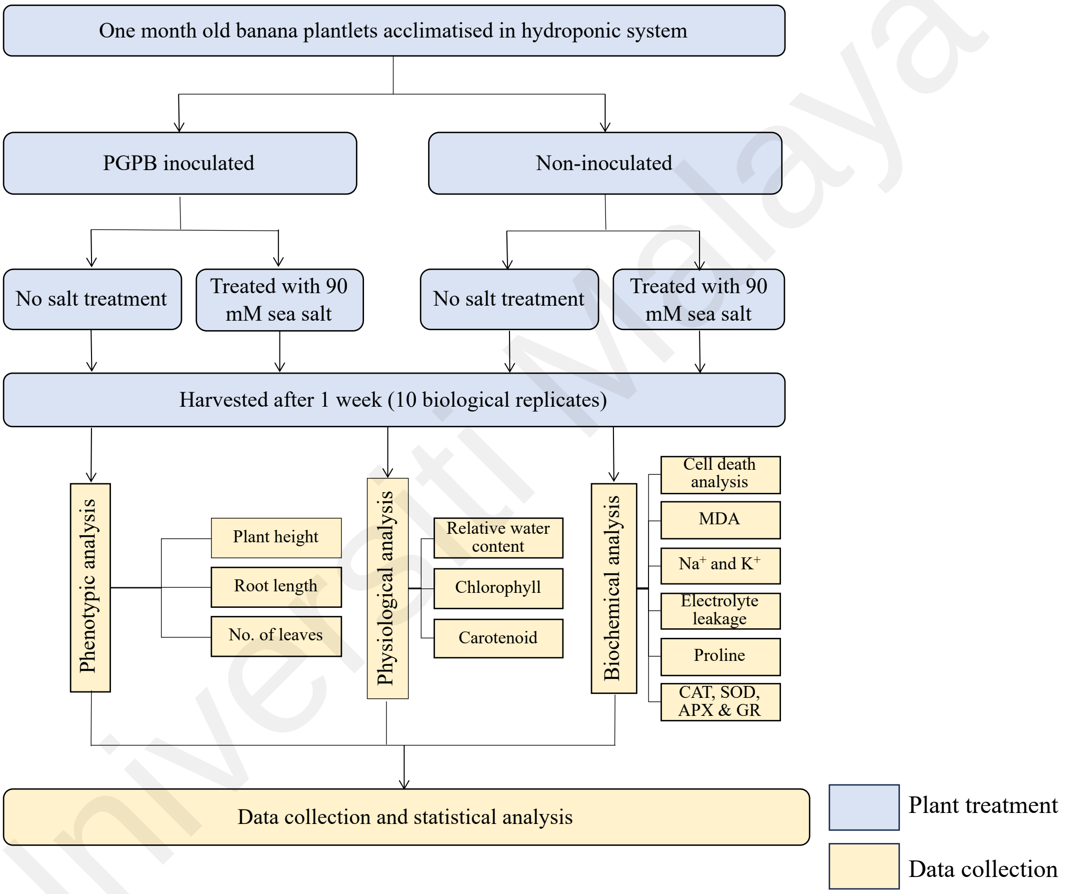


Fig. 4.8: Bacterial root colonization. Field Emission Scanning Electron Microscopy (FESEM) images of banana root 72 h after inoculation with plant growth promoting bacteria; Red arrow indicating colonization of (A) RB1, (B) RB3, (C) RB4, (D) EB1, (E) EB2, (F) EB3 and (G) Control (non-inoculated).

4.7 Inoculation of Banana with PGPB Strains Reduces the Negative Effects of Salt Stress

The first set of experiments were performed using the selected PGPB (RB1, RB3, RB4, EB1, EB2, and EB3) strains, outlined in Fig 4.9A. Subsequently, the greenhouse experiments were conducted using the two best performing PGPB isolates (EB3 and RB3) applied as a consortium as shown in Fig. 4.9B.

(A)



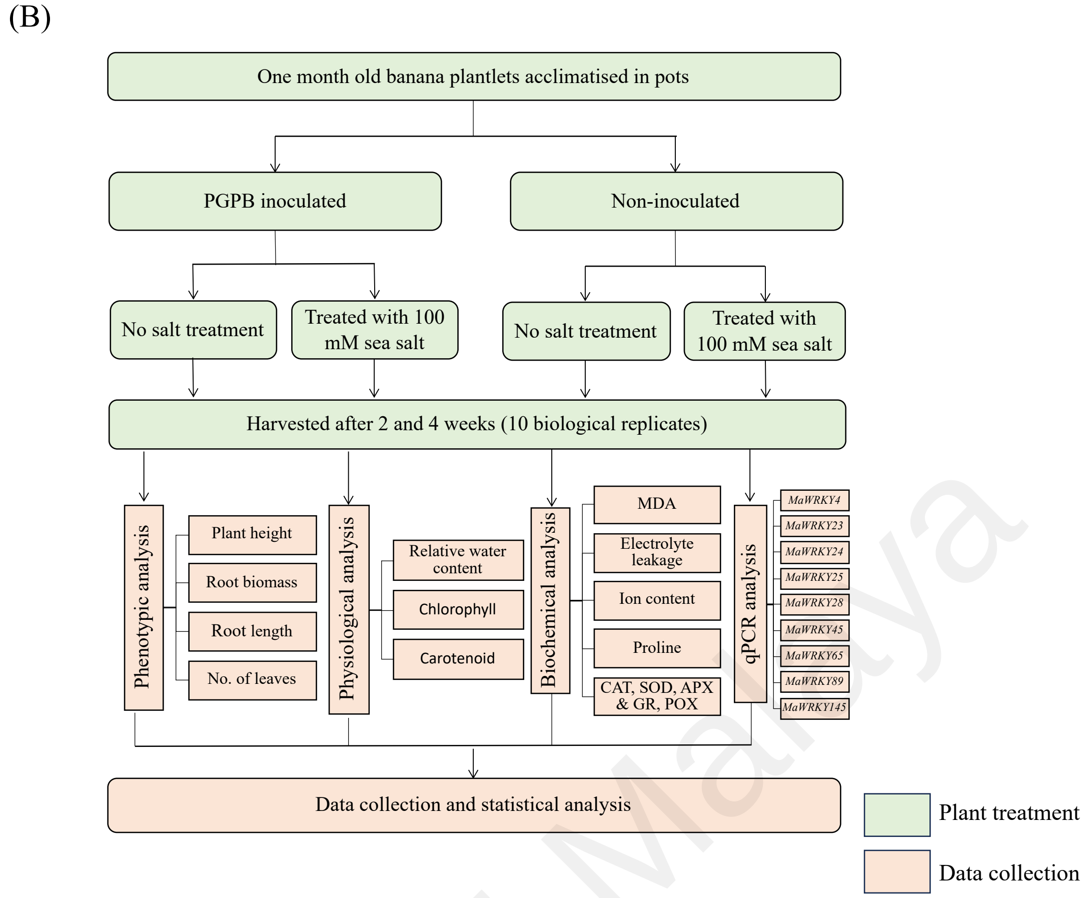
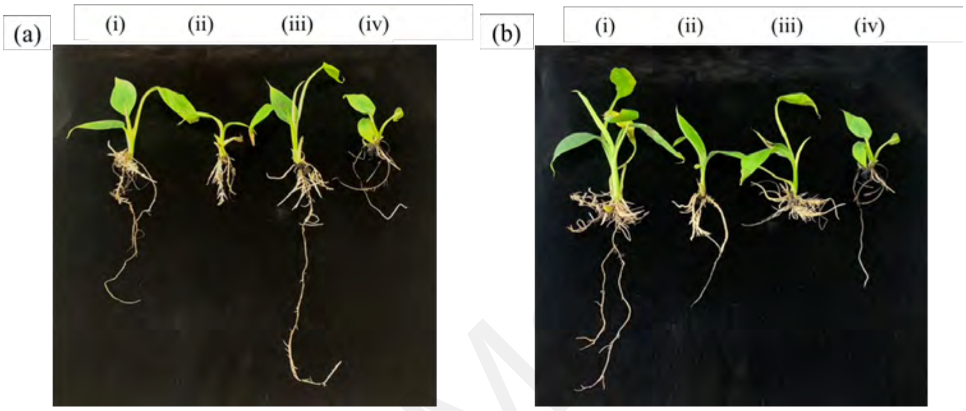


Fig. 4.9: Flowchart of experimental design. (A) Schematic representation of hydroponic experiments. Blue blocks denote plant treatment procedures, while yellow blocks denote analytical processes conducted in the study. (B) Schematic representation of greenhouse salt stress experiments. Green blocks denote plant treatment procedures, while pink blocks denote analytical processes conducted in the study.

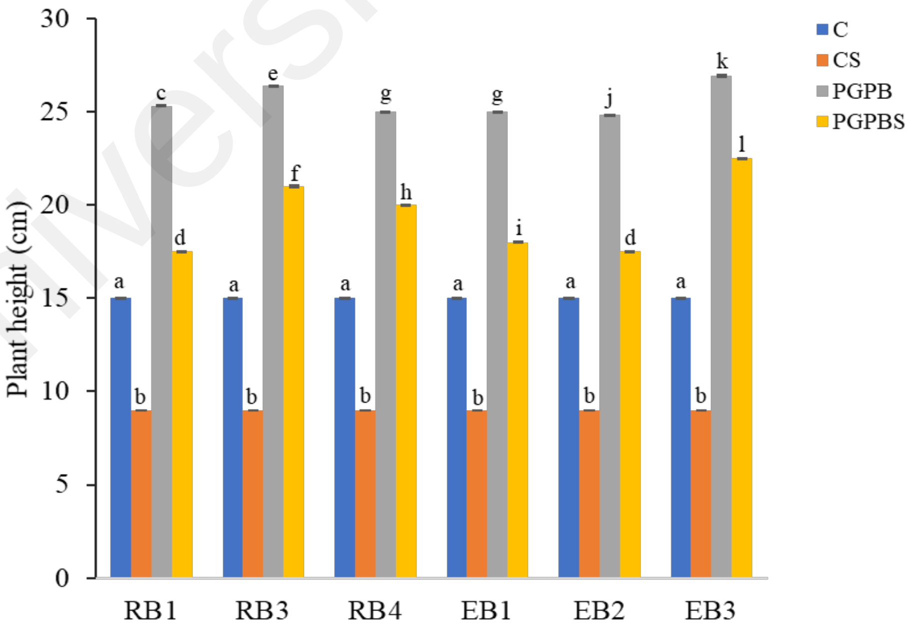
4.7.1 Inoculation of Banana with PGPB Improve Banana Growth Parameters under Salt Stress

Under the influence of aquarium salt (90 mM), the effects of inoculation with the six selected strains (RB1, RB3, RB4, EB1, EB2, and EB3) on banana growth were studied and compared to non-inoculated control plants. The results showed that the inoculated banana plantlets had significantly greater root length and plant height compared to the control plants (Fig 4.10A). Among the treatments, strains EB3 and RB3 showed the highest increases in plant height, with a 2.5-fold and 2.3-fold increase, respectively, compared to the control plants under salt stress (Fig. 4.10B). Similarly, treated plants had roots that were 1.2–1.5 fold longer than control plants (Fig. 4.10C). The number of leaves per plant did not, however, show significant differences between the treatment groups (Fig. 4.10D).

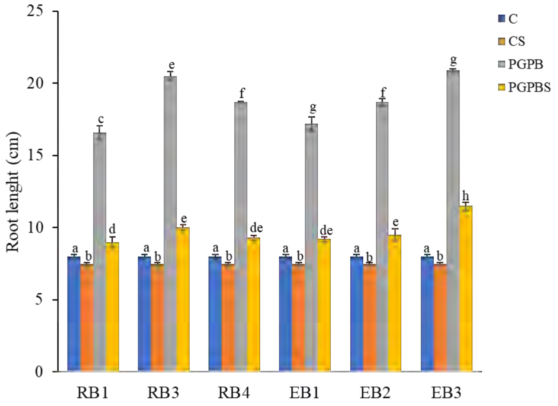
(A)



(B)



(C)



(D)

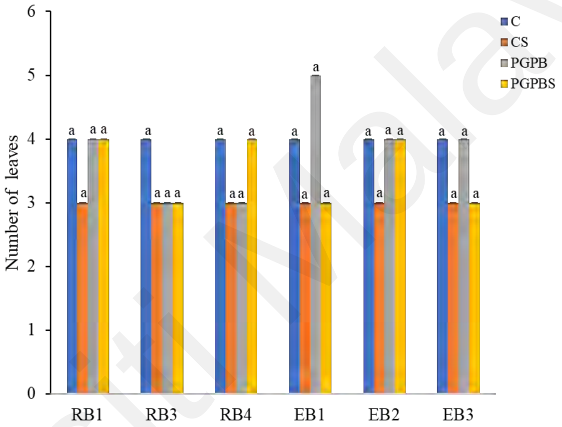
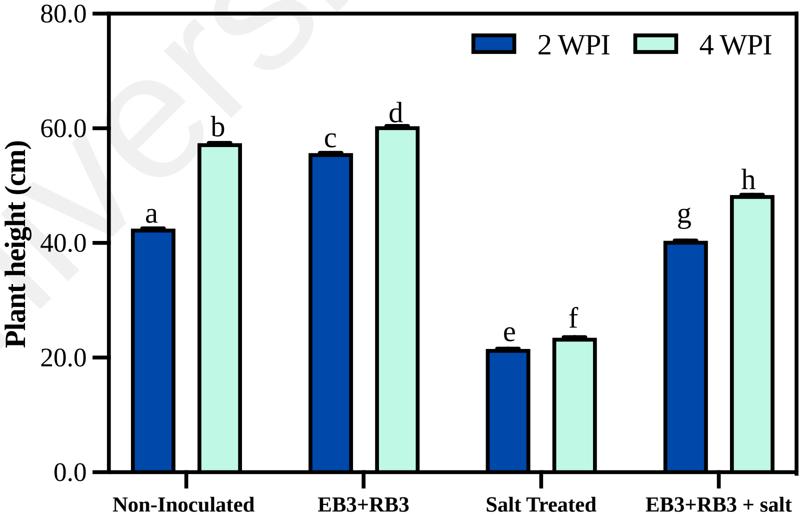


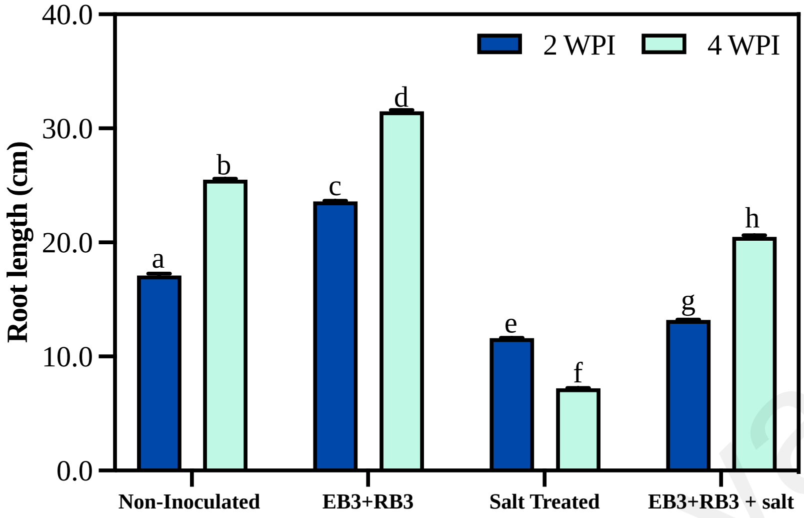
Fig. 4.10: Effect of PGPB inoculation on plant growth parameters in hydroponic analysis. (A) Picture of plantlets root inoculated following exposure to 90 mM sea salt for a period of 7 days. (a) root inoculated with strains (i) RB1, (ii) RB4, (iii) RB3, (iv) non-inoculated. (b) root inoculation with endophytic bacteria (i) EB3, (ii) EB2, (iii) EB1, (iv) non inoculated. Changes in (B) plant height, (C) root length, (D) number of leaves observed in banana plantlets after 7 days of treatment in hydroponic media. The treatments include non-inoculated plantlets in medium without added sea salt (C), non-inoculated plantlets in 90 mM sea salt (CS), plantlets inoculated with isolates without added sea salt (PGPB), and plantlets inoculated with isolates in 90 mM sea salt PGPBS. The values are mean ± SD (n = 10). Different letters indicate significant differences at ($p < 0.05$) using ANOVA and Tukey post-hoc tests.

The selection of *Pseudomonas* sp. EB3 and *Bacillus* sp. RB3 for the greenhouse study was based on their demonstrated effectiveness in protecting banana plants against salinity stress in the hydroponic study. At this stage only one combination was selected due to limited time and resources and the best performing of each type of (rhizobacteria and endophytic bacteria) were used. In the greenhouse analysis, the effect of the PGPB consortium inoculation on growth parameters in salt-stressed banana plants was evaluated. The plants pre-treated with the consortium showed significantly better growth (Fig. 4.11B, C) and biomass (Fig. 4.11D) compared to the stressed plants without consortium pre-treatment. Among the growth parameters assessed, root length displayed the most significant improvement in the consortium-inoculated plants under salt stress conditions. Specifically, the root length of the consortium-inoculated plants was 2.7-fold longer compared to the stressed plants without consortium inoculation.

(A)



(B)



(C)

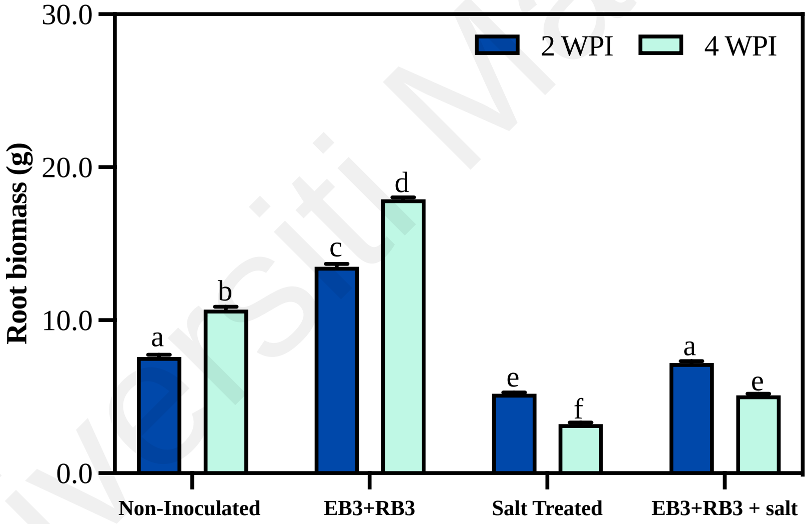


Fig. 4.11: Effect of PGPB inoculation on plant growth parameters under salt stress conditions. Banana plants at four weeks: non-inoculated; EB3+RB3 inoculated; salt treated with 100 mM solution (not inoculated); EB3+RB3 and salt treated with 100 mM solution. Changes in banana plant growth, (B) plant height, (C) root length, and (D) root biomass under salt stress. Salt treatment was for 2 weeks and 4 weeks in soil with PGPB in 100 mM sea salt. WPI represent weeks post inoculation. The standard error is denoted by error bars. The values are mean ± SD (n = 10). Using ANOVA and Tukey post-hoc tests, different letters indicate significant differences at ($p < 0.05$).

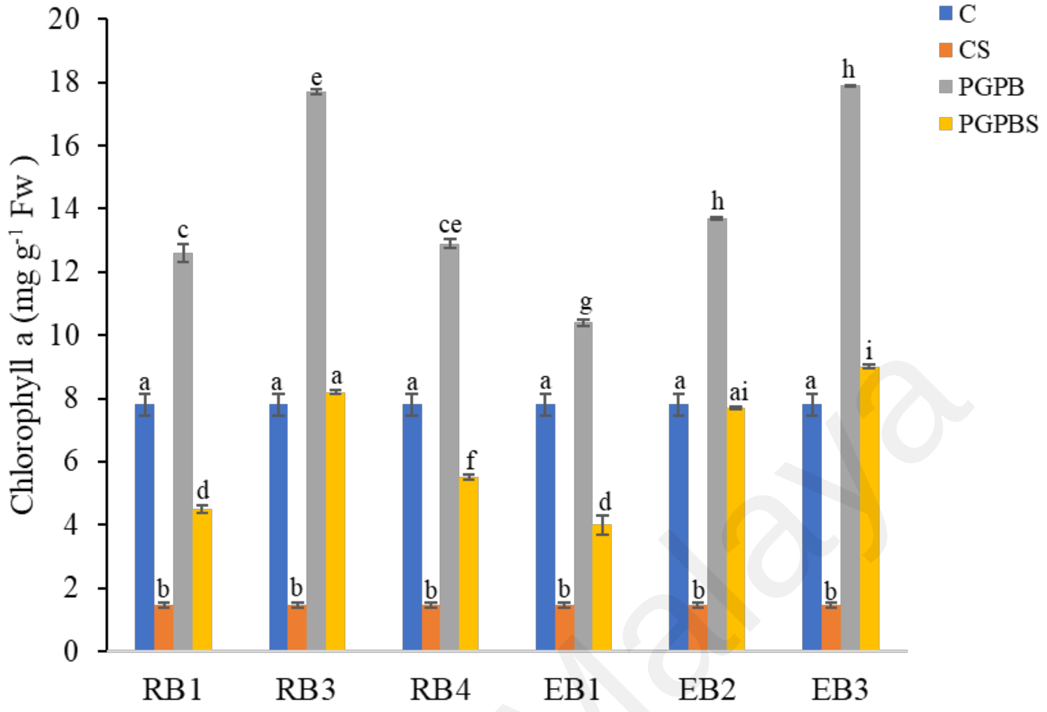
4.7.2 PGPB Inoculation Enhances Banana Physiological Parameters under Salt Stress

The salt stress treatment resulted in a reduction in chlorophyll and carotenoid levels in banana plantlets (Fig. 4.12). However, PGPB reduced the detrimental effects of salt stress on chlorophyll levels and increased levels of chlorophyll in non-stressed conditions. In comparison to non-inoculated plants under salt stress, inoculated plants showed a 2.6–6-fold higher Chl a content; the highest Chl a levels were observed in the EB3 (6–fold) and RB3 (5.4–fold) treatment groups (Fig. 4.12A). Similar to this, during salt stress, inoculated plants had Chl b content that was 3.5-7.6 fold higher than in non-inoculated plants, with strain EB3 (7.6-fold) and strain RB3 (6.8-fold) showing the highest Chl b levels (Fig. 4.12B).

Carotenoids, important for electron transport and photosynthesis, were found to be higher in inoculated banana plants in comparison to control plants, under both non-stressed and salt stress conditions. The inoculated plants exhibited carotenoid levels 1.4-3.2-fold higher than the control plants, with treatments using strain EB3 (3.2-fold) and strain RB3 (2.8-fold) showing the highest carotenoid levels (Fig. 4.12C).

Under salt stress, the osmolyte proline plays a crucial role in maintaining cellular homeostasis. In comparison to control plants, plants inoculated with PGPB exhibited higher proline content under both non-stressed and salt stress conditions. The proline levels in inoculated plants were 1.27-1.83-fold higher than the control plants, with strain EB3 (1.83-fold) and strain RB3 (1.63-fold) showing the highest levels (Fig. 4.12D).

(A)


Chlorophyll a (mg g^{-1} Fw)
0
2
4
6
8
10
12
14
16
18
20
C
CS
PGPB
PGPBS
RB1
RB3
RB4
EB1
EB2
EB3
a
b
c
d
a
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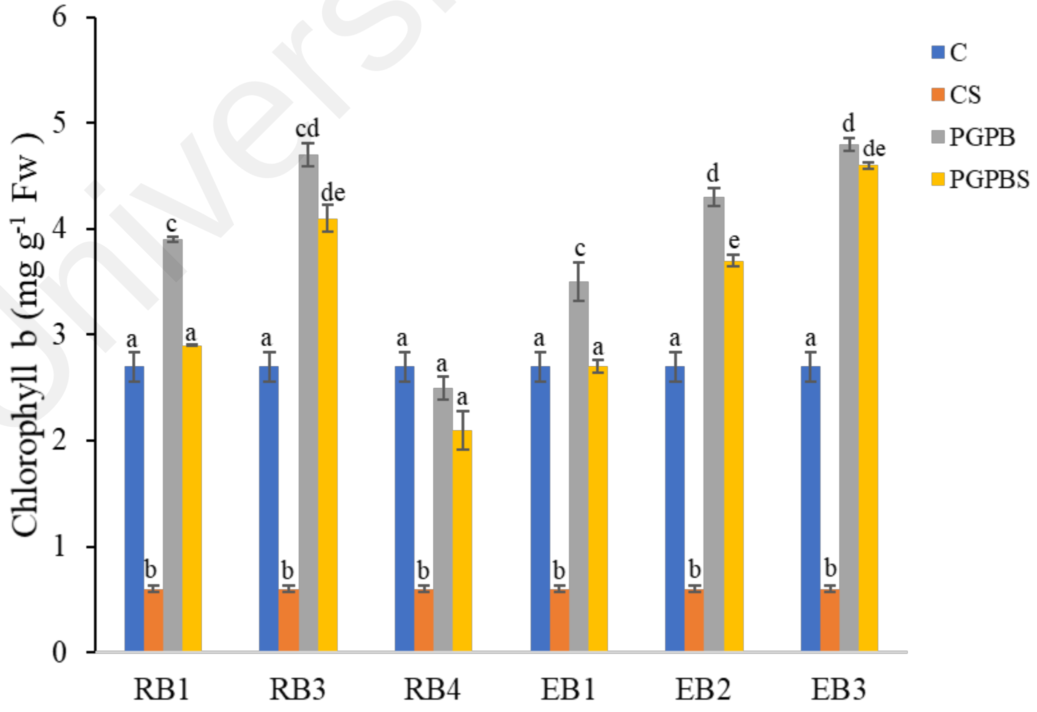
i

(B)


Chlorophyll b (mg g^{-1} Fw)
0
1
2
3
4
5
6
C
CS
PGPB
PGPBS
RB1
RB3
RB4
EB1
EB2
EB3
a
b
c
a
a
b
cd
de
a
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a
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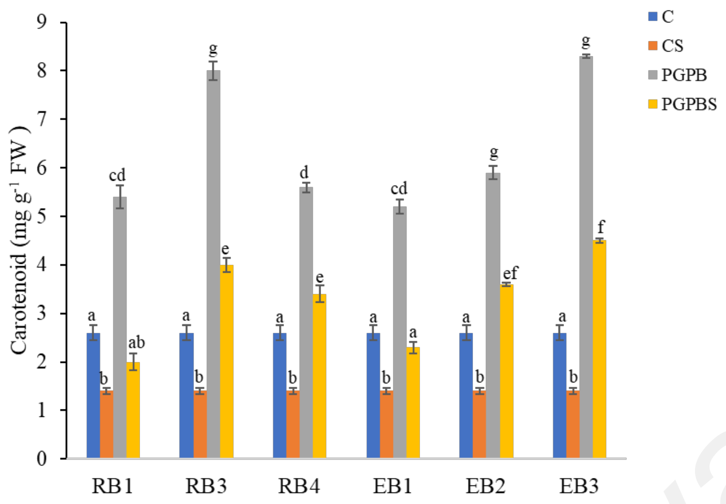
d
de

(C)



(D)

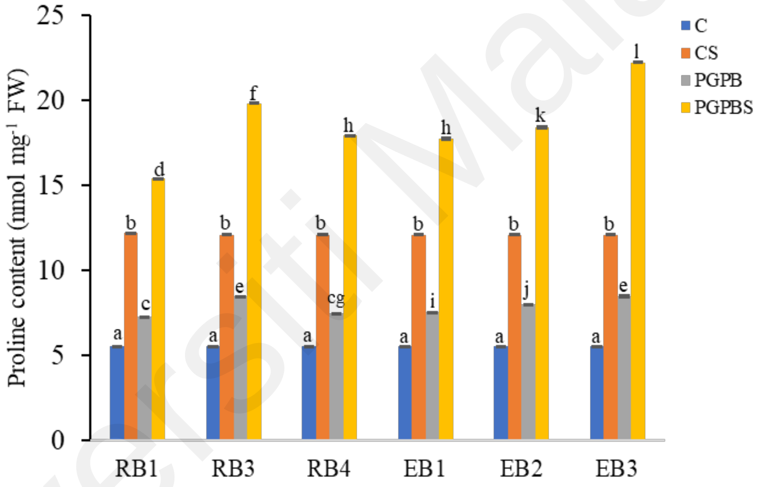
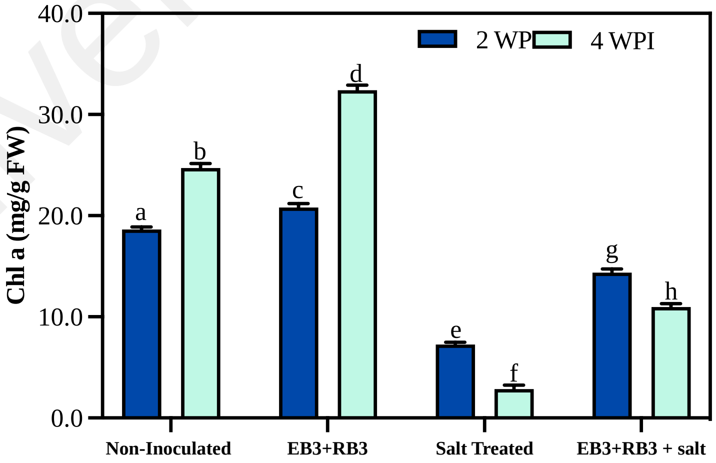


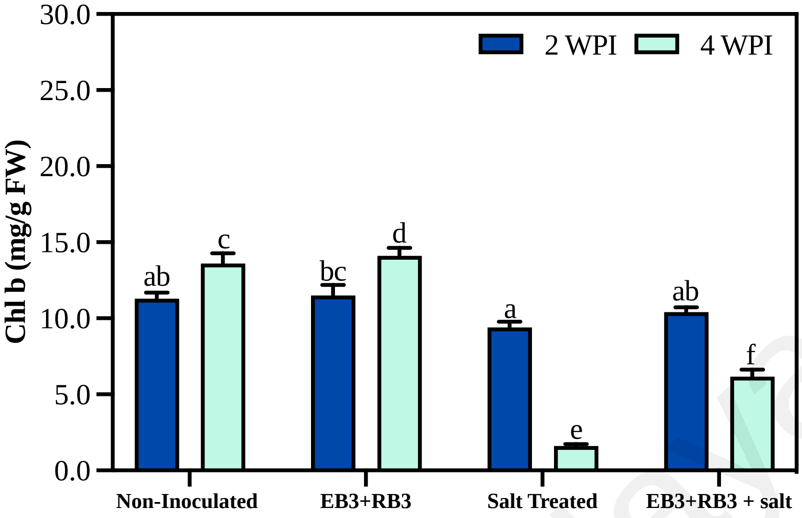
Fig. 4. 12: Effect of PGPB inoculation on chlorophyll, carotenoid, and proline content in plants in hydroponic analysis. Quantification of (A) chlorophyll a, (B) chlorophyll b, (C) carotenoid, (D) proline content in the leaves of banana plantlets after 7 days of treatment in hydroponic media. The treatments include non-inoculated plantlets in medium without added sea salt (C), non-inoculated plantlets in 90 mM sea salt (CS), plantlets inoculated with isolates without added sea salt (PGPB), and plantlets inoculated with isolates in 90 mM sea salt PGPBS. The values are mean ± SD (n = 10). Different letters indicate significant differences at ($p < 0.05$) using ANOVA and Tukey post-hoc tests.

In the greenhouse analysis, the impact of the PGPB consortium inoculation on pigment levels were also assessed (Fig. 4.13). Compared to the non-pretreated groups, banana plants treated with the consortium demonstrated a significantly high levels of chlorophyll and carotenoid contents under non-stressed conditions. Furthermore, in the presence of salt stress, the consortium treated plants showed a substantial enhancement in chlorophyll a and b levels, with a 3.8-fold higher level compared to the non-pretreated groups (Fig. 13.A, B). Similarly, the carotenoid content in the consortium-inoculated plants increased substantially under salinity stress, reaching levels that were 12-fold higher than in the non-inoculated plants (Fig. 4.13C). Moreover, the relative water content, was significantly higher (1.4-fold) in the consortium-inoculated banana plants under both non-stressed and salinity-stressed conditions (Fig. 4.13D). Furthermore, a 2-fold higher proline level was observed in consortium-inoculated plants under salinity stress compared to non-inoculated plants (Fig. 4.13E).

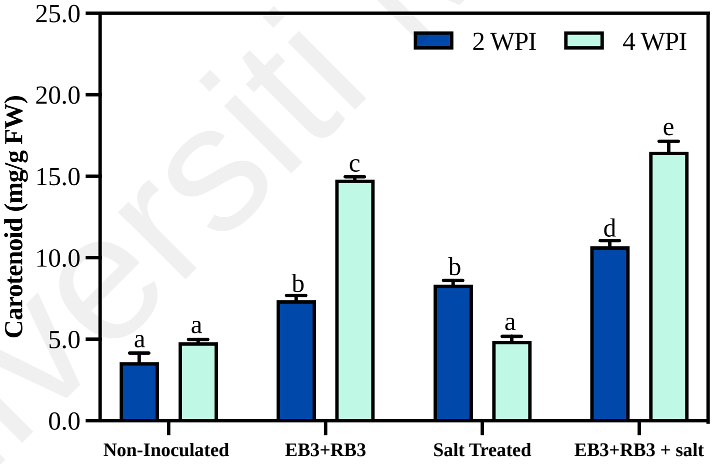
(A)



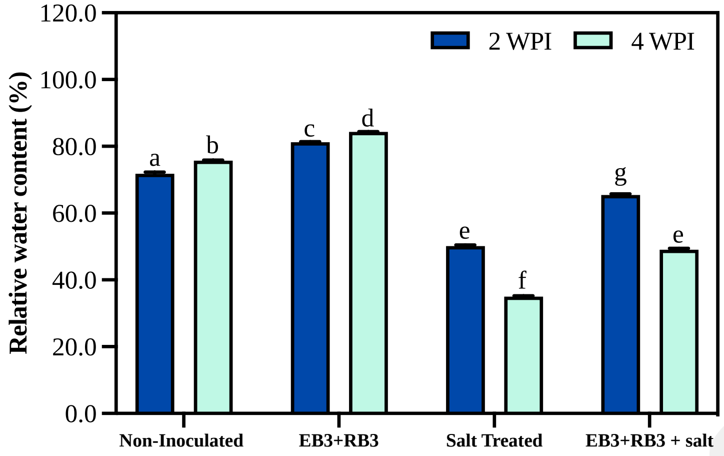
(B)


30.0
25.0
20.0
15.0
10.0
5.0
0.0
Chl b (mg/g FW)
2 WPI
4 WPI
ab
c
bc
d
a
e
ab
f
Non-Inoculated
EB3+RB3
Salt Treated
EB3+RB3 + salt

(C)


25.0
20.0
15.0
10.0
5.0
0.0
Carotenoid (mg/g FW)
2 WPI
4 WPI
a
a
b
c
b
a
d
e
Non-Inoculated
EB3+RB3
Salt Treated
EB3+RB3 + salt

(D)



(E)

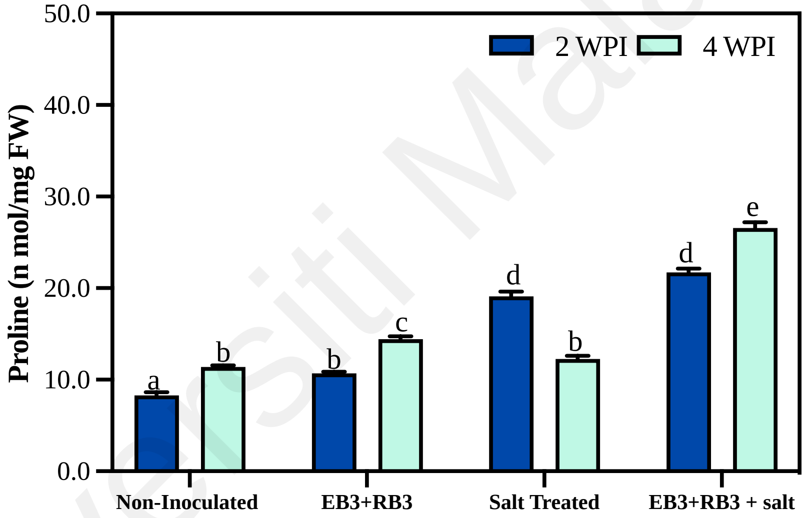


Fig. 4.13: Effect of EB3+RB3 inoculation on chlorophyll, carotenoid, relative water content and proline content in plants under salt stress condition. Estimation of (A) chlorophyll a, (B) chlorophyll b, (C) carotenoid, (D) relative water content and (E) Proline content banana in leaves under salt stress. Salt treatment was for 2 weeks and 4 weeks in soil with PGPB in 100 mM sea salt. WPI represent weeks post inoculation. The standard error is displayed by error bars. The values are mean ± SD (n = 10). Using ANOVA and Tukey post-hoc tests, significant differences at ($p < 0.05$) are shown by different letters.

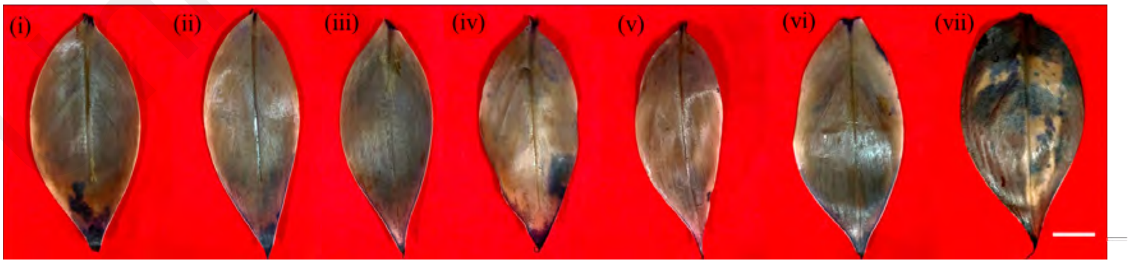
4.7.3 PGPB Inoculated Bananas have Less Membrane Damage under Salt Stress

Salt stress resulted in visible membrane damage in the leaves of both inoculated and non-inoculated plants, as observed by necrotic regions stained with Evans blue dye. In contrast to the non-inoculated control plants, the staining on the leaves lamina of the inoculated plants was less severe (Fig. 4.14A).

In comparison to the control plants, inoculated banana plants displayed lower levels of lipid peroxidation in both non-stressed and salt-stressed conditions, as shown in MDA (Fig. 4.14B). The inoculated plants exhibited MDA levels 0.61-0.45-fold lower than those of the control plants, with the greatest reductions observed in treatments with strain EB3 (0.45-fold) and strain RB3 (0.51-fold).

Salt stress induces the generation of ROS, which was assessed by measuring H_2O_2 levels. Inoculated plants showed significantly lower H_2O_2 levels than the control plants in both non-stressed and salt-stressed conditions (Fig. 4.14C). H_2O_2 levels in the inoculated plants were 0.86-0.23-fold lower than in the control plants, with the lowest levels seen in strain EB3 (0.23-fold) and strain RB3 (0.47-fold) treatments.

(A)



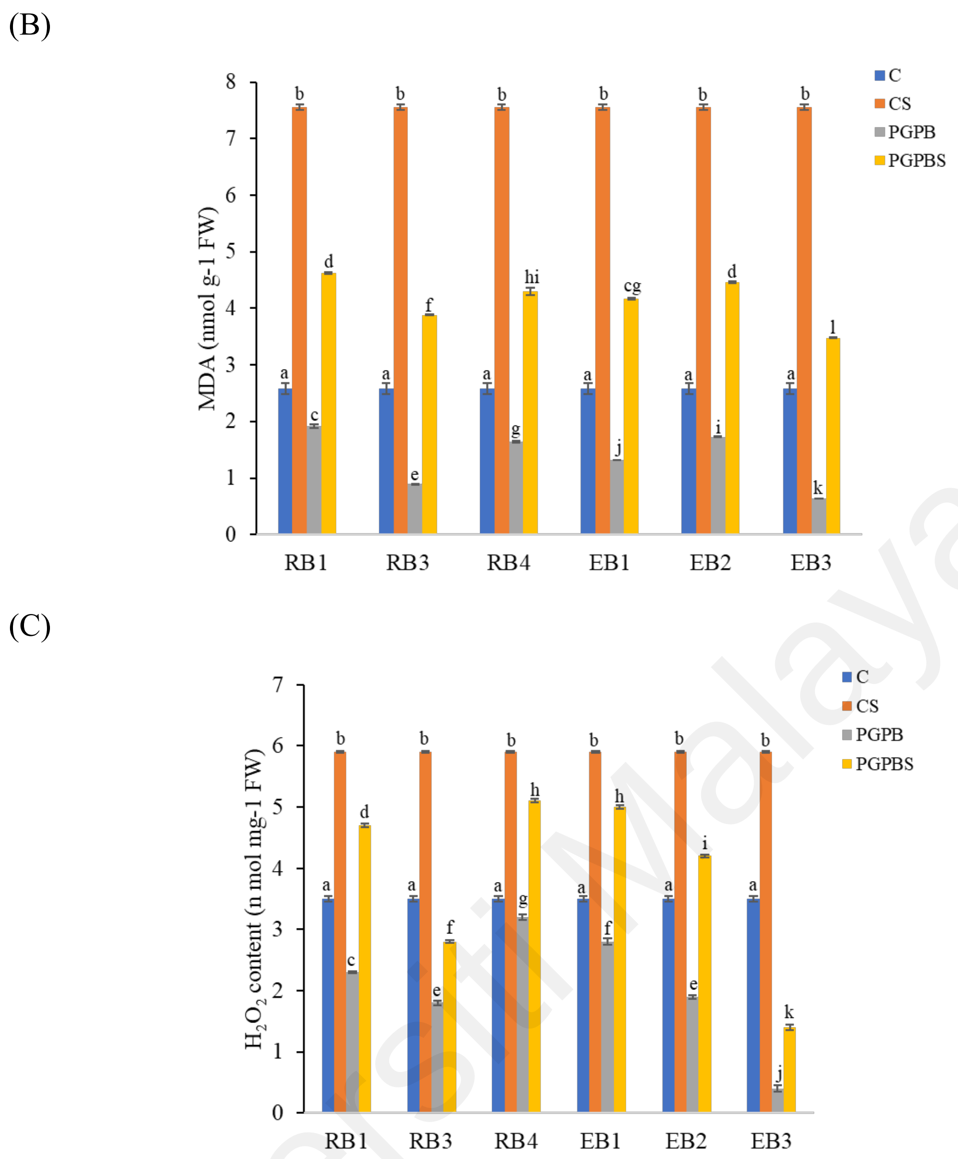


Fig. 4.14: Effects of PGPB inoculation on stress markers in plants. (A) Evan's blue staining in leaves of banana plantlet following exposure to 90 mM sea salt (i) RB1, (ii) RB3, (iii) RB4, (iv) EB1, (v) EB2, (vi) EB3, (vii) Control, (Bar=1.5 cm). (B) Changes in lipid peroxidation measured in terms of MDA content and (C) Estimation of hydrogen peroxide content in banana leaves after 7 days of treatment in hydroponic media. The treatments include non-inoculated plantlets in medium without added sea salt (C), non-inoculated plantlets in 90 mM sea salt (CS), plantlets inoculated with isolates without added sea salt (PGPB), and plantlets inoculated with isolates in 90 mM sea salt PGPBS. The values are mean ± SD (n = 10). Using ANOVA and Tukey post-hoc tests, significant differences at ($p < 0.05$) are shown by different letters.

Consistent with the findings from the hydroponic experiments, the greenhouse analysis also demonstrated that consortium pre-treated banana plants exhibited significantly lower levels of MDA (2-fold reduction) compared to the non-inoculated stressed plants (Fig. 4.15). This further confirms the role of the consortium in reducing lipid peroxidation and oxidative stress in banana plants exposed to salinity stress.

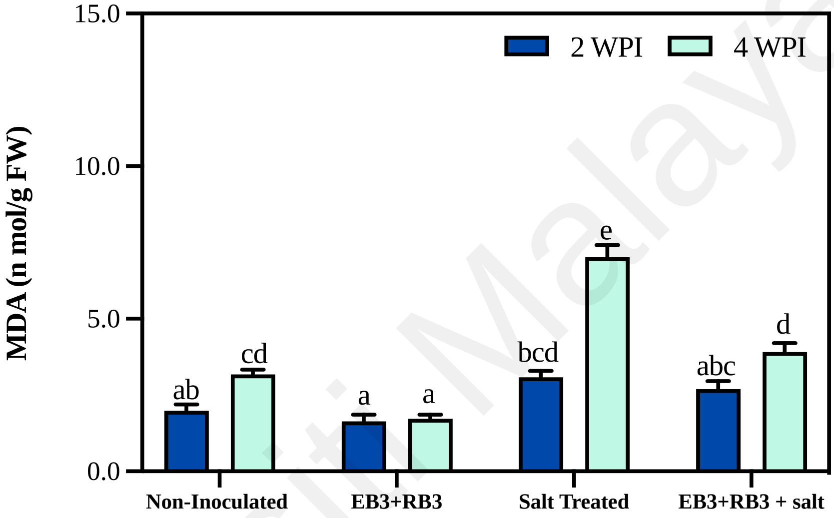


Fig. 4.15: Effect of EB3+RB3 inoculation on lipid peroxidation in plants under salt stress. Estimation of lipid peroxidation changes in banana leaves under salt stress expressed as changes in MDA content. Salt treatment was for 2 weeks and 4 weeks in soil with PGPB in 100 mM sea salt. WPI represent weeks post inoculation. Standard error is shown by error bars. The values are mean ± SD (n = 10). Significant differences at ($p < 0.05$) are shown by different letters using ANOVA and Tukey post-hoc tests.

4.7.4 PGPB Inoculation Improve Ion Content in Banana under Salt Stress

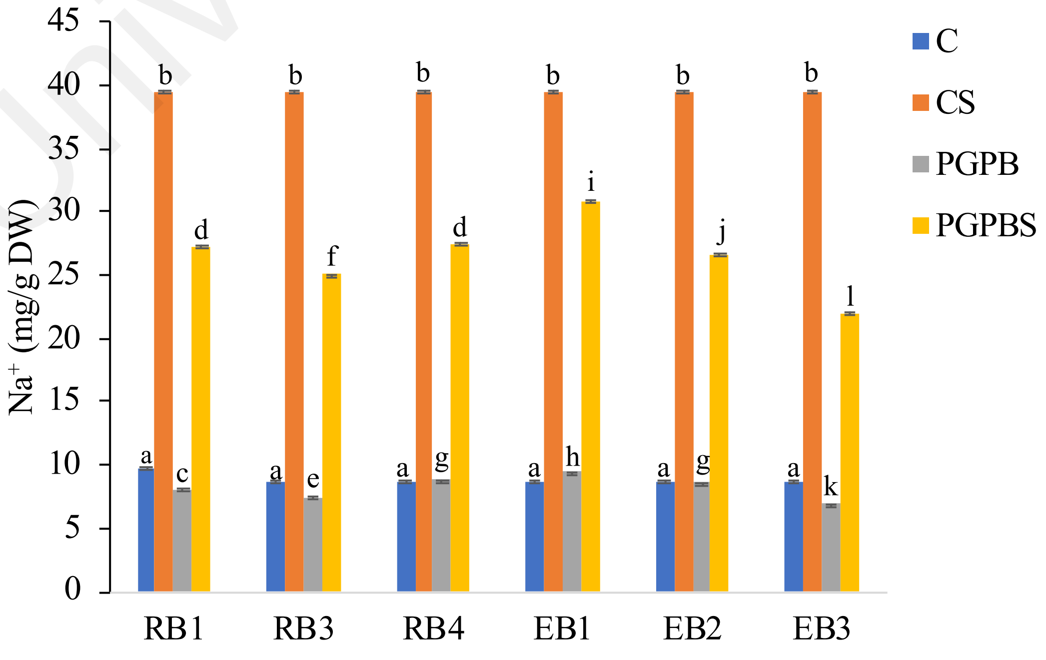
The effect of the PGPB on electrolyte leakage and ion content under salt stress in banana plants conditions was assessed in both hydroponic and greenhouse studies. Salt stress leads to alterations in the cellular ion content, affecting plant growth and development. High sodium (Na^+) content is detrimental to plant growth under salt stress.

The Na^+ concentration of the inoculated plants was significantly lower than in control plants (Fig. 4.16A). The Na^+ content in inoculated plants was 0.78-0.56-fold lower than that in the control plants, with the treatments with strain EB3 and strain RB3 having the lowest content (0.56 and 0.63-fold, respectively).

Potassium (K^+) is essential in water stress tolerance by regulating stomatal movement. Inoculated banana plants displayed higher K^+ content in both non-stressed and salt-stressed conditions, (Fig. 416B). Under salt stress, inoculated plants had K^+ contents that were 1.42–1.84 times higher than those of uninoculated plants. The treatments with strain EB3 and strain RB3 had the highest K^+ contents (1.84–fold) and 1.59–fold, respectively.

Furthermore, salt stress often leads to increased plasma membrane permeability, leading to electrolyte leakage. However, under salt stress, inoculated banana plants exhibited significantly lower electrolyte leakage than the control plants (Fig. 4.16C). Electrolyte leakage values were 0.77-0.51-fold lower in inoculated plants than in control plants, with strain RB3 and strain EB3 treatments producing the lowest values (0.51 and 0.56-fold, respectively).

(A)



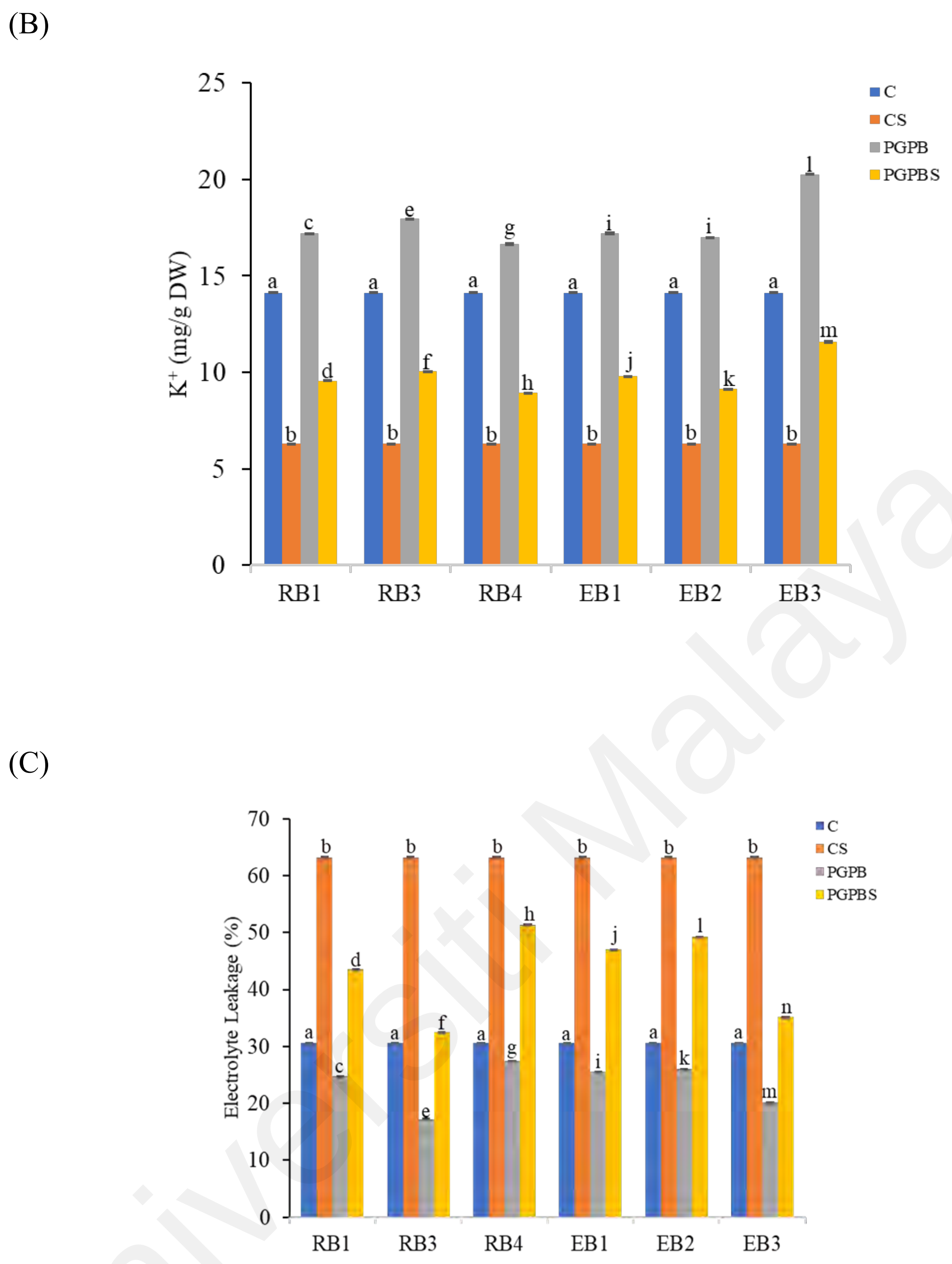
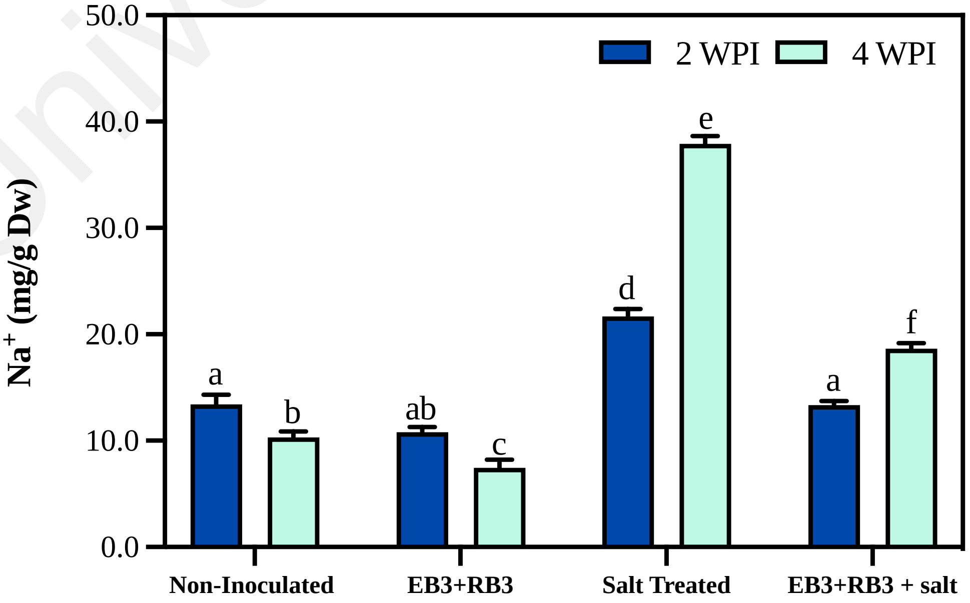


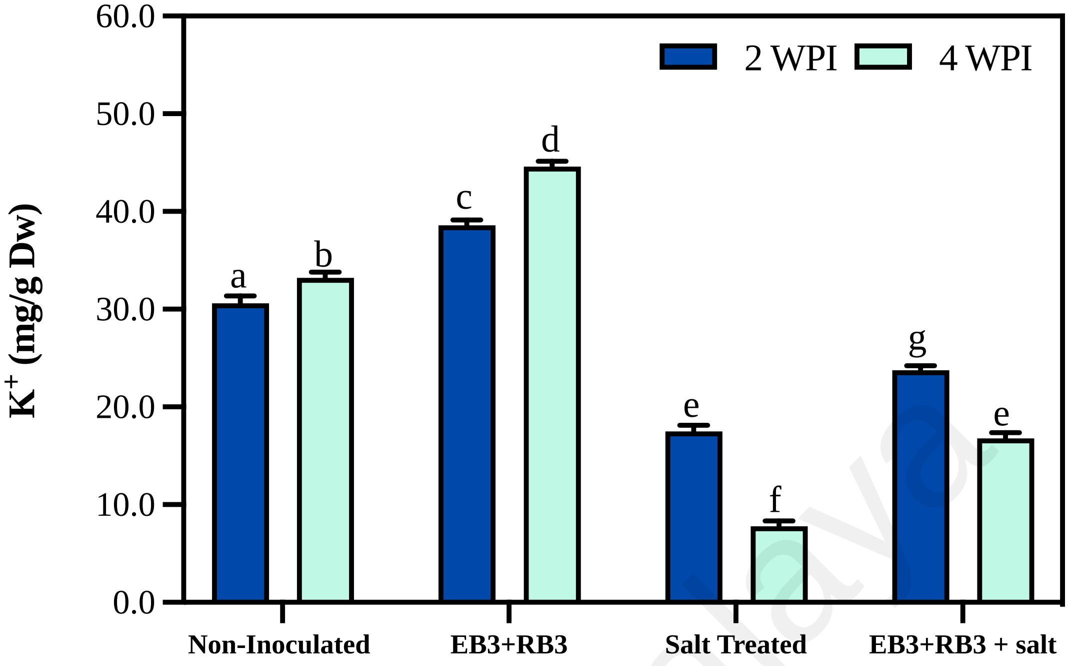
Fig. 4.16: Effect of PGPB inoculation on ion content in plants in hydroponic analysis. Assessment of (A) sodium content, (B) potassium content and (C) electrolyte leakage in leaves of banana plantlets after 7 days of treatment in hydroponic media. The treatments include non-inoculated plantlets in medium without added sea salt (C), non-inoculated plantlets in 90 mM sea salt (CS), plantlets inoculated with isolates without added sea salt (PGPB), and plantlets inoculated with isolates in 90 mM sea salt PGPBS. The values are mean ± SD (n = 10). Using ANOVA and Tukey post-hoc tests, different letters denote significant differences at ($p < 0.05$).

Consistent with the hydroponic analysis, the greenhouse study also demonstrated improvements in ion homeostasis due to the PGPB consortium pretreatment. In the absence of consortium pretreatment, banana plants subjected to salt stress exhibited elevated levels of Cl^- and Na^+ ions, coupled with decrease in concentrations of Mg^+, K^+, and Ca^{2+} ions (Fig. 4.17). This imbalance in ion content is a typical response to salinity stress. However, when the plants were pretreated with the consortium prior to salt stress, significant improvements were observed in ion homeostasis. Consortium inoculation resulted in lower levels of Na^+ (reduced by 1.9-fold) and Cl^- (reduced by 1.6-fold), while the levels of K^+ (increased by 2.2-fold), Mg^+ (increased by 1.5-fold), and Ca^{2+} (increased by 1.5-fold) were higher in comparison to non-inoculated plants under salt stress conditions. Additionally, under salt stress condition, banana plants inoculated with the consortium displayed a significantly lower electrolyte leakage (reduced by 2.1-fold) in comparison to non-inoculated plants (Fig. 4.17F).

(A)

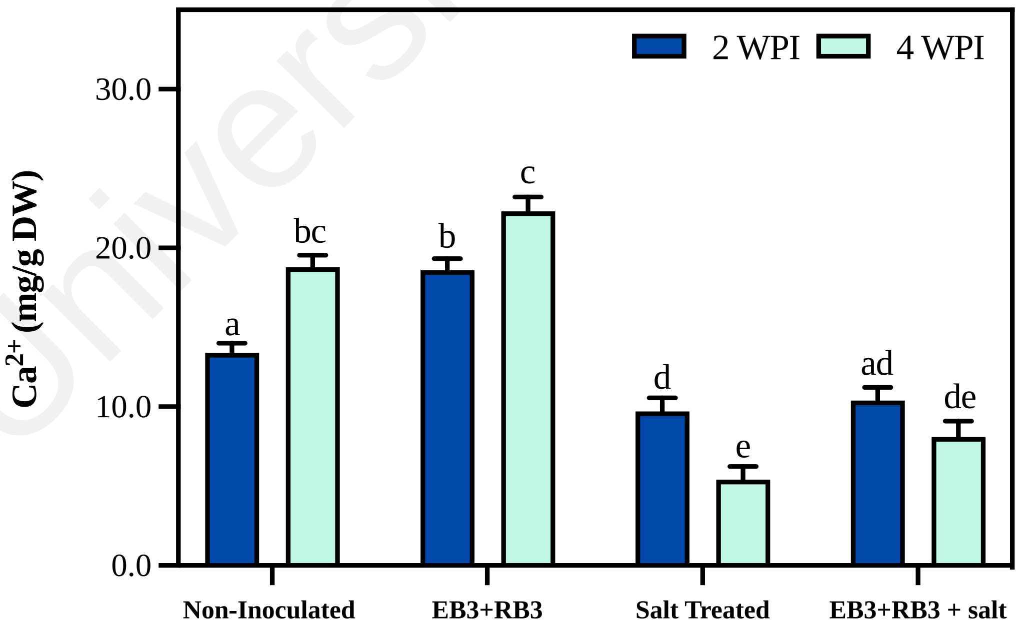


(B)



60.0
50.0
40.0
30.0
20.0
10.0
0.0
K+ (mg/g Dw)
2 WPI
4 WPI
a
b
c
d
e
f
g
e
Non-Inoculated
EB3+RB3
Salt Treated
EB3+RB3 + salt

(C)



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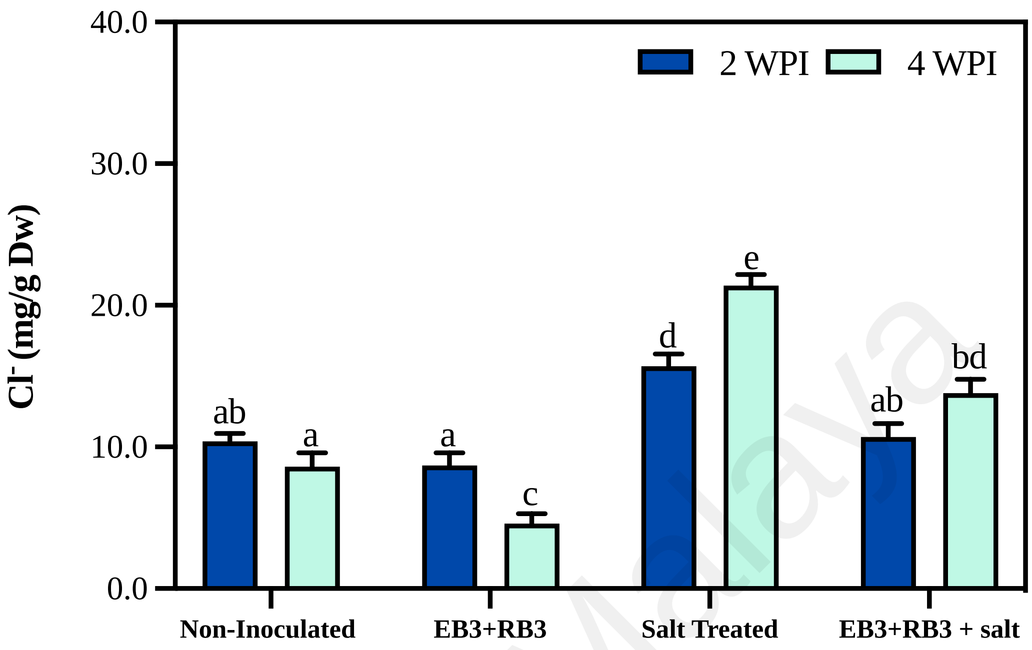
20.0
10.0
0.0
Ca2+ (mg/g DW)
2 WPI
4 WPI
a
bc
b
c
d
e
ad
de
Non-Inoculated
EB3+RB3
Salt Treated
EB3+RB3 + salt

(D)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20.0
10.0
0.0
Cl^- (mg/g Dw)
2 WPI
4 WPI
ab
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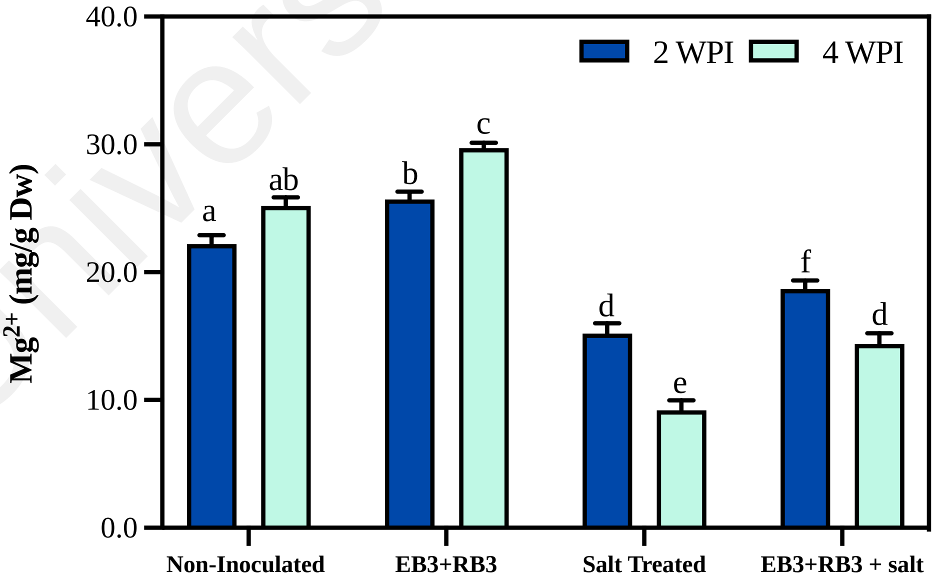
ab
bd
Non-Inoculated
EB3+RB3
Salt Treated
EB3+RB3 + salt

(E)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10.0
0.0
Mg^{2+} (mg/g Dw)
2 WPI
4 WPI
a
ab
b
c
d
e
f
d
Non-Inoculated
EB3+RB3
Salt Treated
EB3+RB3 + salt

(F)

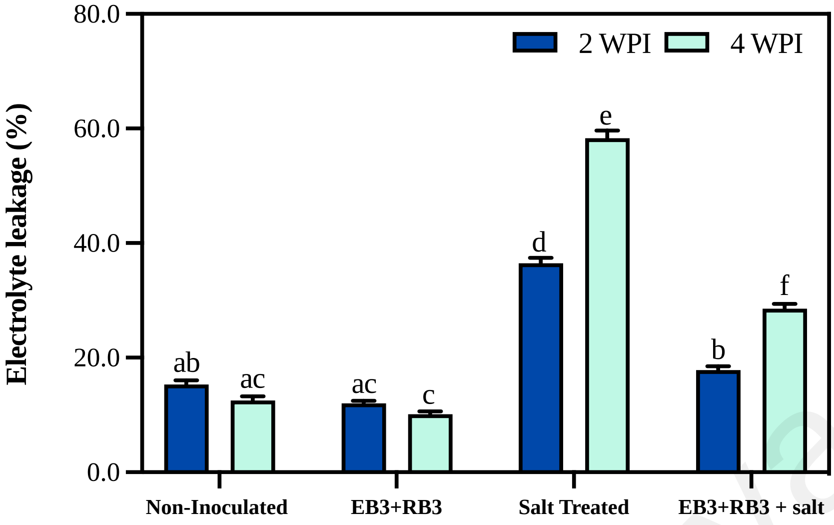


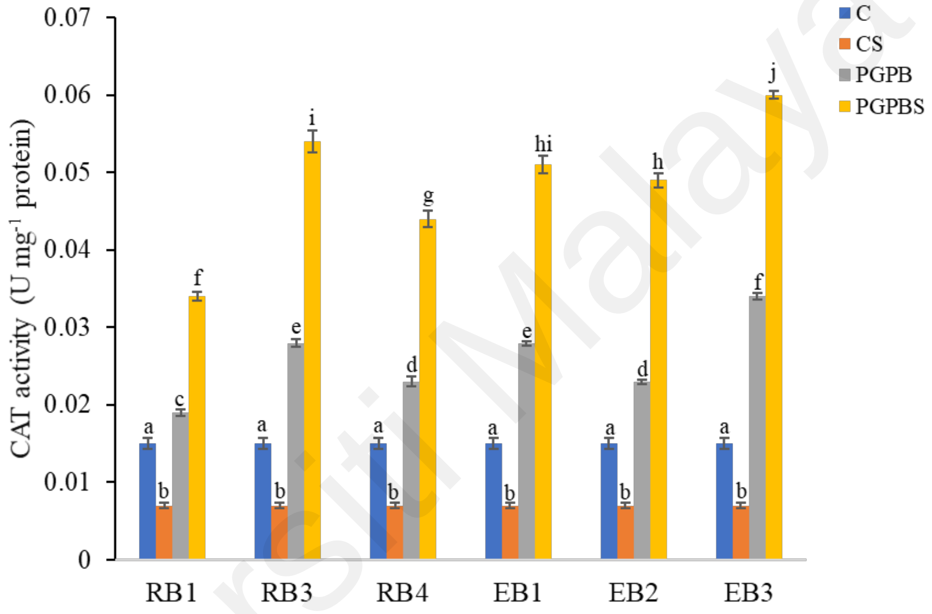
Fig. 4.17: Effect of EB3+RB3 inoculation on ion content in plants under salt stress conditions. Assessment of (A) sodium, (B) potassium, (C) calcium, (D) chlorine, (E) magnesium ion content and (F) electrolyte leakage in leaves of banana under salt stress. Salt treatment duration was for 2 weeks and 4 weeks in soil with PGPB under 100 mM sea salt. WPI represent weeks post inoculation. The standard error are denoted by error bars. The values are mean ± SD (n = 10).) Using ANOVA and Tukey post-hoc tests, different letters denote significant differences at (p < 0.05.

4.7.5 Banana Inoculated with PGPB have Higher Antioxidant Enzymes Activities under Salt Stress

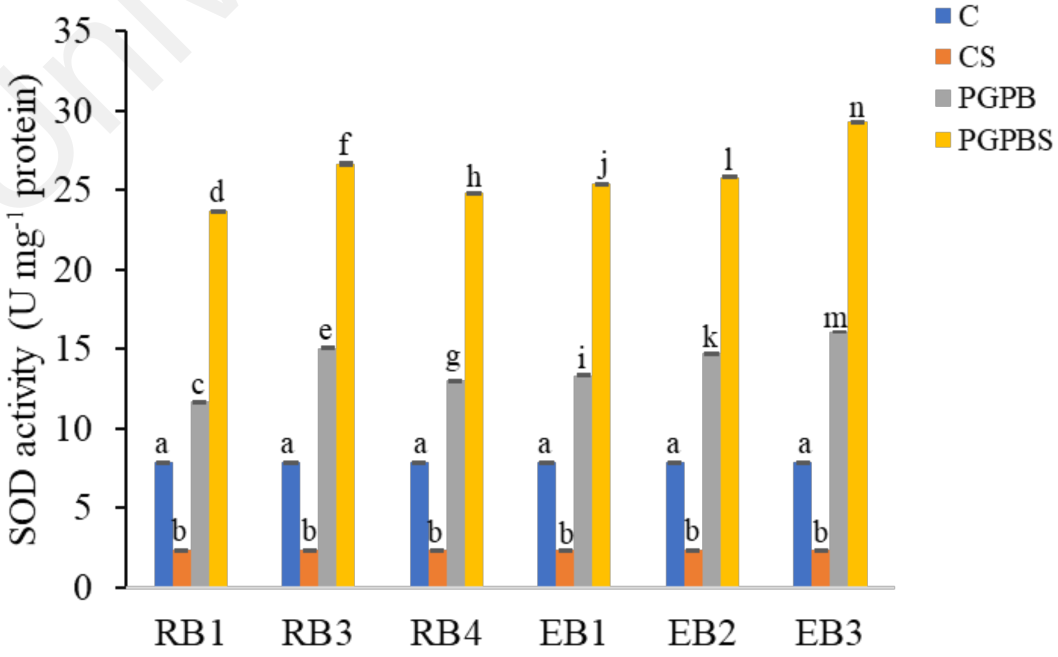
Under salt stress conditions, the antioxidant enzymes play a crucial role in mitigating the levels of ROS in plant cells in banana plants under salt stress, the effects of inoculation with PGPB strains on the levels of antioxidant enzymes (CAT, SOD, APX, and GR) were evaluated. The levels of antioxidant enzyme activities were found to be lowest in non-inoculated plants subjected to salt stress, while inoculated plants showed higher enzyme activities (Fig. 4.18). The inoculated plants had CAT activity levels that ranged from 4.8 to 8.5-fold higher than the control plants, with strain EB3 (8.5-folds) and strain RB3 (7.7-folds) showing the highest values (Fig. 4.18A). SOD activity levels were

10.1-12.5-fold higher in inoculated plants than in control plants, with EB3 (12.5-fold) and RB3 (11.3-fold) exhibiting the highest values (Fig. 4.18B). The inoculated plant's APX activity levels were 5-7.5-fold higher than the control plants, with EB3 (7.5-fold) and EB2 (7-fold) exhibiting the greatest levels (Fig. 4.18C). When compared to control plants, GR activity levels in inoculated plants were 3.4–4–fold higher, with EB3 (4–fold) and RB3 showing the greatest levels (Fig. 4.18D).

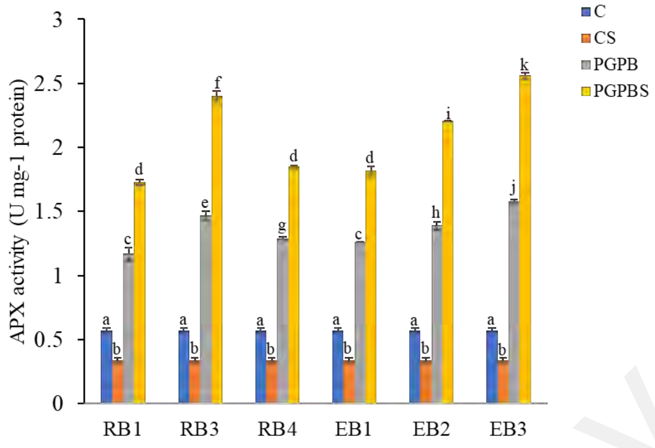
(A)



(B)



(C)



(D)

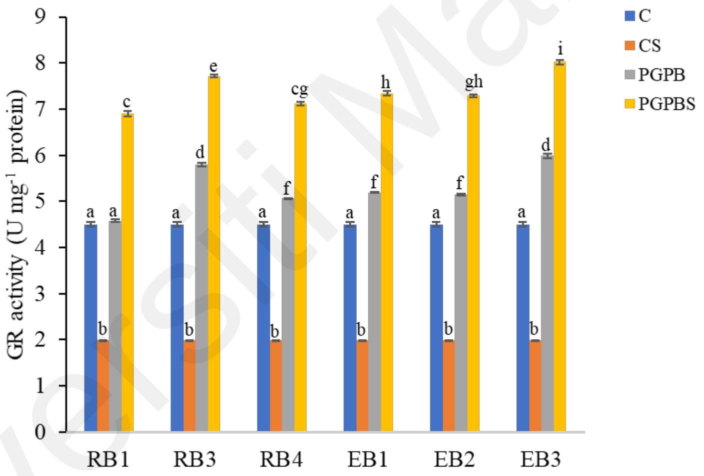
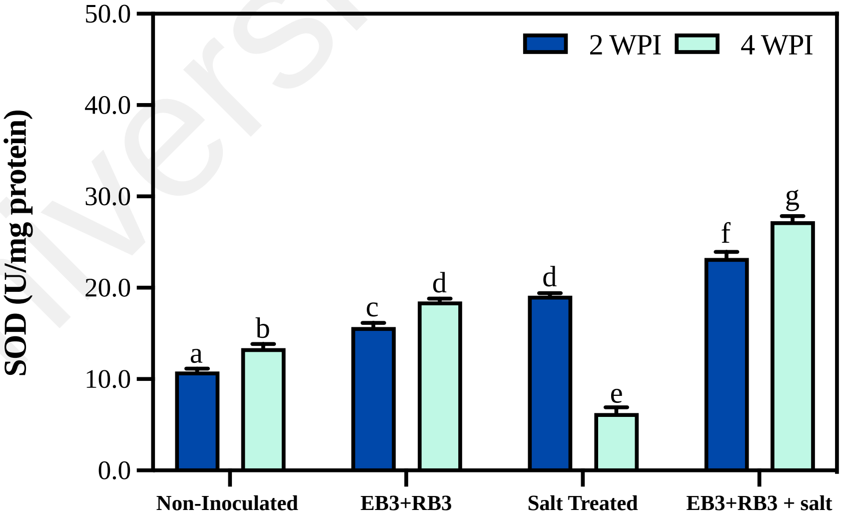


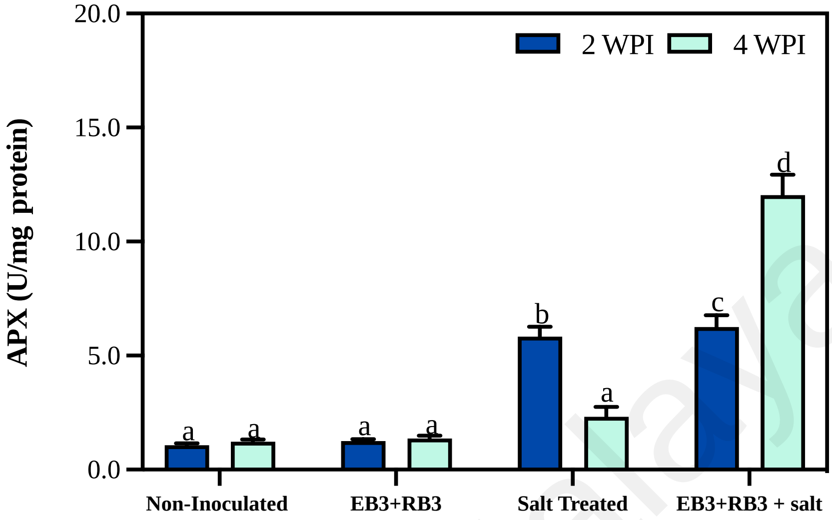
Fig. 4.18: Effect of PGPB inoculation on antioxidant enzymes activities in plants in hydroponic analysis. Activities of (A) catalase (B) superoxide dismutase (C) ascorbate peroxidase and (D) glutathione reductase in leaves of banana plantlets after 7 days of treatment in hydroponic media. The treatments include non-inoculated plantlets in medium without added sea salt (C), non-inoculated plantlets in 90 mM sea salt (CS), plantlets inoculated with isolates without added sea salt (PGPB), and plantlets inoculated with isolates in 90 mM sea salt PGPBS. The values are mean ± SD (n = 10). Different letters indicate significant differences at ($p < 0.05$) using ANOVA and Tukey post-hoc tests.

In the greenhouse analysis, both under normal and salt-stressed conditions, the levels of crucial antioxidant enzymes, such as SOD, APX, GR, POD, and CAT, were found to be significantly higher in consortium-inoculated banana plants compared to non-inoculated plants (Fig. 4.19). Notably, the consortium-inoculated plants exhibited the highest activity of antioxidant enzymes under stressful conditions. It is worth noting that enzyme activities in the non-consortium pretreated plants were relatively high at earlier time points (2 weeks) under salinity stress but decreased at later time points (4 weeks) under salt stress. In contrast, with the pretreatment of the PGPB consortium, banana plants consistently showed significantly higher antioxidant enzyme activity throughout the duration of stress.

(A)



(B)


APX (U/mg protein)
20.0
15.0
10.0
5.0
0.0
2 WPI
4 WPI
a
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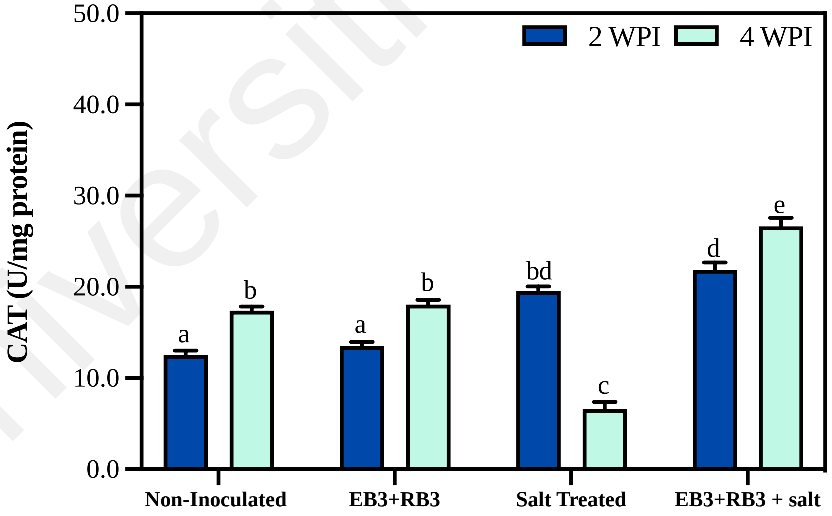
c
d
Non-Inoculated
EB3+RB3
Salt Treated
EB3+RB3 + salt

(C)


CAT (U/mg protein)
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10.0
0.0
2 WPI
4 WPI
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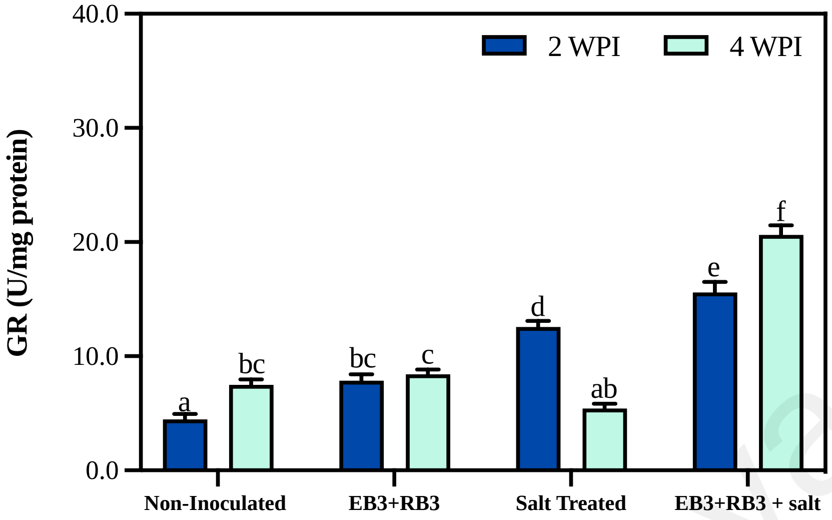
Non-Inoculated
EB3+RB3
Salt Treated
EB3+RB3 + salt

(D)



(E)

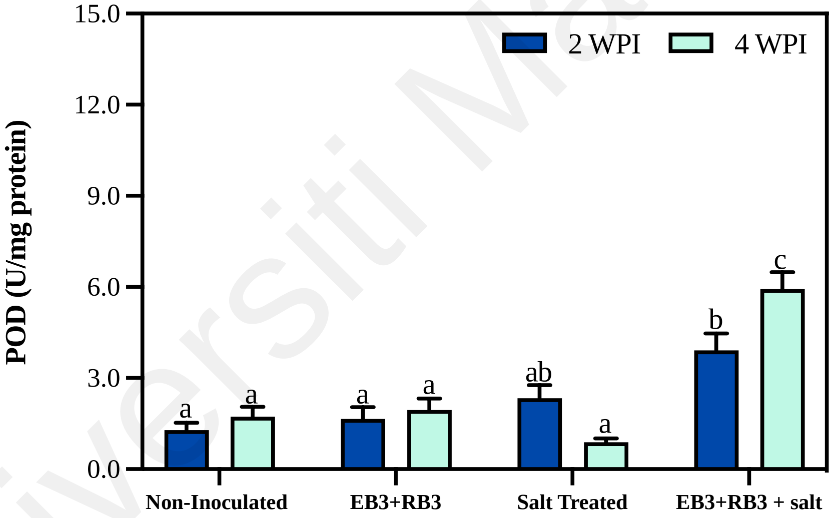


Fig. 4.19: Effect of EB3+RB3 inoculation on antioxidant enzymes activities in plants under salt stress conditions. Activities of (A) superoxide dismutase, (B) ascorbate peroxidase, (C) catalase (D) glutathione reductase and (E) peroxidase in banana leaves under salt stress. Salt treatment was for 2 weeks and 4 weeks in soil with PGPB in 100 mM sea salt. WPI represent weeks post inoculation. The standard error are shown with error bars. The values are mean ± SD (n = 10). Using ANOVA and Tukey post-hoc tests, different letters denote significant differences at ($p < 0.05$).

4.8 PGPB Consortium Inoculation Mitigates Foc-TR4 Stresses in Banana

The experiments were conducted using the two best performing PGPB isolates from hydroponic analysis (EB3 and RB3) applied as a consortium as outlined in Fig. 4.20

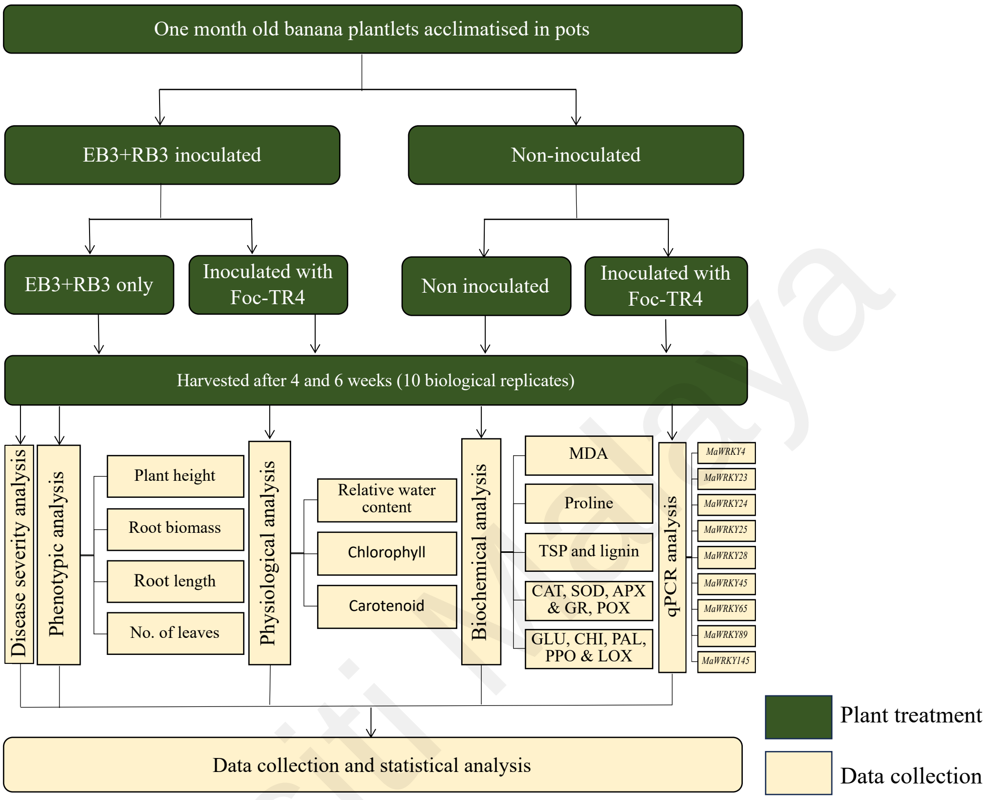


Fig. 4.20: Flowchart of experimental design for Foc-TR4 experiments. Green blocks denote plant treatment procedures, while orange blocks denote analytical processes conducted in the study.

4.8.1 PGPB Consortium have Antagonistic Activity against Foc-TR4

The halotolerant PGPB consortium consisting of *Bacillus* sp. RB3 and *Pseudomonas* sp. EB3 exhibited antagonistic activity against the fungal pathogen *Fusarium oxysporum* f. sp. *cubense* tropical race 4 (Foc-TR4). The ability of both bacterial strains to inhibit the pathogen's growth was demonstrated by the inhibition zones surrounding their colonies (Fig. 4.21). The efficacy of the consortium against Foc-TR4 was determined to be 71.4%. Among the individual strains, *Bacillus* sp. RB3 demonstrated stronger inhibition with a value of 65.7%, while *Pseudomonas* sp. EB3 had a value of 53.2%.

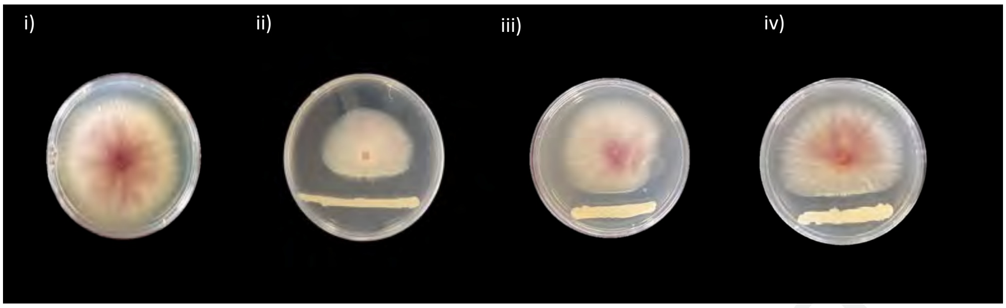


Fig. 4.21: Dual Culture Assay. Antagonistic activity of PGPB against *Fusarium oxysporum* f. sp. *cubense* tropical race 4 (Foc-TR4) in a dual culture assay (A) Foc-TR4 (control); (B) consortium of *Bacillus* sp. RB3 and *Pseudomonas* sp. against Foc-TR4 (C) *Bacillus* sp. RB3 against Foc-TR4 (D) *Pseudomonas* sp. EB3 against Foc-TR4.

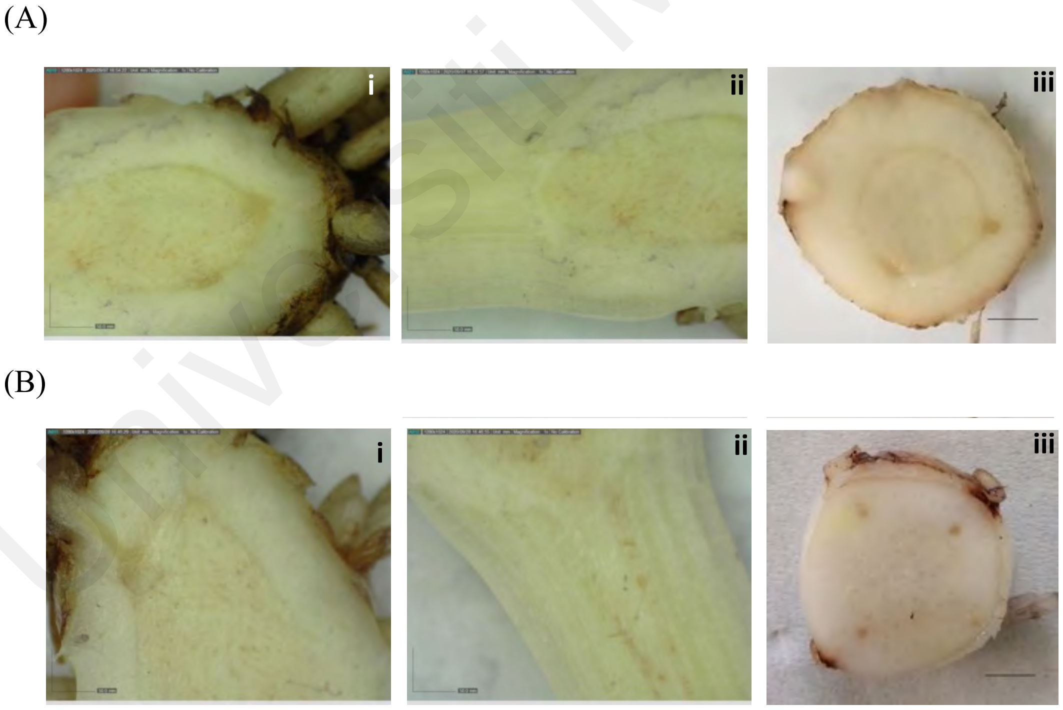
4.8.2 PGPB Consortium Reduces Fusarium Wilt Disease Severity in Banana

The impact of the EB3+RB3 consortia on growth parameters were examined in both non-Foc-TR4 inoculated and Foc-TR4 inoculated plants. Plants inoculated with Foc-TR4 in the absence of PGPB displayed characteristics disease symptoms, such as root browning, leaf yellowing, and necrosis of the root tissue at 4- or 6-weeks post inoculation (wpi) (Table 4.3, Fig. 4.22). At 4 wpi, the internal disease severity index (DSI) for the Fusarium-infected plants was 37.5% based on the extent of root tissue necrosis, with an external DSI of 25% based on the degree of wilting (Table 4.3). The Foc-TR4 infected plants showed increased DSI by 6 wpi, with an internal DSI of 75% and an external DSI of 87.5%. On the other hand, the consortium inoculated plants have a less severe effect from Foc-TR4 infection. Plants co-inoculated with Foc-TR4 in a consortium showed no exterior DSI and a milder internal DSI of 12.5% at 4 wpi (Table 4.3). With an internal DSI of 37.5% and an external DSI of 25%, these plants showed signs of minor yellowing of the leaves and lesions on the roots and stems by 6 wpi (Table 4.3).

Table 4. 3: Disease scoring of banana plants infected with *Fusarium oxysporum f. sp. cubense* tropical race 4 (Foc-TR4) at two-time points

Treatment	Percentage disease severity (%) 4 weeks Internal	4 weeks External	6 weeks Internal	6 weeks External
Non-inoculated	0^a	0^a	0^a	0^a
EB3+RB3	0^a	0^a	0^a	0^a
Foc-TR4 inoculated	37.5 ± 0.1^b	25 ± 0.1^b	75 ± 0.1^c	87.5 ± 0.1^c
EB3+RB3 + Foc-TR4	12.5 ± 0.1^a	0^a	37.5 ± 0.11^b	25 ± 0.16^b

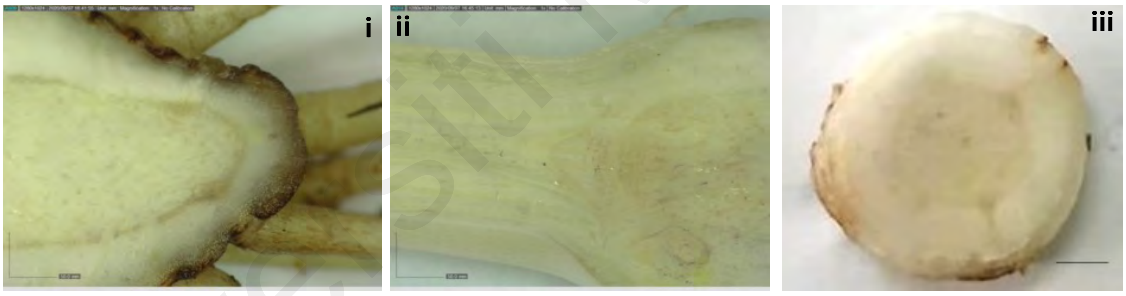
Values correspond to disease severity according to the evaluation scale of 0–4. The values are mean ± sd (n=10). Using ANOVA and Tukey post-hoc tests, different letters denote significant differences at $p < 0.05$).



(C)

(D)

(E)



(F)

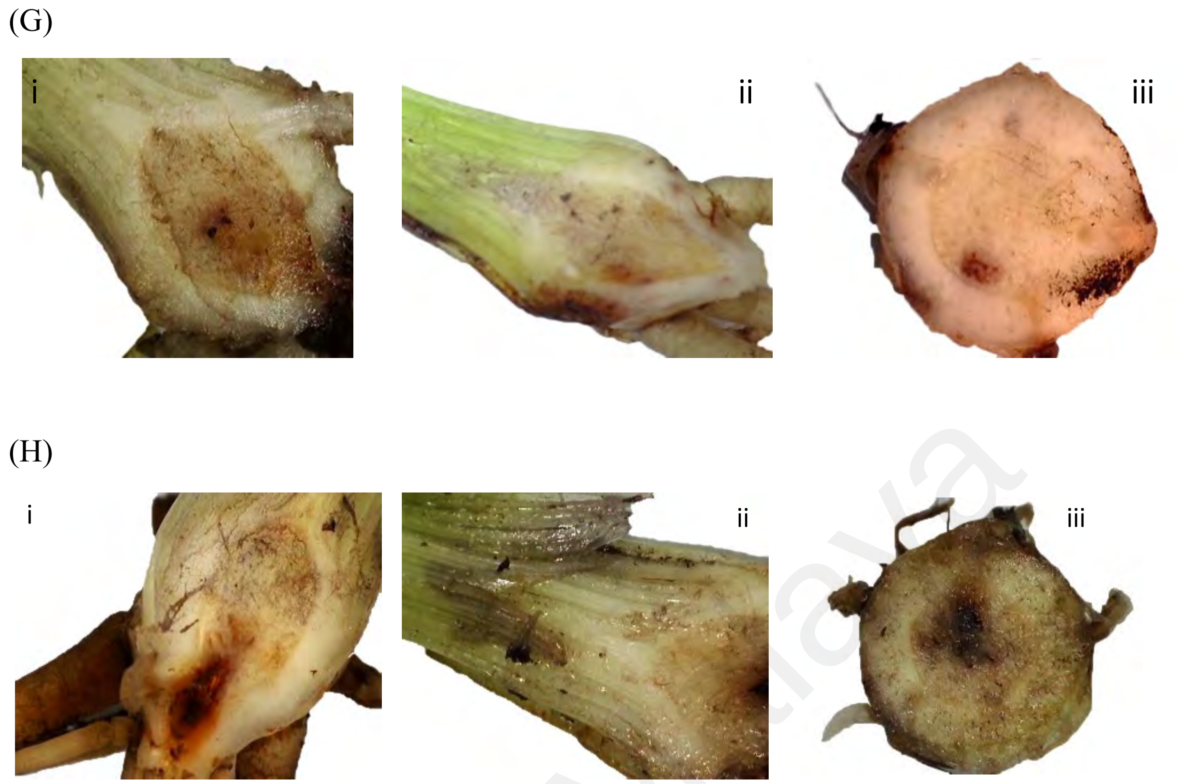
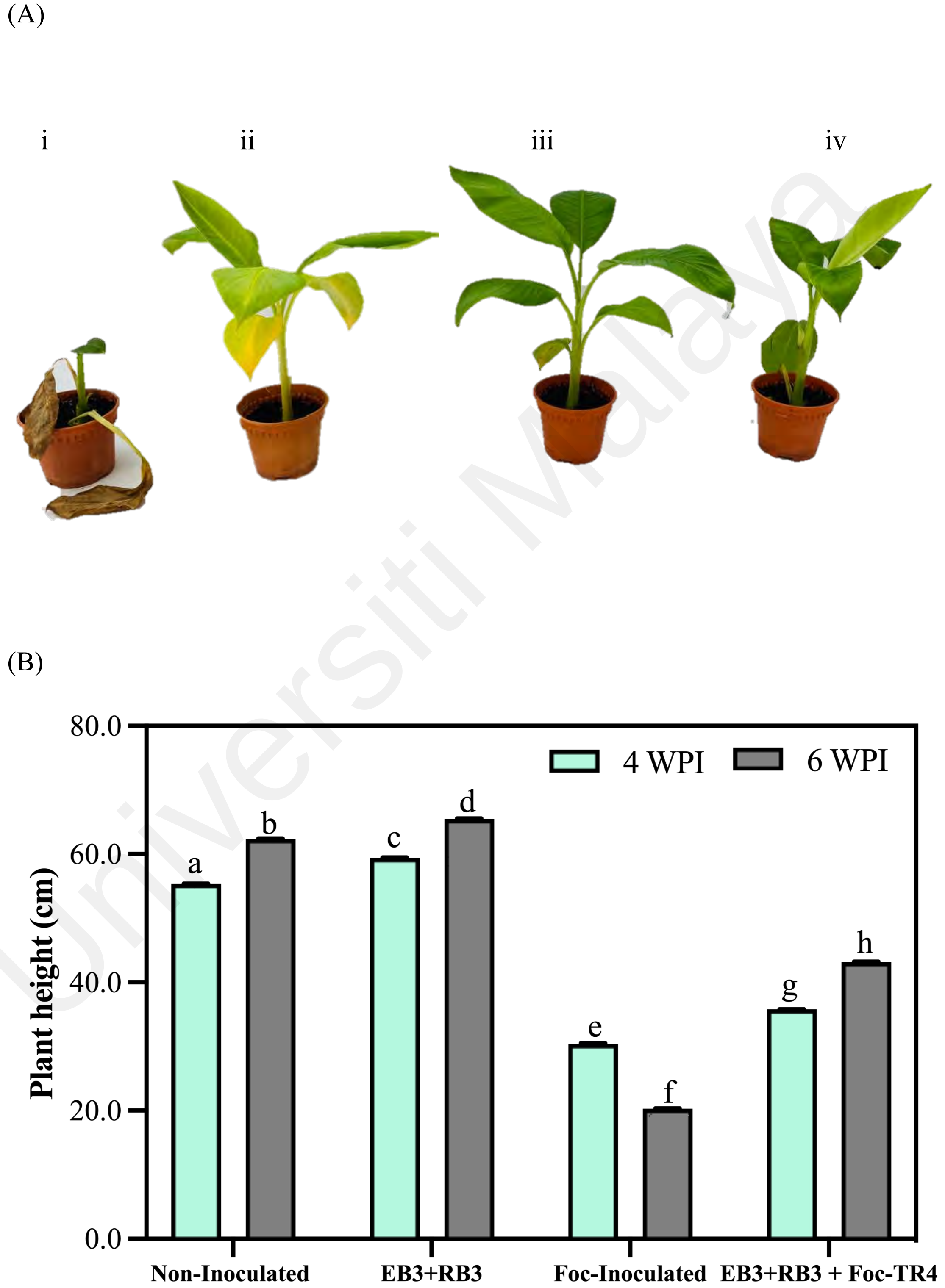


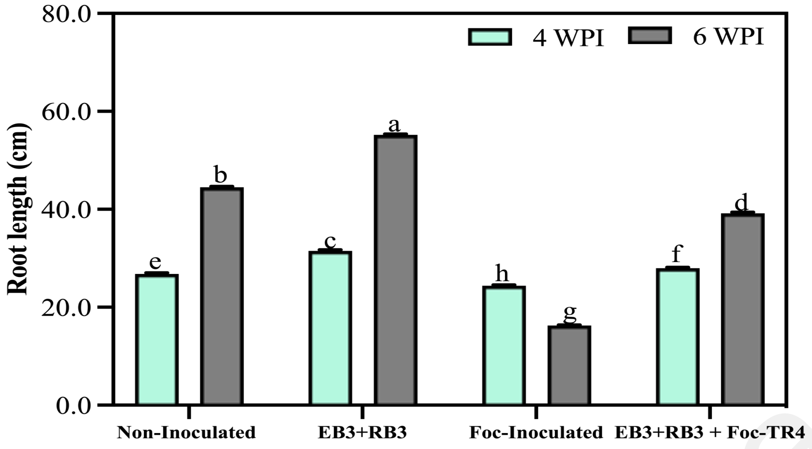
Fig. 4.22: Cross-section of plant parts under stereomicroscope. (A) 4 weeks non-inoculated, (B) 6 weeks non-inoculated, (C) 4 weeks Foc-TR4 inoculated, (D) 6 weeks Foc-TR4 inoculated; (E) 4 weeks PGPB-inoculated, (F) 6 weeks PGPB non-inoculated, (G) 4 weeks PGPB and Foc-TR4 inoculated, (H) 6 weeks PGPB and Foc-TR4 inoculated; (i) cross section of rhizome, scale bar=50 mm; ii) cross section of the stem, scale bar= 50 mm; iii) cross-section of corm, scale bar= 1 cm.

4.8.3 PGPB Consortium Enhanced Banana Growth Under Foc-TR4 Stress

The impact of Foc-TR4 stress on the growth of banana plants was evident, with notable reductions observed in root length, plant height, and root biomass (Fig. 4.23). However, the detrimental effects of Foc-TR4 stress were significantly mitigated in plants that received the PGPB consortium inoculation prior to stress treatments. Consortium-inoculated plants displayed remarkably improved growth and biomass compared to the stressed plants without consortium pre-treatment. Among the growth parameters, the most notable enhancement was observed in root length. Under Foc-TR4 stress conditions, the root length of consortium-inoculated plants was 2.4-fold longer compared to the stressed plants without consortium inoculation (Fig. 4.23C).



(C)



(D)

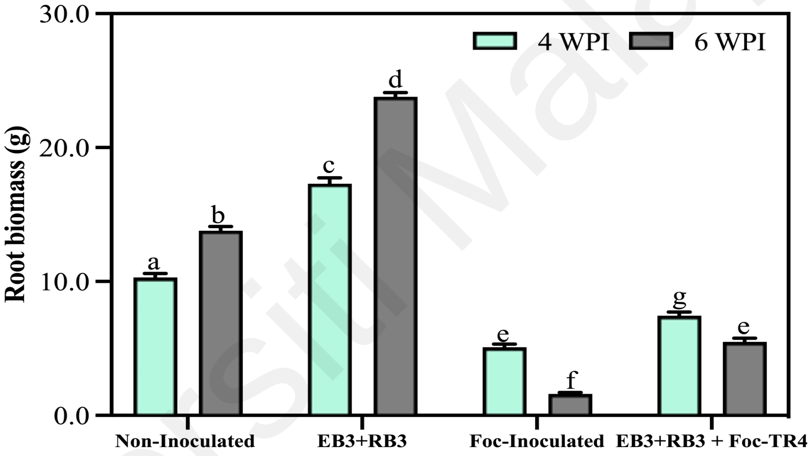
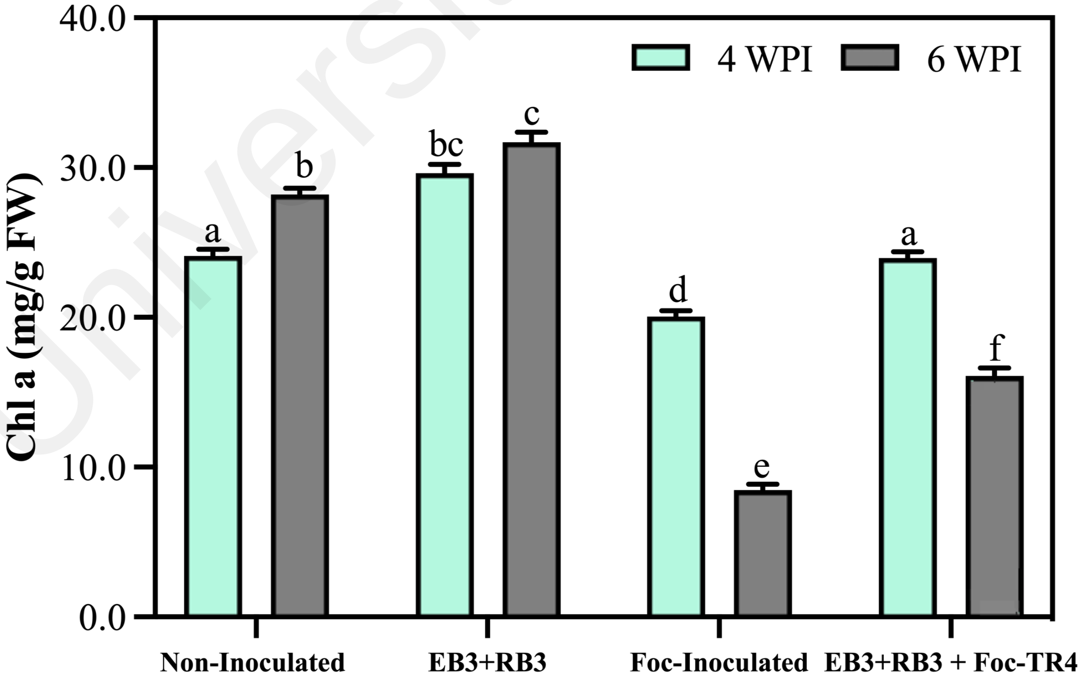


Fig. 4.23: Effect of EB3+RB3 inoculation on plant growth parameters in plants under Foc-TR4 stress conditions. (A) Picture of banana plants at six weeks: (i) Foc-TR4 inoculated only, (ii) EB3+RB3 with Foc-TR4 inoculated, (iii) EB3+RB3 inoculated only and (iv) Non-inoculated (no PGPB and no Foc-TR4). Changes in banana plant growth (B) Plant height, (C) Root length, and (D) root biomass under Foc-TR4 stress. Fusarium treatments were for 4 and 6 weeks in soil with PGPB and with Foc-TR4. WPI represent weeks post inoculation. The standard error are shown with error bars. The values are mean ± SD (n = 10). Using ANOVA and Tukey post-hoc tests, different letters shows significant differences at ($p < 0.05$).

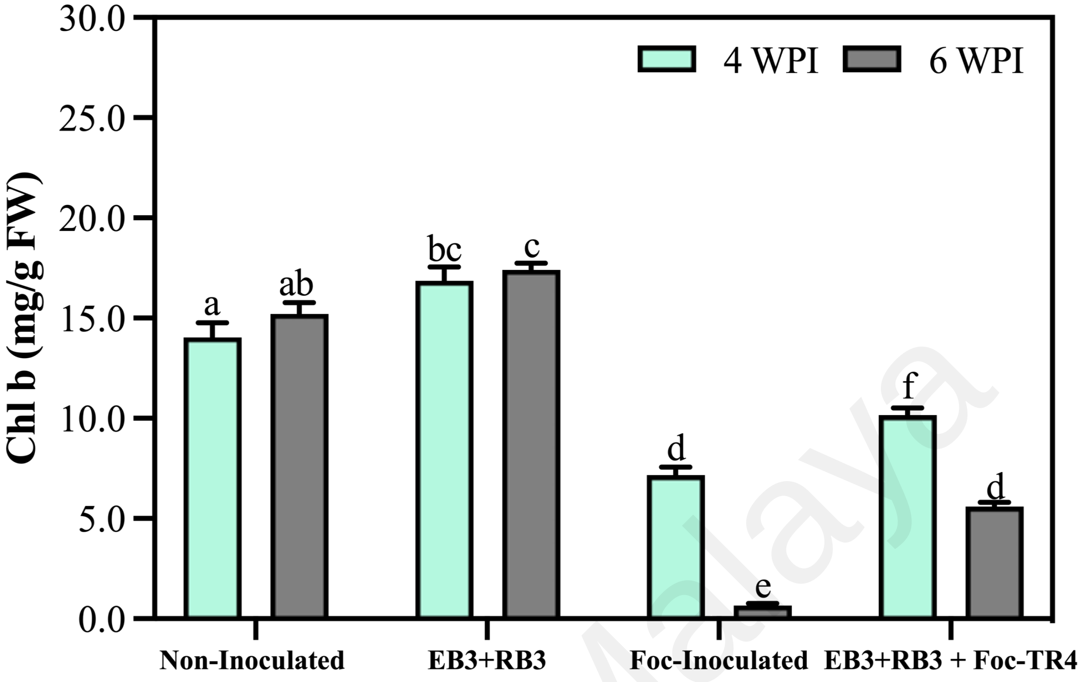
4.8.4 PGPB Consortium Improved Banana Physiological Parameters Under Foc-TR4 Stress

The impact of Fusarium inoculation on chlorophyll and carotenoid contents in banana plants was evident, with a significant decrease observed in these pigments. However, the pretreatment of banana with the PGPB consortium led to a notable improvement in pigment levels, particularly under non-stressed conditions (Fig. 4.24). In non-stressful conditions, the non-pretreated groups' levels of chlorophyll and carotenoid content were considerably lower than those of the banana plants that received pretreatment from the consortium. The most substantial increase in pigment content was observed for carotenoids, with a 9.5-fold increase in the pretreated and stressed plants. The consortium pre-treated plants showed higher relative water content in addition to pigment enhancement in both non-stressed and Foc-TR4 stress conditions (Fig. 4.24D).

(A)

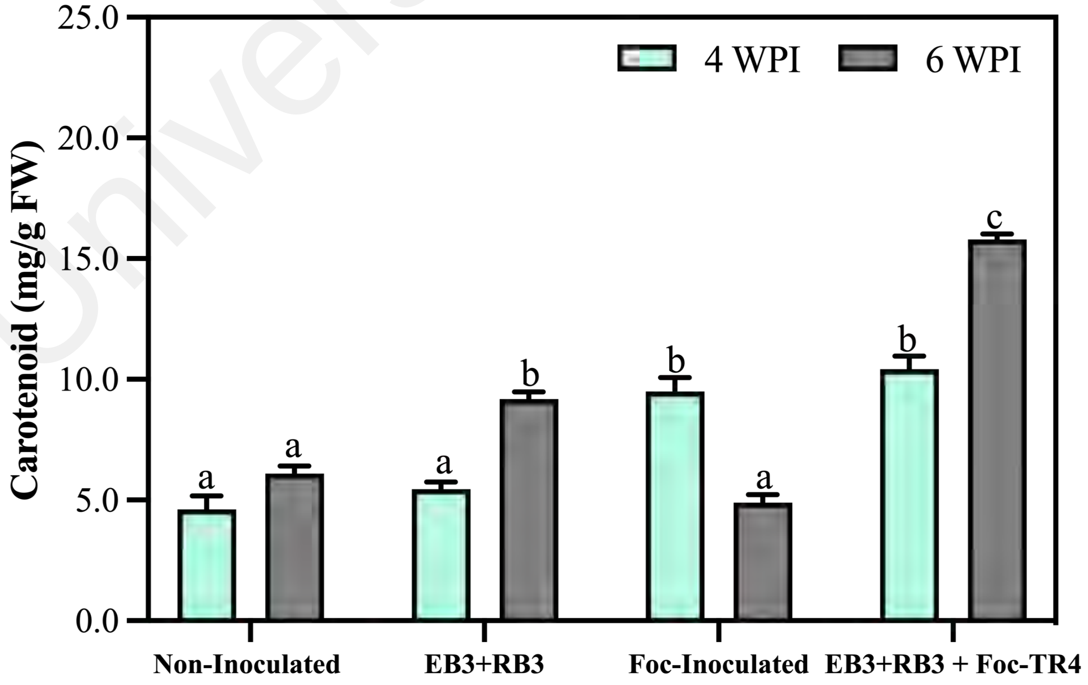


(B)



4 WPI
6 WPI
Chl b (mg/g FW)
30.0
25.0
20.0
15.0
10.0
5.0
0.0
a
ab
bc
c
d
e
f
d
Non-Inoculated
EB3+RB3
Foc-Inoculated
EB3+RB3 + Foc-TR4

(C)



4 WPI
6 WPI
Carotenoid (mg/g FW)
25.0
20.0
15.0
10.0
5.0
0.0
a
a
a
b
b
a
b
c
Non-Inoculated
EB3+RB3
Foc-Inoculated
EB3+RB3 + Foc-TR4

(D)

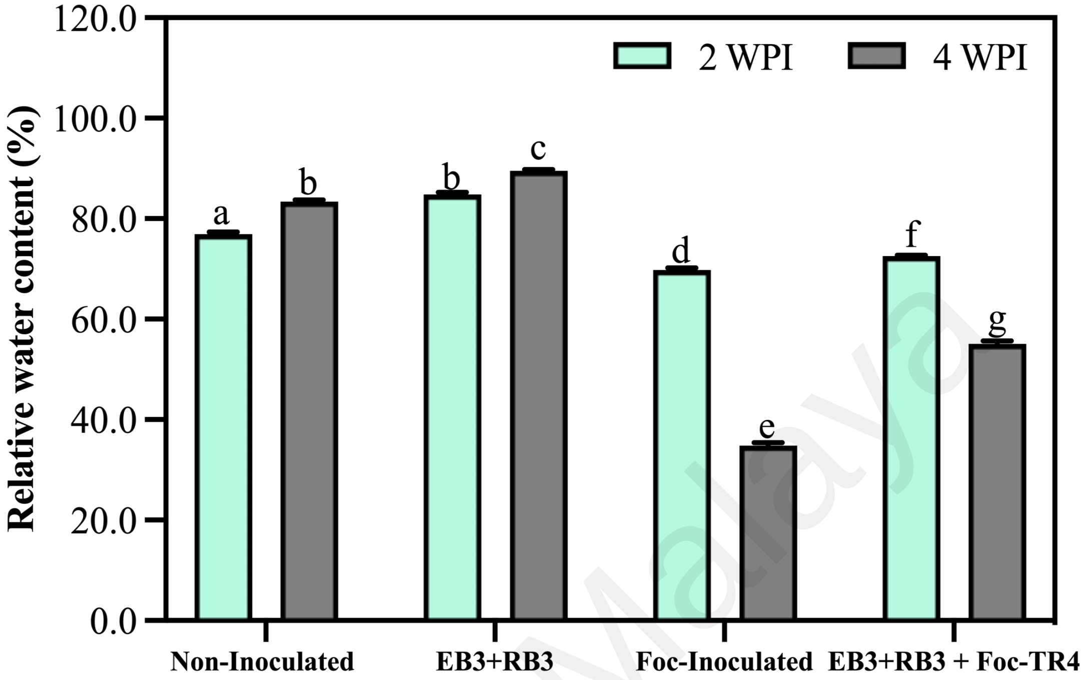


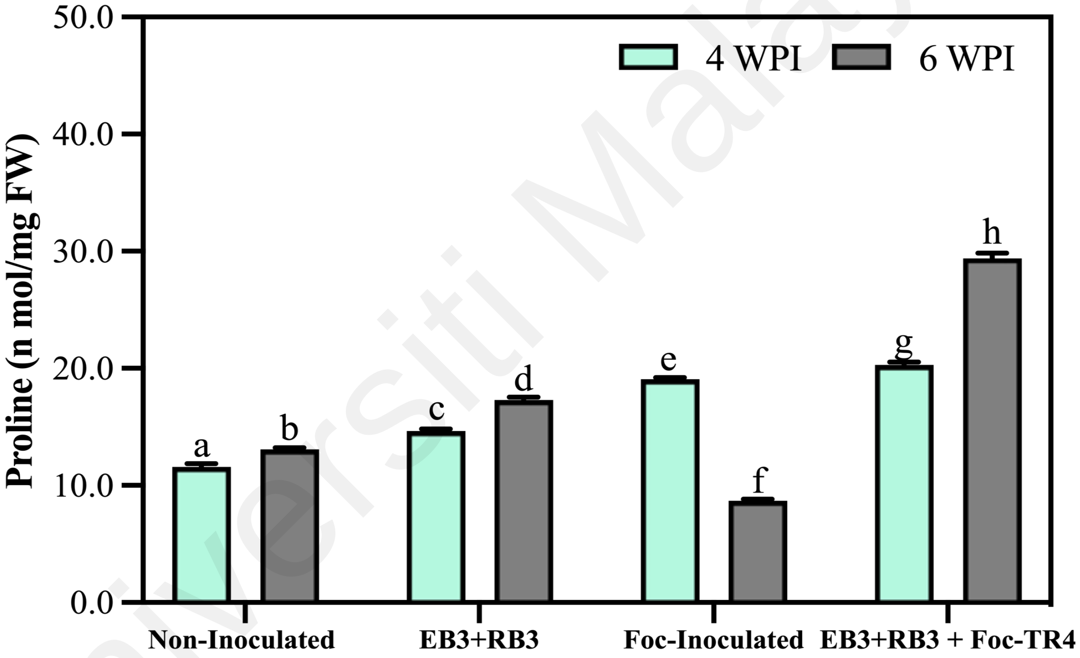
Fig. 4.24: Effect of EB3+RB3 inoculation on chlorophyll, carotenoid, and relative water content in plants under Foc-TR4 stress condition. Assessment of (A) chlorophyll a, (B) chlorophyll b, (C) carotenoid, and (D) relative water content in banana leaves under Foc-TR4 stress. Fusarium treatments were for 4 and 6 weeks in soil with PGPB and with Foc-TR4. WPI represent weeks post inoculation. The standard error is displayed by error bars. The values are mean ± SD (n = 10). Using ANOVA and Tukey post-hoc tests, different letters represent significant differences at ($p < 0.05$).

4.8.5 PGPB Consortium Enhanced Osmotic Balance and Reduced Oxidative Damage in Banana under Foc-TR4 Stress

In banana plants, the presence of Foc-TR4 stress disrupt the osmotic balance, leading to the buildup of proline, as a response to stress (Fig. 4.25A). The pretreatment of banana plants with the PGPB consortium further enhances the proline content, showing a significant increase in proline levels compared to non-inoculated plants. When compared to non-inoculated plants, consortium-inoculated plants under Foc-TR4 stress showed the largest increase in proline levels (3.5-fold higher).

Regarding lipid peroxidation, which is indicated by the production of malondialdehyde (MDA), the presence of Foc-TR4 stress leads to increased MDA levels in non-inoculated plants (Fig. 4.25B). However, in contrast, the plants that were pretreated with the PGPB consortium, exhibited a 2-fold lower levels of MDA compared to non-inoculated stressed plants.

(A)



(B)

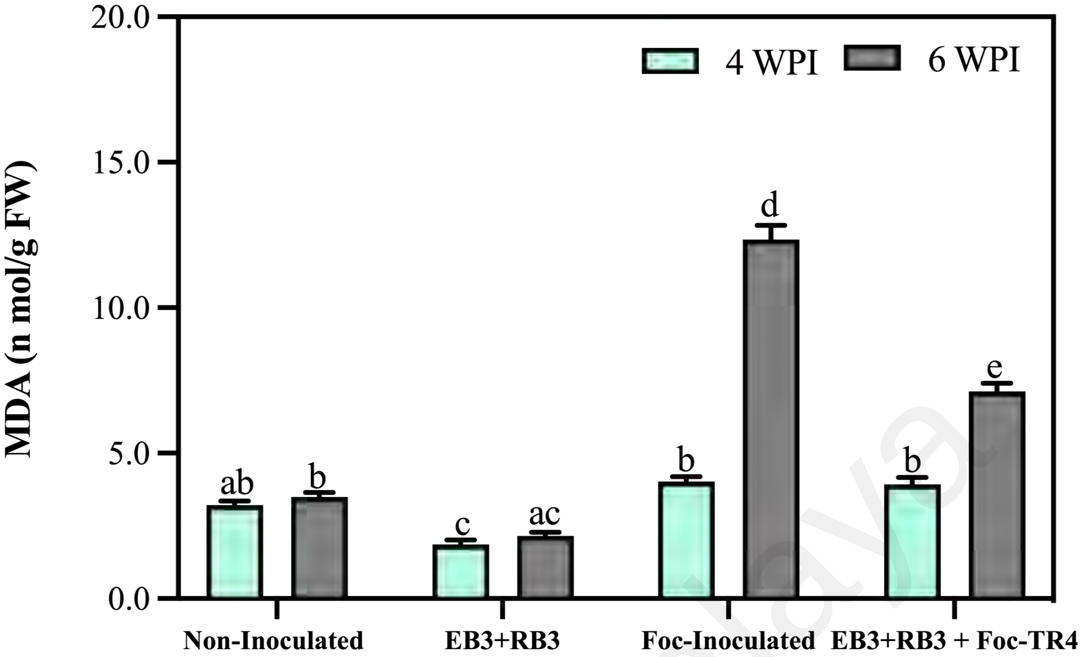


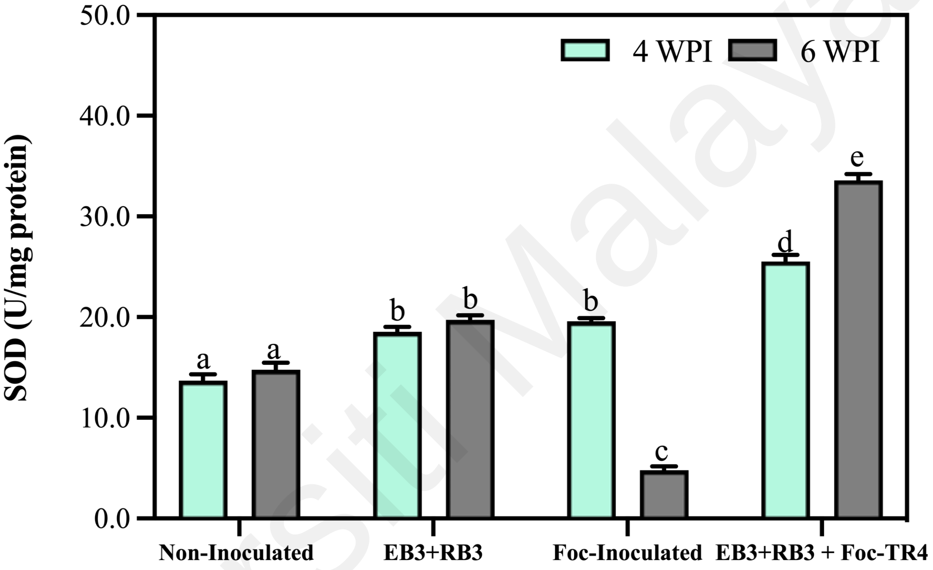
Fig. 4.25: Effect of EB3+RB3 inoculation on proline and lipid peroxidation in plants under Foc-TR4 stress. Estimation of (A) proline and (B) lipid peroxidation measured in terms of MDA content in banana leaves under Foc-TR4 stress. Fusarium treatments were for 4 and 6 weeks in soil with PGPB and with Foc-TR4. WPI represent weeks post inoculation. The standard errors are shown by error bars. The values are mean ± SD (n = 10). Using ANOVA and Tukey post-hoc tests, different letters represent significant differences at ($p < 0.05$).

4.8.6 PGPB Consortium Inoculated Banana have Higher Antioxidant Enzymes Activities under Foc-TR4 Stress

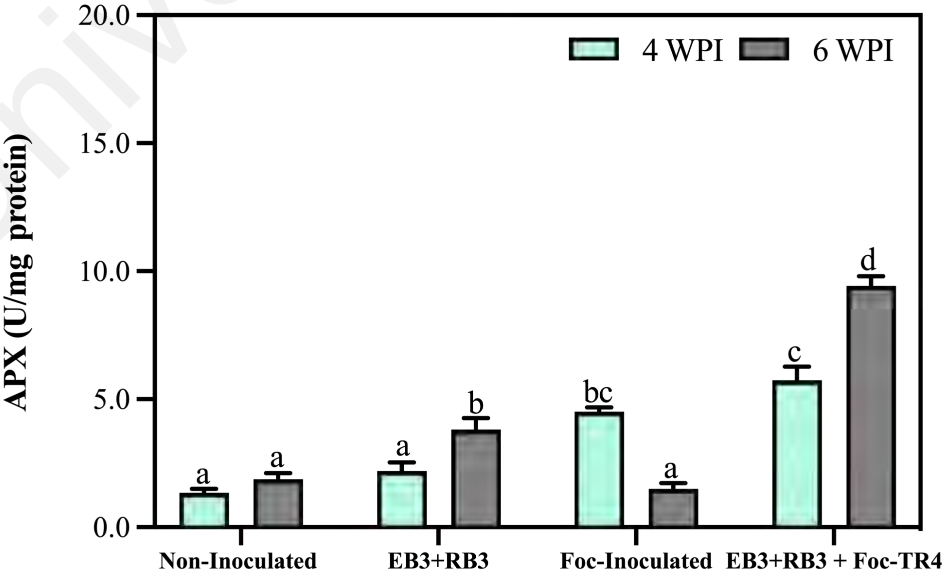
Antioxidant enzymes play a crucial role in regulating the levels of ROS in plant cells under various stress conditions, including Foc-TR4 stress. In this study, consortium-inoculated banana plants exhibited significantly higher levels of antioxidant enzyme activities including SOD, APX, GR, POD, and CAT compared to non-inoculated plants, both under non-stressed and Foc-TR4 stress conditions. Under stressed conditions, plants inoculated in consortiums exhibited the highest activity of the antioxidant enzymes (Fig.

4.26). Interestingly, it was observed that in non-pretreated plants, the enzyme activities were relatively high at earlier time points (4 weeks) under Foc-TR4 stress, but the activities decreased at later time points (6 weeks). Nonetheless, banana plants continuously shown noticeably increased antioxidant enzyme activity with the pretreatment for the whole of the stress period (Fig. 4.26).

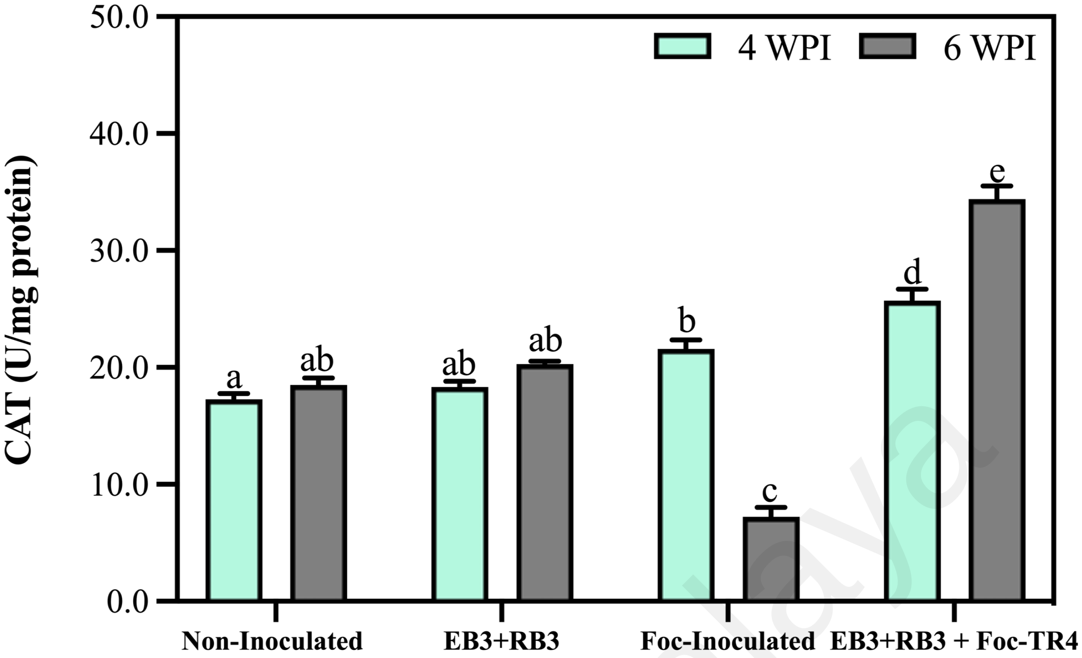
(A)



(B)

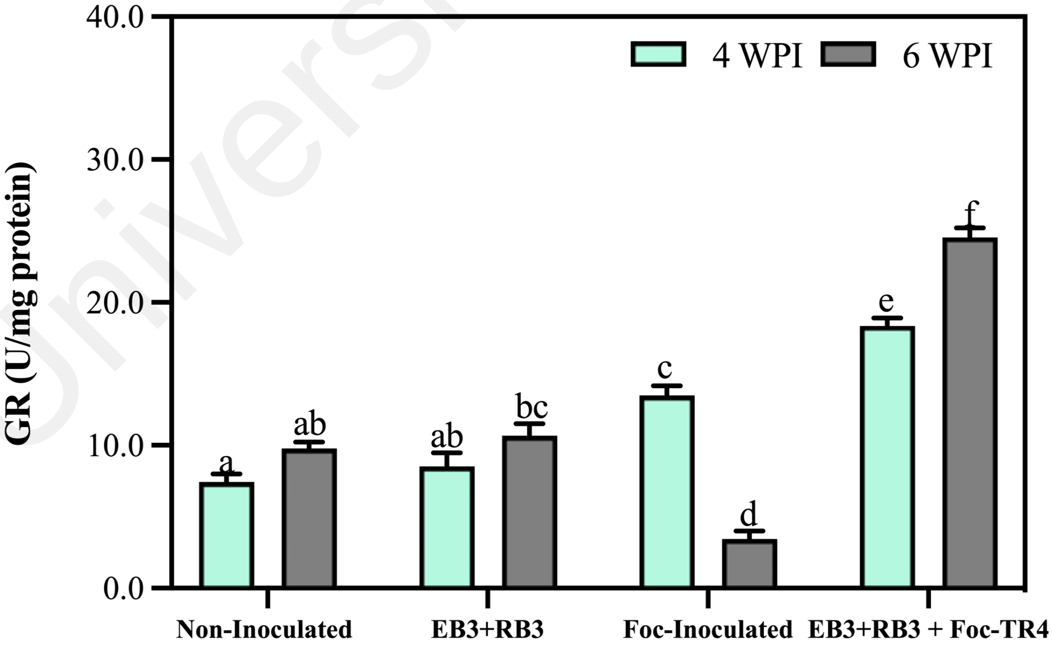


(C)



50.0
40.0
30.0
20.0
10.0
0.0
CAT (U/mg protein)
4 WPI
6 WPI
a
ab
ab
ab
b
c
d
e
Non-Inoculated
EB3+RB3
Foc-Inoculated
EB3+RB3 + Foc-TR4

(D)



40.0
30.0
20.0
10.0
0.0
GR (U/mg protein)
4 WPI
6 WPI
a
ab
ab
bc
c
d
e
f
Non-Inoculated
EB3+RB3
Foc-Inoculated
EB3+RB3 + Foc-TR4

(E)

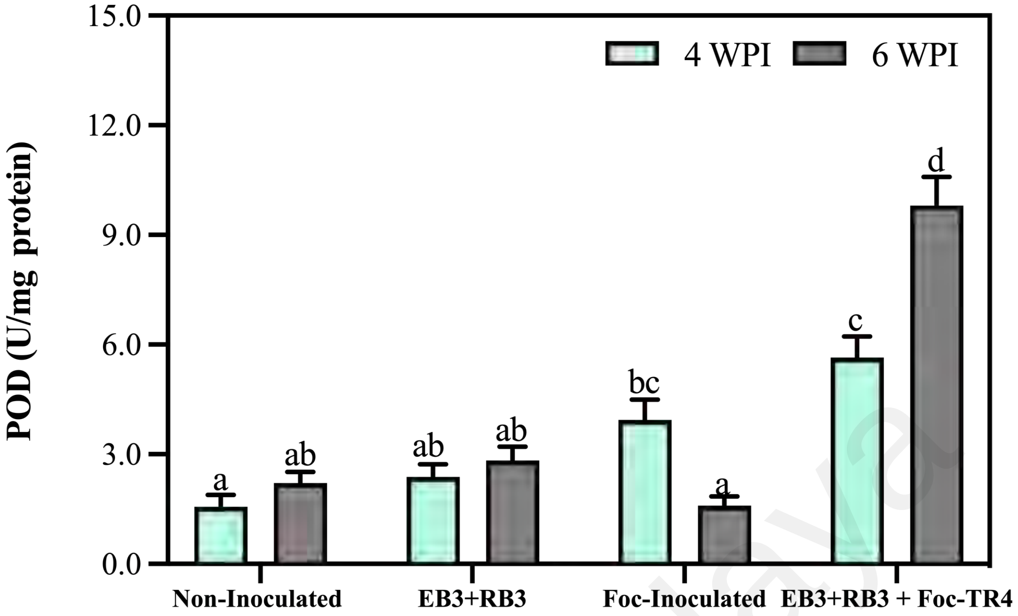


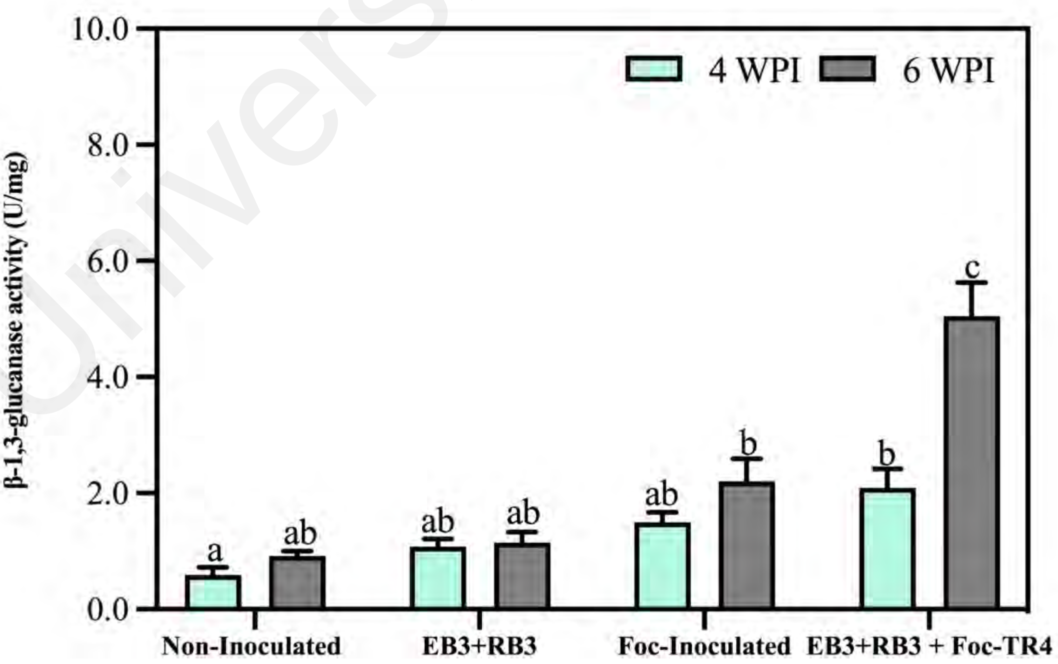
Fig. 4.26: Effect of EB3+RB3 inoculation on antioxidant enzymes activities in plants under Foc-TR4 stress. Activities of (A) superoxide dismutase, (B) ascorbate peroxidase, (C) catalase (D) glutathione reductase and (E) peroxidase in banana leaves under Foc-TR4 stress. Fusarium treatments were for 4 and 6 weeks in soil with PGPB and with Foc-TR4. WPI represent weeks post inoculation. The standard error are shown by error bars. The values are mean ± SD (n = 10). Using ANOVA and Tukey post-hoc tests, different letters represent significant differences at ($p < 0.05$).

4.8.7 PGPB Consortium Enhanced Banana Defense-Related Enzyme Activities, TSP and LTGA Under Foc-TR4 Stress

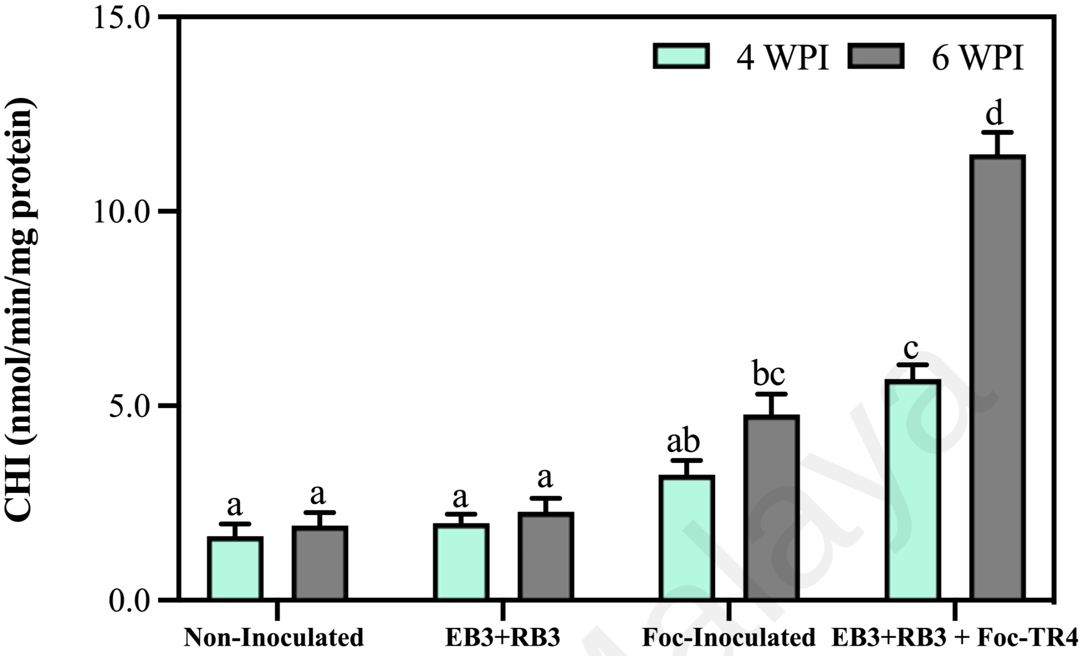
The pretreatment of banana plants with the EB3+RB3 consortium had a significant impact on defense-related enzyme activities, and the levels of total soluble phenolics (TSP) and lignin-like total phenolics (LTGA), particularly in the context of Foc-TR4 inoculation. In the experiment involving non-inoculated plants, the consortium-inoculated plants exhibited significantly higher levels of defense-related enzymes, including PPO, CHI, PAL, GLU, and LOX, compared to non-inoculated plants at both 4 and 6 weeks (Fig. 4.27). Moreover, in comparison to plants lacking consortium or Foc-TR4 inoculation, the non-inoculated plants showed increased levels of defense-related enzyme activity when they were later challenged with Foc-TR4. However, the consortium-inoculated plants that were then subsequently challenged with Foc-TR4 exhibited the highest levels of defense-related enzymes among all the groups at both 4- and 6-wpi.

Regarding phenolic compounds and lignin, the pretreatment with the PGPB consortium did not induce significant changes in their levels when plants were not inoculated with Foc-TR4. However, when the consortium-pretreated plants were subsequently inoculated with Foc-TR4, there was a substantial increase in TSP levels (2.1-fold) and LTGA levels (1.2-fold) compared to plants inoculated with Foc-TR4 alone (Fig. 4.28, A and B).

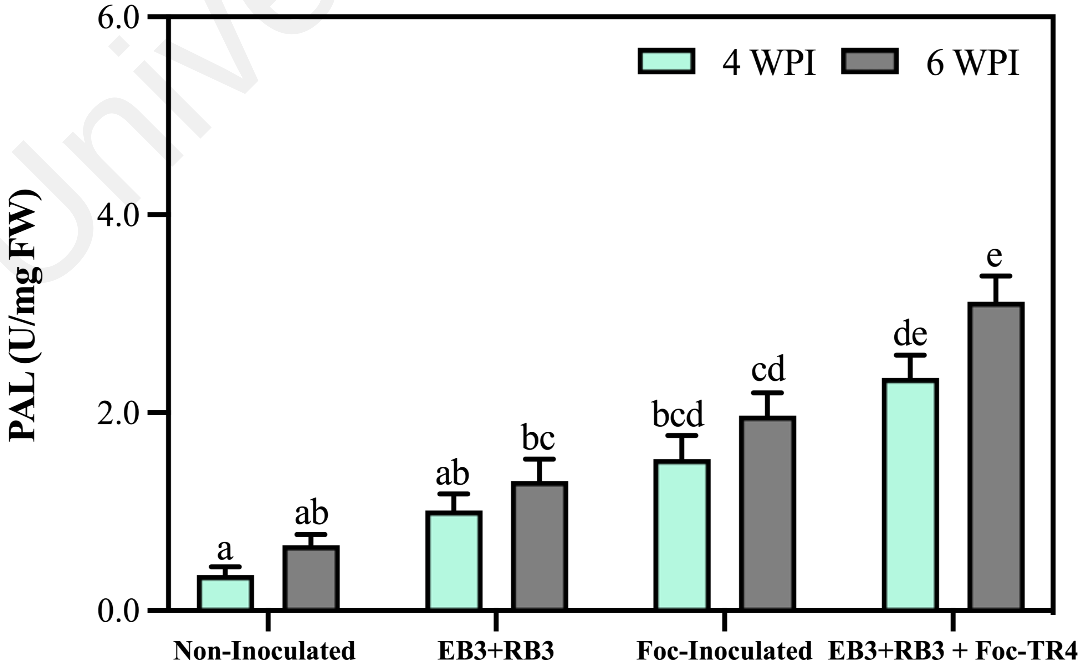
(A)



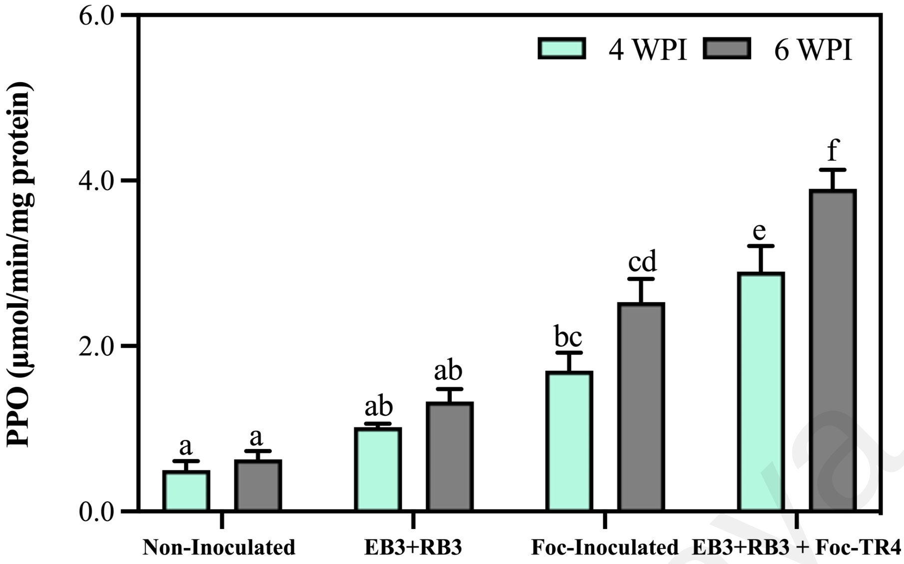
(B)


4 WPI
6 WPI
CHI (nmol/min/mg protein)
15.0
10.0
5.0
0.0
a
a
a
a
ab
bc
c
d
Non-Inoculated
EB3+RB3
Foc-Inoculated
EB3+RB3 + Foc-TR4

(C)


4 WPI
6 WPI
PAL (U/mg FW)
6.0
4.0
2.0
0.0
a
ab
ab
bc
bcd
cd
de
e
Non-Inoculated
EB3+RB3
Foc-Inoculated
EB3+RB3 + Foc-TR4

(D)



(E)

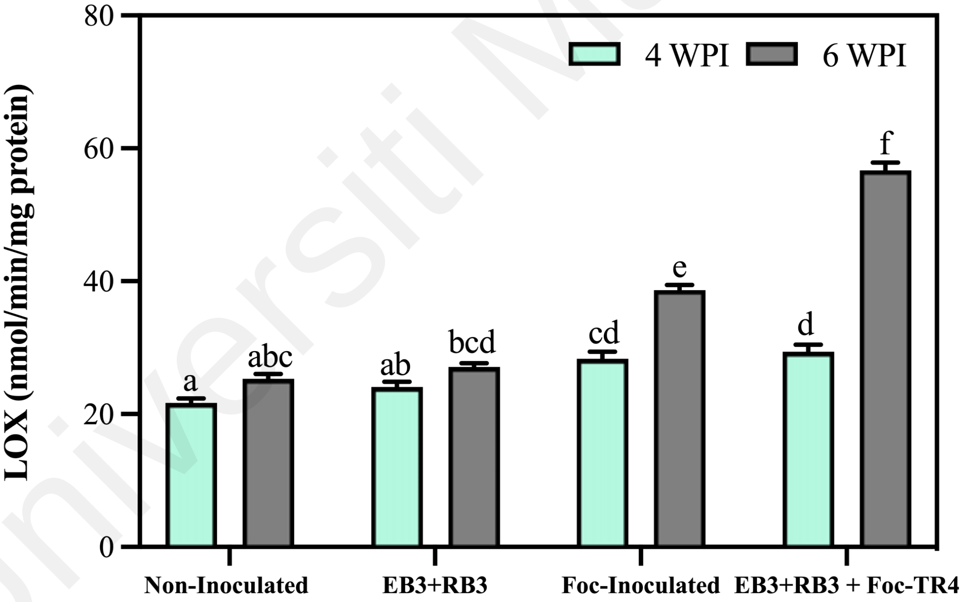
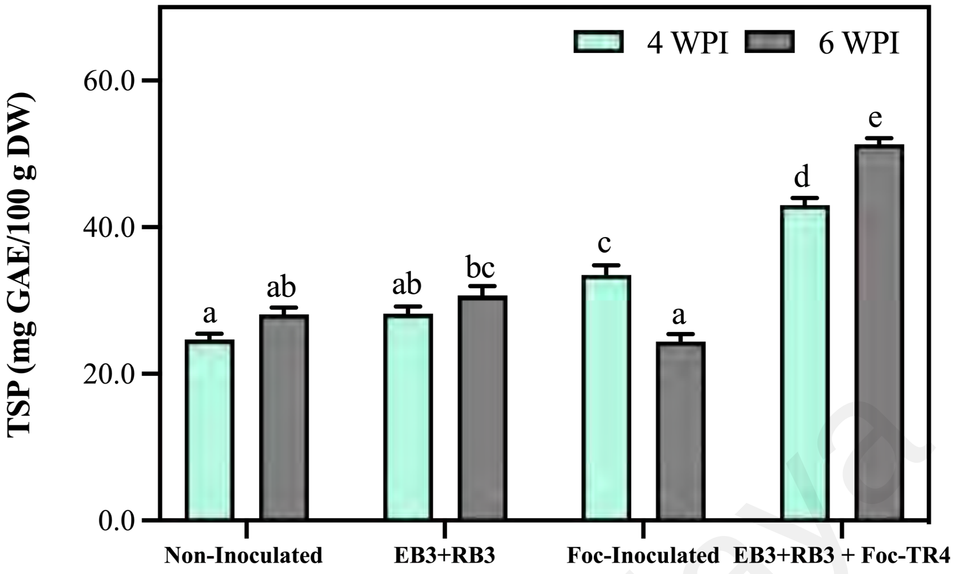


Fig. 4.27: Effect of EB3+RB3 inoculation on defense enzymes activities in plants under Foc-TR4 stress. Activities of (A) glucanase, (B) chitinase, (C) phenyl ammonia lyase, (D) polyphenol oxidase and (E) lipoxygenase in banana leaves under Foc-TR4 stress. Fusarium treatments were for 4 and 6 weeks in soil with PGPB and with Foc-TR4. WPI represent weeks post inoculation. The standard errors are shown by the error bars. The values are mean ± SD (n = 10). Using ANOVA and Tukey post-hoc tests, different letters represent significant differences at ($p < 0.05$).

(A)



(B)

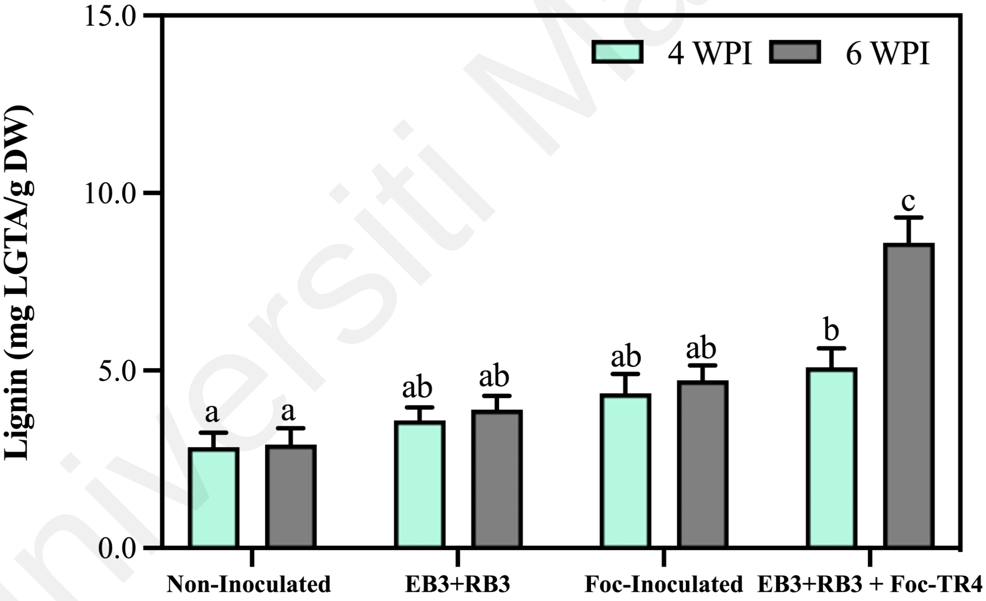
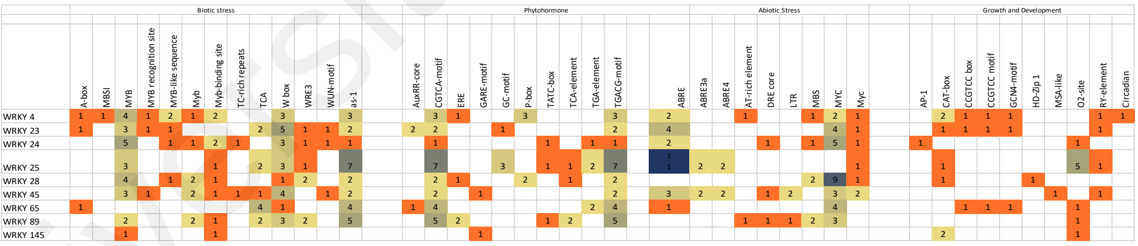


Fig. 4.28: Effect of EB3+RB3 inoculation on total soluble phenolics and lignin content in plants under Foc-TR4 stress. Estimation of (A) total soluble phenolics and (B) lignin content, in leaves of banana under Foc-TR4 stress. Fusarium treatments were for 4 and 6 weeks in soil with PGPB and with Foc-TR4. WPI represent weeks post inoculation. . The standard errors are denoted by the error bars.. The values are mean ± SD (n = 10). Using ANOVA and Tukey post-hoc tests, different letters represent significant differences at ($p < 0.05$).

4.9 Cis-Elements of Banana WRKY Genes

A total of 35 cis-regulatory elements were identified in the promoters of nine selected banana WRKY genes (Fig. 4.29). These elements were categorized into four main groups based on their functions: abiotic stress-responsive, biotic stress-responsive, growth and development-responsive and phytohormone-responsive. Abiotic stress-responsive cis-elements: LTR, MBS, AT-rich element, DRE core, ABRE, and MYC. Biotic stress-responsive cis-elements: MYB, W-box, as-1, MBSI, A-box, WUN-motif, TCA, Tc-rich repeats, STRE and WRE3. Phytohormone-responsive cis-elements: ERE, CCTCA-motif, AuxRR-core, GC-motif, GARE-motif, P-box, TCA element, TGA-element, TATC-box, and TGACG-motif. Growth and development-responsive cis-elements: MSA-like, CCGTC-box, HD-Zip 1, AP-1, RY-element, CAT-box, CGN4-motif, O2-site, and Circadian.



	Biotic stress													Phytohormone										
	A-box	MBSI	MYB	MYB recognition site	MYB-like sequence	Myb	Myb-binding site	TC-rich repeats	TCA	W box	WRE3	WUN-motif	as-1	AuxRR-core	CGTCA-motif	ERE	GARE-motif	GC-motif	P-box	TATC-box	TCA-element	TGA-element	TGACG-motif	ABRE
WRKY 4	1	1	4	1	2	1	2			3			3		3	1			3				3	2
WRKY 23	1		3	1	1				2	5	1	1	2	2	2			1					2	4
WRKY 24			5		1	1	2	1		3	1	1	1		1					1		1	1	2
WRKY 25			3				1		2	3	1		7		7			3		1	1	2	7	1 1
WRKY 28			4		1	2	1			1	2		2		2	1			2		1		2	
WRKY 45			3	1		2	1	1	1	4		1	2		2		1						2	3
WRKY 65	1								4	1			4	1	4							2	4	1
WRKY 89			2			2	1		2	3	2		5		5	2				1	2		5	
WRKY 145			1				1										1							

	Abiotic Stress								Growth and Development									
	ABRE3a	ABRE4	AT-rich element	DRE core	LTR	MBS	MYC	Myc	AP-1	CAT-box	CCGTCC box	CCGTCC motif	GCN4-motif	HD-Zip 1	MSA-like	O2-site	RY-element	Circadian
WRKY 4			1			1	2	1		2	1	1	1				1	1
WRKY 23							4	1		1	1	1	1				1	
WRKY 24				1		1	5	1	1							1		
WRKY 25	2	2						1		1						5	1	
WRKY 28						2	9	1		1				1				
WRKY 45	2	2		1	2		3	2							1		1	
WRKY 65							4				1	1	1			1		
WRKY 89			1	1	1	2	3									1		
WRKY 145										2						1		

Fig. 4.29: Promoter analysis of *MaWRKY* transcription factors. Frequency of cis regulatory elements related to biotic stress, phytohormone, abiotic stress and growth and development, the orange represents the lowest frequency, and the blue represents the highest frequency.

4.10 Differential Expression of *MaWRKYs* in Roots of Banana Plants

The expression of nine selected stress responsive *MaWRKY* genes was analyzed in the roots of banana plants under different conditions. The results showed that inoculation with the consortium, both under non-stressed conditions and in combination with stress treatments, led to increased expression levels of all nine *MaWRKY* genes (Fig. 4. 30-31). Under non-stressed conditions, plants inoculated with the consortium showed induced expression of *MaWRKY25*, *MaWRKY45, MaWRKY145 MaWRKY24*, *MaWRKY28, MaWRKY89*, and *MaWRKY65* at 2, 4, and 6 weeks. However, *MaWRKY4* and *MaWRKY23* did not show significant induction. Under salinity stress conditions, the highest levels of expression were observed in consortium-inoculated plants for *MaWRKY89* at 4 weeks, *MaWRKY23* at 4 weeks, and *MaWRKY145* at 2 weeks. Similarly, under Foc-TR4 stress conditions, consortium-pretreated plants showed the highest levels of expression for *MaWRKY4*, *MaWRKY45*, and *MaWRKY65* at 6 weeks.

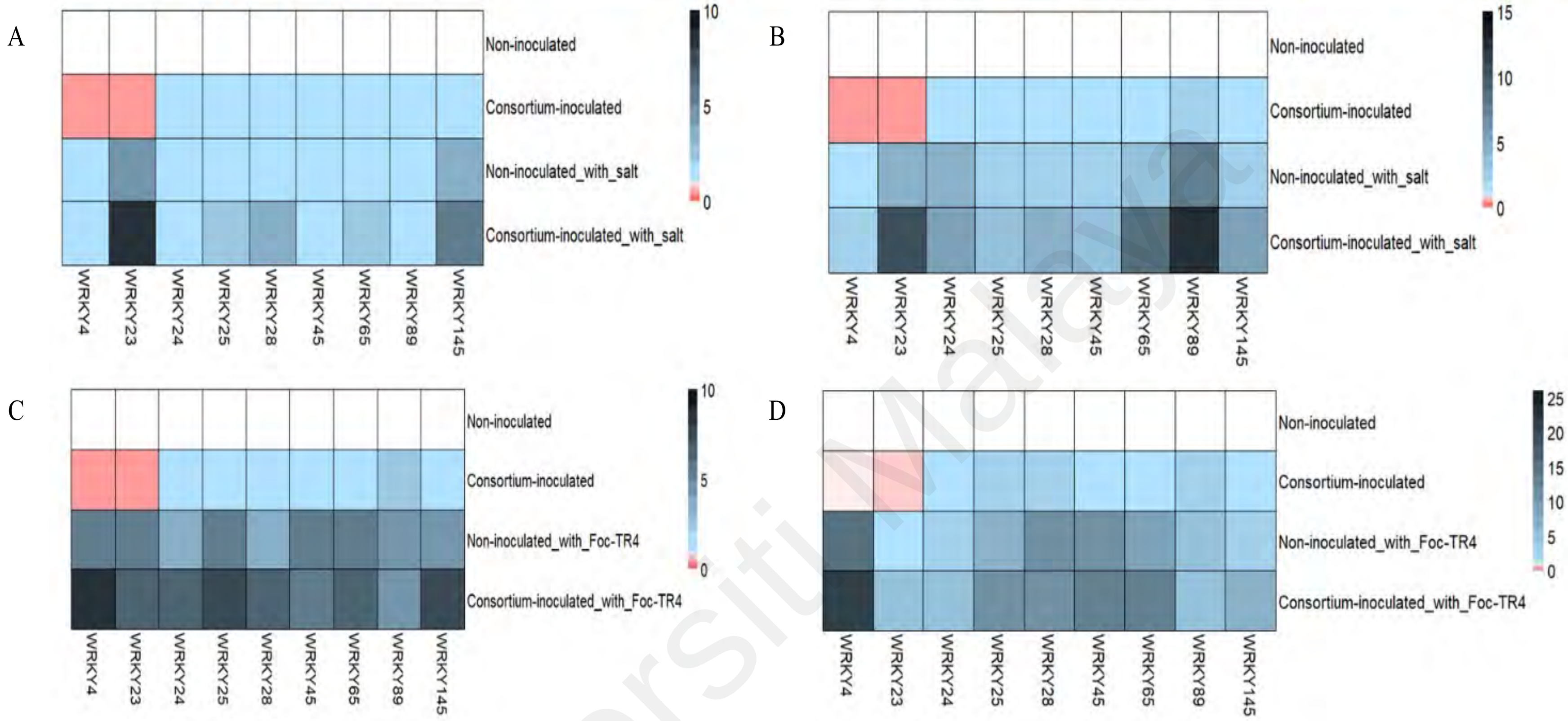
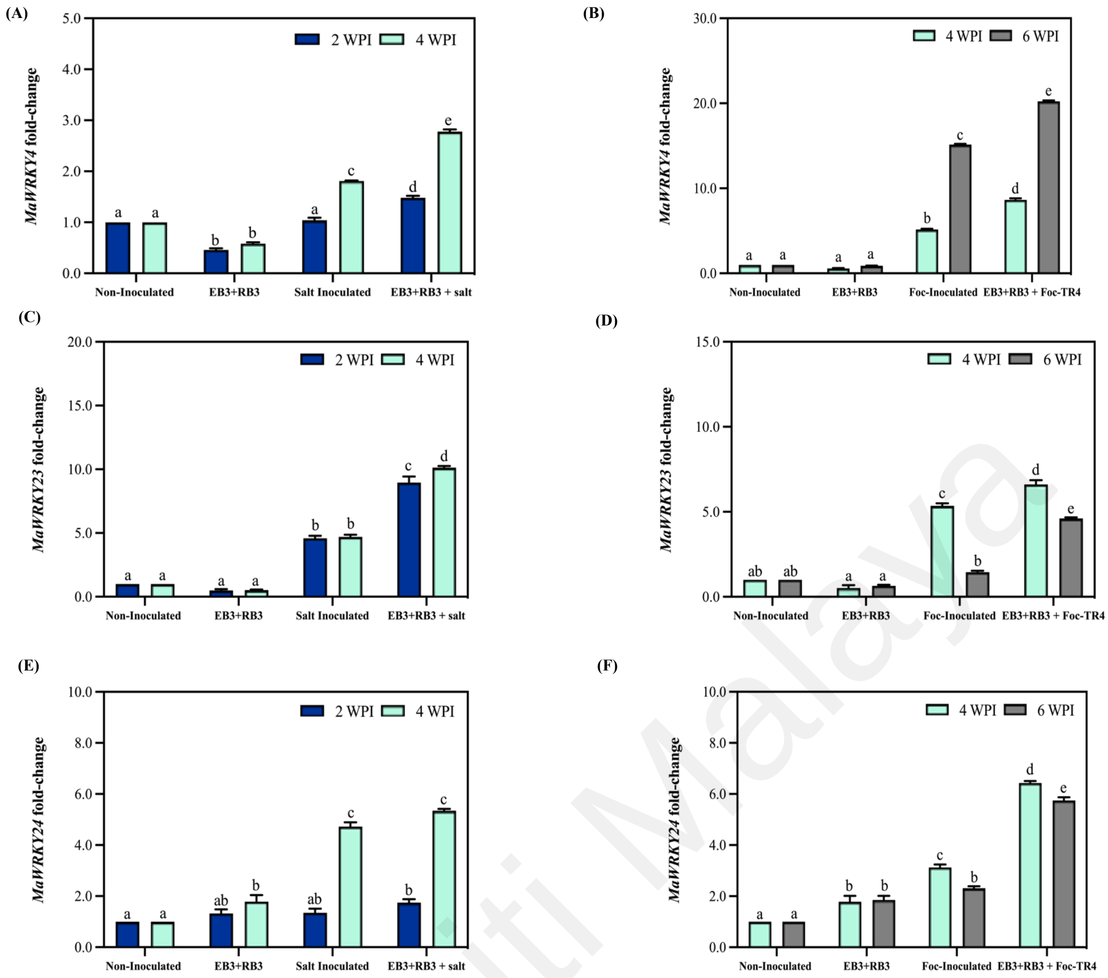
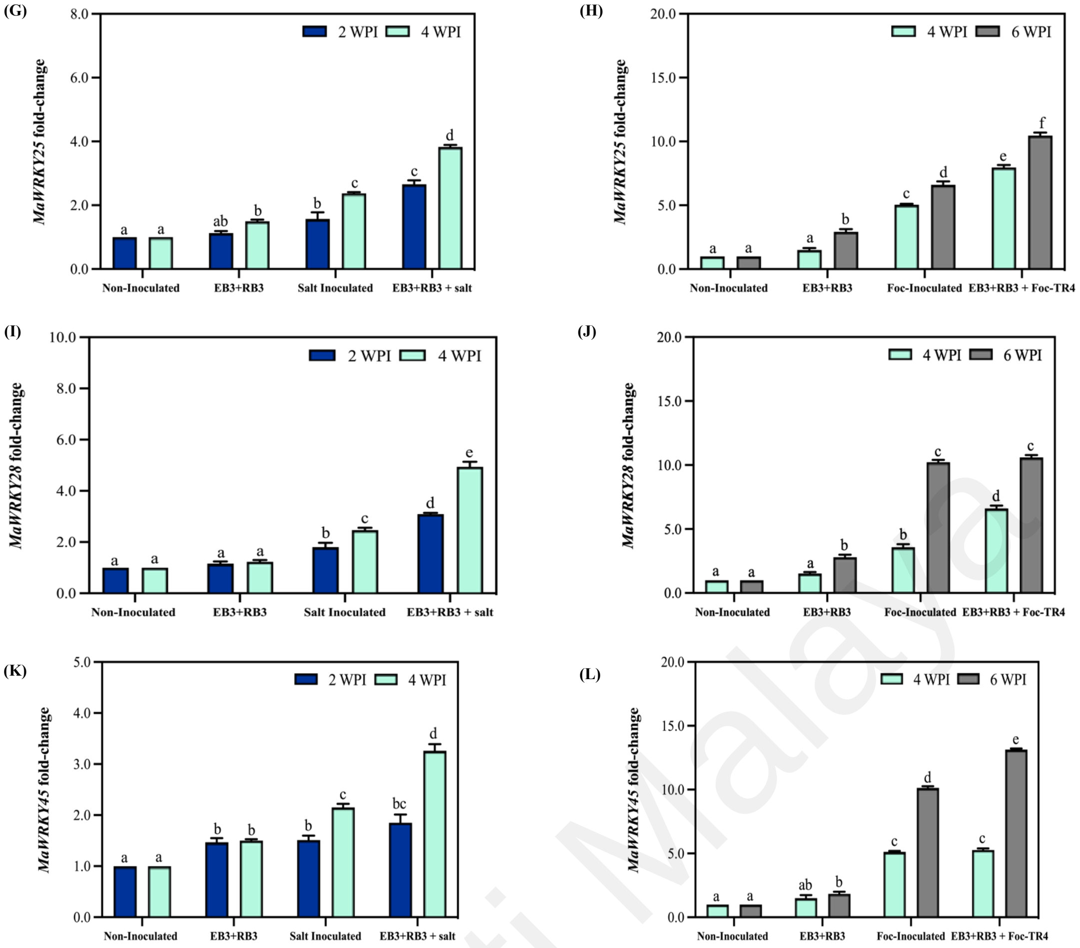


Fig. 4.30: Heat map analysis of *MaWRKY* gene expression in banana plants. (A) Heat map showing *MaWRKY* expression profile in roots of banana plants under salt stress at 2 weeks. (B) Heat map showing *MaWRKY* expression profile in roots of banana plants under salt stress at 4 weeks. (C) Heat map showing *MaWRKY* expression profile in roots of banana plants under Foc-TR4 stress at 4 weeks. (D) Heat map showing *MaWRKY* expression profile in roots of banana plants under Foc-TR4 stress at 6 weeks. The degree of expression is shown by the color bar to the right of the graph which ranges from red to blue suggesting the amount of expression is growing. Salt treatment was for 2 weeks and 4 weeks in soil with PGPB in 100 mM sea salt. Fusarium treatments were for 4 and 6 weeks in soil with PGPB with Foc-TR4. The non-inoculated, non-treated banana roots (without PGPB, without Foc-TR4 and not treated with salt) were used as calibrator (value set as 1.0) for all comparisons. The relative expression of *MaWRKY* TFs was determined using $2^{-\Delta\Delta CT}$ method. Error bars indicate the standard error. The values are mean ± SD (n = 3). Different letters represent significant differences at ($p < 0.05$) using ANOVA and Tukey post-hoc tests.


(A)
MaWRKY4 fold-change
5.0
4.0
3.0
2.0
1.0
0.0
2 WPI
4 WPI
a
a
b
b
a
c
d
e
Non-Inoculated
EB3+RB3
Salt Inoculated
EB3+RB3 + salt
(B)
MaWRKY4 fold-change
30.0
20.0
10.0
0.0
4 WPI
6 WPI
a
a
a
a
b
c
d
e
Non-Inoculated
EB3+RB3
Foc-Inoculated
EB3+RB3 + Foc-TR4
(C)
MaWRKY23 fold-change
20.0
15.0
10.0
5.0
0.0
2 WPI
4 WPI
a
a
a
a
b
b
c
d
Non-Inoculated
EB3+RB3
Salt Inoculated
EB3+RB3 + salt
(D)
MaWRKY23 fold-change
15.0
10.0
5.0
0.0
4 WPI
6 WPI
ab
ab
a
a
c
b
d
e
Non-Inoculated
EB3+RB3
Foc-Inoculated
EB3+RB3 + Foc-TR4
(E)
MaWRKY24 fold-change
10.0
8.0
6.0
4.0
2.0
0.0
2 WPI
4 WPI
a
a
ab
b
ab
c
b
c
Non-Inoculated
EB3+RB3
Salt Inoculated
EB3+RB3 + salt
(F)
MaWRKY24 fold-change
10.0
8.0
6.0
4.0
2.0
0.0
4 WPI
6 WPI
a
a
b
b
c
b
d
e
Non-Inoculated
EB3+RB3
Foc-Inoculated
EB3+RB3 + Foc-TR4


(G)
MaWRKY25 fold-change
2 WPI
4 WPI
0.0
2.0
4.0
6.0
8.0
a
a
ab
b
b
c
c
d
Non-Inoculated
EB3+RB3
Salt Inoculated
EB3+RB3 + salt
(H)
MaWRKY25 fold-change
4 WPI
6 WPI
0.0
5.0
10.0
15.0
20.0
a
a
a
b
c
d
e
f
Non-Inoculated
EB3+RB3
Foc-Inoculated
EB3+RB3 + Foc-TR4
(I)
MaWRKY28 fold-change
2 WPI
4 WPI
0.0
2.0
4.0
6.0
8.0
10.0
a
a
a
a
b
c
d
e
Non-Inoculated
EB3+RB3
Salt Inoculated
EB3+RB3 + salt
(J)
MaWRKY28 fold-change
4 WPI
6 WPI
0.0
5.0
10.0
15.0
20.0
a
a
a
b
b
c
d
c
Non-Inoculated
EB3+RB3
Foc-Inoculated
EB3+RB3 + Foc-TR4
(K)
MaWRKY45 fold-change
2 WPI
4 WPI
0.0
1.0
2.0
3.0
4.0
5.0
a
a
b
b
b
c
bc
d
Non-Inoculated
EB3+RB3
Salt Inoculated
EB3+RB3 + salt
(L)
MaWRKY45 fold-change
4 WPI
6 WPI
0.0
5.0
10.0
15.0
20.0
a
a
ab
b
c
d
c
e
Non-Inoculated
EB3+RB3
Foc-Inoculated
EB3+RB3 + Foc-TR4

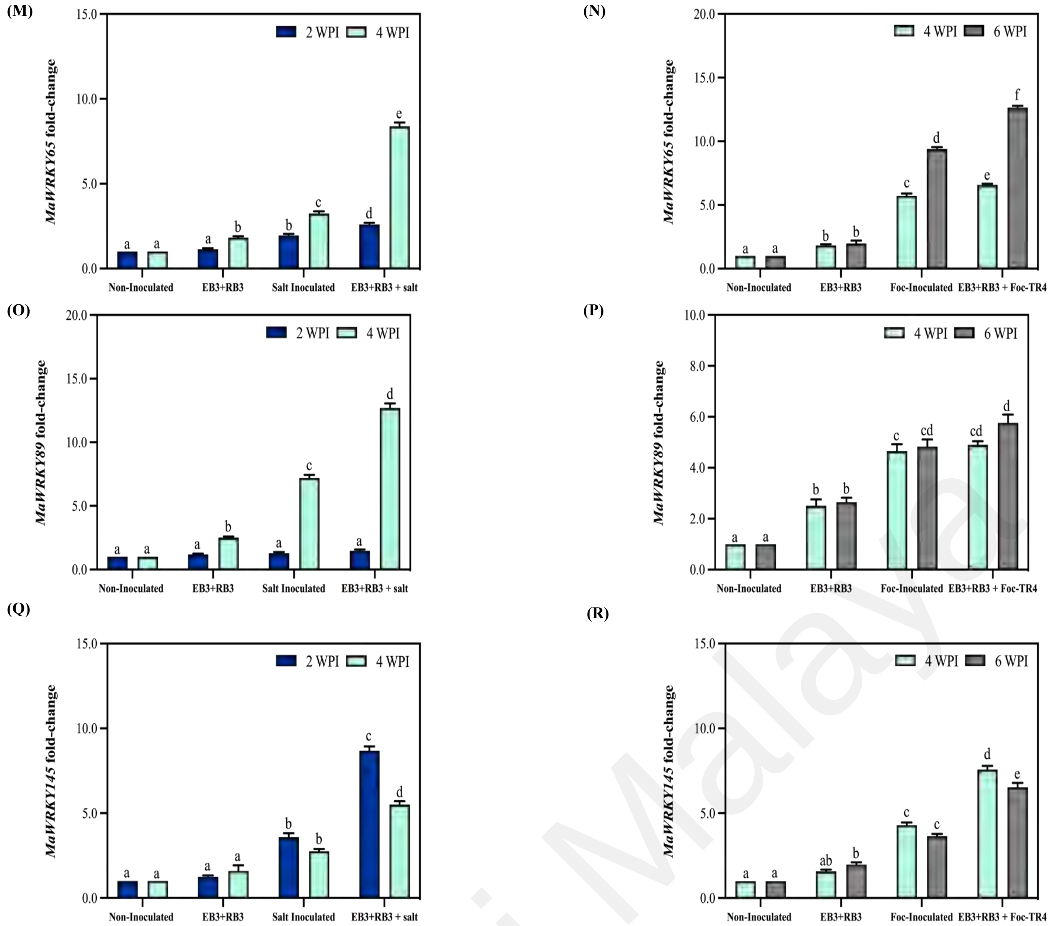


Fig. 4.31: Relative Expression Levels of *MaWRKY* in plants. Relative expression of (A, B) *WRKY4*, (C, D) *WRKY23*, (E, F) *WRKY24*, (G, H) *WRKY25*, (I, J) *MaWRKY28*, (K, L) *MaWRKY45*, (M, N) *MaWRKY65*, (O, P) *MaWRKY89* and (Q, R) *MaWRKY145* in roots of banana plants. Salt treatment was for 2 weeks and 4 weeks in soil with PGPB in 100 mM sea salt. Fusarium treatments were for 4 and 6 weeks in soil with PGPB and with Foc-TR4. The non-inoculated, non-treated banana roots (without PGPB, without Foc-TR4 and not treated with salt) were used as calibrator (Value set as 1.0) for all comparisons. The relative expression of *MaWRKY* TFs was determined using $2^{-\Delta\Delta CT}$ method. The standard errors are denoted by the error bars. The values are mean ± SD (n = 3). Using ANOVA and Tukey post-hoc tests, Different letters represent a significant difference at ($p < 0.05$).

CHAPTER 5: Discussion

Mangrove forests are predominantly situated in the intertidal zone or estuarine margins of wetlands, where they are regularly subjected to tidal flooding due to their proximity to the coast. These environments offer distinct ecological settings characterized by elevated salinity levels and nutrient accumulation from sediment deposition. As a result, they provide a conducive habitat for the growth and survival of halotolerant bacteria. Mangrove plants themselves have adapted to these conditions, possessing strong root systems capable of withstanding salt, as well as waxy and succulent leaves that aid in water storage and minimize evaporation (Sasmito et al., 2020). Furthermore, the microbiota in the rhizosphere and endosphere of mangrove plants play a crucial role in their ability to thrive in this challenging ecosystem.

The first aim of this thesis research was to isolate halotolerant bacteria from mangrove plant rhizosphere and sample collection at Carey Island, Selangor, Malaysia, led to the isolation of 406 rhizobacteria and 29 endophytic bacteria from the rhizosphere and roots of Carey Island three mangrove species: *Rhizophora apiculata* (red mangrove), *Avicennia alba* (black mangrove) and *Sonneratia alba* (white mangrove) (Fig. 3.1, Appendix B). The high number of bacteria isolated could be attributed to the anoxic and oxic processes present in Carey Island (Bakrin Sofawi et al., 2017) which support the growth of numerous bacteria. Furthermore, the proximity of plantations to the collection site may have contributed to the higher microbial density observed in this study. Previous research by Long et al. (2016) indicated that plantations alter the microbiota of mangrove habitats and increase microbial density through the release of organic compounds via root exudation and oxygen leakage.

The other aims of this study were to identify and characterize halotolerant bacteria for their plant growth promoting activities and the effects of the PGPB on physiological, biochemical, and molecular changes in banana. The PGPB isolates were identified *Bacillus* sp. and *Pseudomonas* sp. The two strains selected for use as a consortium, demonstrated the ability to protect banana plants against salinity stress and Foc-TR4 infection, indicating their potential as a biocontrol agent.

5.1 The Mangrove Forest in Carey Island Hosts Halotolerant Bacteria

A significant number of the halotolerant bacteria, specifically 16 out of 29 endophytic bacteria and 88 out of 213 rhizobacteria, were found to tolerate aquarium sea salt concentration of 400 mM. These isolates were predominantly obtained from the roots and soil surrounding *S. alba*, a white mangrove species. It is worth noting that *S. alba* is situated on higher ground than red or black mangroves but still experiences regular inundation by seawater. Analysis of the rhizosphere soil across the three mangrove sites revealed that the zone occupied by white mangroves exhibited the highest levels of nitrogen (0.45%) and organic carbon (49.55%), in comparison to the black and red mangroves, in addition to the highest number of bacterial isolates. Conversely, only six out of the 213 rhizobacteria were found in the rhizosphere of black mangrove (*A. alba*), and ten were isolated from red mangrove (*R. apiculata*). Furthermore, only three endophytic bacteria and one endophytic bacterium were isolated from the roots of *A. alba* and *R. apiculata* respectively. The lower number of bacteria in these sites might be attributed to the continuous flooding experienced by black and red mangroves at the shoreline. The sediment deposits, including nutrients such as organic carbon, constantly fluctuate due to tidal ebb and flow, thereby impacting the overall microbial population. These results highlight the significance of nitrogen and organic carbon as energy sources and macronutrients for microbial growth and biomass in the mangrove ecosystem.

Out of the 213 rhizobacterial isolates and 29 endophytic bacterial isolates, a total of 88 rhizobacteria and 16 endophytic bacteria exhibited growth in an aquarium sea salt solution with a high concentration of 400 mM NaCl. This salinity level surpasses those reported in previous studies on PGPB, which identified three isolates capable of growing in up to 75 mM NaCl (Sapre et al., 2022) and a *Streptomyces* spp. that tolerated up to 300 mM NaCl (Nozari et al., 2021). The bacterial isolates were further identified through 16S rRNA gene sequencing and subsequent phylogenetic analysis. Based on these analyses, the isolates were classified within the genera *Bacillus* and *Pseudomonas* (Fig. 4.4 - 4.6, Appendix F-G). Among these two genera, *Bacillus* was the most abundant, which can be attributed to its ability to form endospores (Liu et al., 2022), which enables adaptation to harsh environments like mangroves. On the other hand, the presence of *Pseudomonas* can be attributed to its role in maintaining ecological balance within the mangrove ecosystem through the degradation of complex molecules (Biswas et al., 2020). It should be noted that the immense diversity within these two genera makes it challenging to accurately identify novel isolates at the species level without aid of several genes or whole genome sequencing (Dunlap, 2019). However, it is well-known that representatives of Bacillus and Pseudomonas species are commonly found in plant rhizospheres, and many of them possess the ability to promote plant growth and withstand high salinities. Recent examples include *P. protegens*, which solubilizes zinc and enhances dehydrogenase and phosphatase activities in rhizospheric soil, thus conferring salt tolerance to wheat plants growing in up to 150 mM NaCl (Singh et al., 2022), and *B. subtilis*, which improves soybean growth at 150 mM NaCl by modulating glyoxalase and antioxidant defense systems, maintaining ion homeostasis, and facilitating osmotic adjustment (Hasanuzzaman et al., 2022). These studies highlight the potential of the isolated bacterial species to enhance plant tolerance to high salinity and promote growth even under challenging conditions.

Among the endophytic and rhizobacterial isolates that displayed salt tolerance (400 mM) as well as nitrogen fixation and phosphate solubilization abilities, three endophytic bacteria (EB1-EB3) and five rhizobacteria (RB1-RB5) were selected for additional phylogenetic analysis to determine their closest relatives among the type members of their respective genera. Interestingly, the 16S rRNA gene sequences of strain RB2 and EB2 were found to be identical, sharing 99.93% similarity with the rhizobacterium *Bacillus siamensis* KCTC13613 (Table 4.2). This finding is intriguing, as RB2 was isolated from rhizosphere soil and EB2 from root tissues.

The possibility of shared sequences between endophytic and rhizobacterial isolates may be attributed to their biphasic life cycles, alternating between soil and plant habitats as reviewed by Afzal et al. (2019). Moreover, several bacterial genera, including *Pseudomonas* and *Bacillus*, are known to exhibit biphasic life cycles, alternating between the endosphere and rhizosphere (Miliute et al., 2015), potentially explaining the observed sequence similarity between strain RB2 and strain EB2 in this study. Endophytic bacteria are known to release cellulases and pectinases (cell wall-destroying enzyme) in the rhizosphere, facilitating their entry into host tissues while maintaining low cell densities (2-6 CFU/GFW) to avoid being detected by the plant defense system (Compant et al., 2005; Rosenblueth & Martínez-Romero, 2006). Further research such as proteomic analysis, may explore the biphasic life cycle of these isolates, shedding light on their unique ecological adaptations within the mangrove ecosystem.

5.2 Successful Colonization of Banana Plantlets by Halotolerant *Bacillus* and *Pseudomonas* Isolates

In this study, a root colonization study was conducted using field emission scanning electron microscopy (FESEM). The results provided a three-dimensional view of banana roots, revealing the establishment and colonization of the isolates on the roots of banana Berangan plants grown in a saline environment (MS media, pH-6.4, ECe-13.23 dS/m). Notably, the bacterial isolates were found to alleviate the symptoms of salinity stress in comparison to uninoculated plants, suggesting their potential for enhancing salt tolerance in important crops like bananas. Among the bacterial isolates, all the *Bacillus* strains displayed a biofilm-like structure and an aggregation matrix-like colonization pattern on the banana roots (Fig. 4.8). The degree of biofilm-like structures may be related to the amount of root exudates produced by specific zones (root hair, elongation, and tip) of the host plants, which are utilized by bacterial isolates as a carbon source (Posada et al., 2018). In contrast, the colonization pattern exhibited by *Pseudomonas* isolates appeared sparser, which may be attributed to their endophytic nature.

Pseudomonas isolates need to first gain entry into the root tissue before proliferating (Gamez et al., 2019). These findings highlight the diverse strategies employed by different bacterial isolates to colonize and interact with the banana root system. Genetic engineering approaches for enhancing salt tolerance in bananas have been explored, but such methods are time-consuming, expensive, and require significant efforts for selection and clonal multiplication of improved lines (Shekhawat et al., 2011; Tak et al., 2017). On the other hand, the utilization of halotolerant PGPB has shown promise in mitigating salinity stress in important plants such as maize (Nozari et al., 2021) and wheat (Hajiabadi et al., 2021). Despite bananas being a globally significant fruit crop and highly susceptible to salinity, limited research has been conducted on utilizing halotolerant bacteria to mitigate stress in these crops. The successful protection of bananas under high saline conditions by PGPB in this study highlights their potential for reclaiming saline soil and enhancing agricultural productivity.

5.3 Halotolerant *Bacillus* and *Pseudomonas* Isolates from Mangrove Forest are Identified as Plant Growth Promoting Bacterial Agents

In the preliminary evaluation of the eight selected strains (RB1-RB5 and EB1-EB3), their plant growth promoting activities were assessed to determine their suitability for subsequent root colonization and plant health studies. Considering the nutrient-limited conditions of the mangrove habitat, it was expected that the isolates would possess activities that facilitate the absorption of essential micro- and macronutrients such as nitrogen, phosphorus, and iron. Nitrogen is critical element for plant growth, as it is a key component of amino acids, nucleic acids, and chlorophyll. Previous studies have highlighted the capability of Bacillus and Pseudomonas isolates to enhance nitrogen content in non-leguminous plants (Fox et al., 2016; Thatoi et al., 2020). These bacterial species produce hydrolytic enzymes that aid in the breakdown of plant cell walls, facilitating root colonization. For instance, *Bacillus* sp. Sb42 has exhibited enhanced nitrogen assimilation in rice (Mutalib et al., 2012), while *Pseudomonas putida* BP25 has shown improved nitrogen uptake in black pepper (Sheoran et al., 2015), resulting in improved plant growth and biomass. Therefore, the halotolerant *Bacillus* and *Pseudomonas* strains obtained from the mangrove habitat hold great potential for promoting growth in salinity-stressed crops, including bananas. Their ability to facilitate nutrient acquisition and enhance plant growth makes them promising candidates for agricultural applications in challenging environments.

In addition to their nitrogen-fixing abilities, the selected halotolerant PGPB isolates also demonstrated a varying degrees of phosphate solubilizing activity. This trait can be highly advantageous for plant growth, especially under stressful conditions. Phosphate can often react and become insoluble inside plant tissues, limiting its accessibility to plants (Varga et al., 2020). However, certain bacteria can solubilize phosphate, making it available for plant uptake. Of particular interest, was the endophytic isolate EB1, which exhibited the highest phosphate solubilization activity (Fig. 4.1). This observation aligns with previous studies reporting similar phosphate solubilization activity in endophytic bacteria (Varga et al., 2020). The high activity in strain EB1 may be attributed to its interaction with the plant host, where the bacteria utilize specific mechanisms to solubilize phosphate and provide it to the plant. Additionally, the biphasic behavior of endophytic bacteria, alternating between the rhizosphere and endosphere of the plant, could contribute to this activity, allowing them to adapt to specific environments and nutrient availability. The observed phosphate solubilization activity in the selected halotolerant PGPB isolates, particularly in the endophytic isolate EB1, highlights their potential to enhance nutrient availability and promote plant growth in challenging environments. These findings contribute to our understanding of the beneficial interactions between bacteria and plants, providing insights into their roles in nutrient cycling and stress tolerance.

Siderophores, low molecular weight iron chelators produced by many bacteria, play a dual role in facilitating bacterial iron uptake and inducing iron starvation in the rhizosphere to impede the growth of phytopathogens (Egamberdieva et al., 2023). In this study, the selected mangrove rhizobacterial isolates exhibited significant siderophore production, ranging from 40% to 50%. The siderophore production levels of the selected endophytic bacterial isolates, however, were less than 10%. These findings highlight the potential of rhizobacteria from mangrove ecosystems as valuable PGPB due to their strong siderophore-producing capabilities. Previous research on maize subjected to salinity stress have highlighted the beneficial effects of salt-tolerant isolates, such as *Arthrobacter pascens* and *Bacillus* sp. derived from the halophyte plant Salt-bush (*Atriplex leucoclada*). These isolates not only produce siderophores but also exhibit phosphate solubilization activities, contributing to plant protection (Ullah & Bano, 2015). Similarly, successful inoculation of soybean with halotolerant isolates of *Pseudomonas pseudoalcaligenes* and *Bacillus subtilis* and obtained from saline soil, resulted in improved salt tolerance through their siderophore production ability (Yasmin et al., 2020) Collectively, these studies underscore the importance of siderophore-producing PGPB in enhancing plant stress tolerance and highlight their potential as valuable resources for sustainable agriculture.

The production of IAA and the enzyme ACC deaminase can contribute to enhanced stress tolerance in plants. In this study, all eight selected mangrove PGPB strains exhibited increased IAA production over a 48-h period. IAA not only functions as a phytohormone but also plays a crucial role in biofilm formation, which facilitates the colonization of bacterial strains on plant roots (Ansari & Ahmad, 2019). Furthermore, the PGPB strains exhibited ACC deaminase activity, an enzyme involved in mitigating salinity stress in plants by decreasing the level of "stress ethylene" which is inhibitory to plant growth. ACC deaminase enzymatically converts the ethylene precursor ACC into ammonia and α-ketobutyrate, which bacteria can use as a source of nutrients (Singh et al., 2015). Although the endophytic isolates had lower levels of ACC deaminase activity than the majority of the rhizobacterial isolates (which produced up to 3902 nmol α-ketobutyrate protein^{-1}), these levels were still within the range of 800-1500 nmol α-ketobutyrate protein^{-1} reported in comparable studies (Gupta & Pandey, 2019). The production of IAA and ACC deaminase activity exhibited by selected mangrove PGPB strains supports their potential as effective plant growth promoters in banana cultivation. These strains possess valuable traits that can enhance root development, nutrient uptake, and salinity tolerance in plants. Harnessing the beneficial effects of these PGPB strains offers promising prospects for sustainable agriculture and the cultivation of salinity-stressed crops like bananas.

5.4 Halotolerant PGPB Strains Enhanced Banana Growth under Salinity and Foc-TR4 Stresses

Salinity and Foc-TR4 stresses have detrimental effects on banana growth and productivity, particularly in tropical and subtropical regions where bananas are a crucial crop for food security and economic development (Ismaila et al., 2022; Wei et al., 2022). To mitigate these negative impacts, the application of halotolerant PGPB offers an environmentally friendly approach that complements the use of synthetic agrochemicals (Vocciante et al., 2022). In this study, six selected PGPB strains (*Pseudomonas* spp. EB1, EB3, and *Bacillus* spp. EB2, RB1, RB3, RB4) were investigated for their protective effects against salinity stress in banana plants under hydroponic conditions. The choice of a 90 mM aquarium sea salt concentration for inducing salinity stress was based on previous research that demonstrated adverse effects on banana plantlets under similar conditions (Patel et al., 2019). The results of the hydroponic study demonstrated that the inoculation of banana plants with each PGPB strain led to improved growth even under saline conditions. Notably, the treated plants exhibited higher plant height and root length compared to non-inoculated plants, indicating the potential of these PGPB strains to mitigate the negative effects of salinity stress on banana growth. Moving to the greenhouse study, the pre-treatment of banana plants with a consortium of *Bacillus* sp. RB3 and *Pseudomonas* sp. EB3 through the application of a liquid inoculum to the soil surrounding young plantlets was examined. Under salinity stress and Foc-TR4 inoculation, the treated banana plants displayed significant improvements in various growth parameters. These improvements included enhanced plant height, root length, and root biomass compared to the control group, suggesting that the RB3-EB3 consortium effectively alleviated the negative impacts of both salinity stress and Foc-TR4 infection. The findings of this study underscore the potential of halotolerant PGPB as a promising tool to enhance banana growth and productivity under challenging environmental conditions. The ability of these PGPB strains to protect banana plants from the negative impacts of salinity stress and Foc-TR4 infection, as demonstrated in both hydroponic and greenhouse studies, highlights the practical applicability of this eco-friendly approach in banana cultivation.

5.5 Halotolerant PGPB Strains Enhanced Bananas Health under Salinity and Foc-TR4 Stresses

The assessment of various plant health parameters in both the hydroponic and greenhouse studies revealed a consistent pattern that supported the beneficial effects of the plant growth-promoting behaviors displayed by the halotolerant PGPB strains. Notably, the presence of PGPB strains promoted higher levels of carotenoids, chlorophylls, and relative water content in the plants, indicating an improvement in photosynthetic efficiency and plant hydration status. However, under salinity and Foc-TR4 stresses, the levels of these pigments were lower compared to non-stressed, non-inoculated plants. The reduction in pigment levels under salinity stress can be attributed to lower nutrient intake and higher sodium accumulation, which lead to chlorophyll degradation and the inhibition of chlorophyllase enzyme function (J. Li et al., 2015). Similarly, the reduction in pigment levels under Foc-TR4 stress is a result of the damage inflicted by the pathogen on the plant's photosystems as the infection spreads (Bauriegel et al., 2011; Santini et al., 2008). The observed alleviation of stress effects in plants inoculated with PGPB strains aligns with previous studies and can be attributed to various mechanisms exhibited by these strains, such as phosphate solubilization, ACC deaminase activity, and production of siderophores and IAA. These mechanisms have been reported to enhance plant tolerance to salinity stress and pathogen infection. For instance, studies have shown that PGPB inoculation with *Kocuria* *rhizophila*, *Bacillus velezensis*, and *Kosakonia radicincitans* increased salt stress tolerance in black gram plants (John et al., 2023), and inoculation with *Pantoea agglomerans* R1 and *Pseudomonas fragi* R4 improved salinity tolerance in French bean plants (Gupta et al., 2023). Moreover, examples of studies have demonstrated the potential of PGPB strains to confer pathogen resistance, such as the inoculation of *B. subtilis* against fungus *Clarireedia jacksonii* in turfgrass plants (Kaur et al., 2023), and the inoculation of *P. putida* against fungus *Macrophomina phaseolina* in mung bean plants (Khan et al., 2023). These findings highlight the potential application of these PGPB strains as bioinoculants to enhance the productivity and resilience of banana crops in salinity-prone areas and in the presence of fungal infection.

5.6 PGPB-Mediated Ion Homeostasis Enhanced Salt Tolerance in Banana

Salt stress poses significant challenges to plant growth and development due to the disruption of ion homeostasis. The regulation of ion fluxes, particularly the maintenance of a high K^+ to Na^+ ratio, is crucial for plants to tolerate salinity stress (Fahad et al., 2015). Excessive accumulation of Na^+ and Cl^- ions can disrupt essential metabolic processes and hinder nutrient uptake, leading to nutrient deficiencies and metabolic toxicity in plants (Rubio et al., 2020). In the hydroponic study, the application of halotolerant PGPB strains to banana plants under normal and saline conditions showed reduced Na^+ levels and increased K^+ content (Fig. 4.16). Building upon these findings, the greenhouse investigation examined the synergistic effects of RB3 and EB3 when applied as a consortium. Remarkably, the consortium treatment significantly enhanced the content of essential cations, such as K^+, Mg^+, and Ca^{2+}, while concurrently reducing the content of Na^+ and Cl^- in both non-stressed and salt-stressed banana plants (Fig. 4.17). This shift in ion accumulation towards favorable cations and away from detrimental Na^+ and Cl^- ions is a crucial attribute in conferring salt tolerance to plants. Similar observations have been reported in other plant species and bacterial consortia. For example, a consortium of *Bacillus aryabhattai* and *Arthrobacter woluwensis* was found to enhance K^+ accumulation while reducing Na^+

accumulation in salt-stressed soybean plants (Khan et al., 2021). Likewise, PGPB consortia consisting of *Pseudarthrobacter oxydans* and *Bacillus zhangzhouensis* have shown improved plant growth, increased uptake of K^+, Mg^+, and Ca^{2+}, and decreased Na^+ uptake in Swiss chard (Redondo-Gómez et al., 2021) and tomato plants (Kapadia et al., 2021) under salt stress conditions. The reduced uptake of detrimental ions and the enhanced uptake of beneficial ions contribute to the mitigation of ionic stress and support plant protection in saline soils. The ability of the PGPB strains to modulate ion content in banana plants highlights its potential as an effective strategy for improving salt tolerance.

5.7 PGPB Inoculation Enhances Banana Resilience against Salinity and Foc-TR4 Stresses

Both the salinity stress and pathogen infections are known to induce the production of ROS, leading to cellular damage and lipid peroxidation (Khaledi et al., 2016; Noctor & Foyer, 1998). Banana plants, like many other crop plants respond to salinity and Foc-TR4 stress conditions by exhibiting elevated levels of H_2O_2 and increased activity of antioxidant enzymes (Fung et al., 2019; Mazumdar et al., 2019). In this study, all six tested PGPB-inoculated strains demonstrated a reduction in MDA levels, a marker of lipid peroxidation and H_2O_2 levels compared to non-inoculated plants, both under normal and saline conditions (Fig. 4.14B). This trend continued in the greenhouse study, where the EB3+RB3 consortium reduce the MDA levels under both salt and Foc-TR4 stresses (Fig. 4.15). The decrease in MDA levels suggests that the PGPB treatment contributes to the maintenance of membrane integrity and reduces oxidative stress. The presence of the PGPB strains in banana plants subjected to salt stress and Foc-TR4 conditions resulted in significant enhancements in various biochemical parameters associated with plant defense mechanisms. Specifically, the levels of the non-enzymatic antioxidant proline, as well as the activities of antioxidant enzymes (SOD, CAT, GR, APX, and POD), were elevated in the PGPB-inoculated plants in both hydroponic and greenhouse studies. These findings suggest that the PGPB treatment enhances the antioxidant capacity of the plants, enabling a more effective defense response against the accumulation of ROS. Such enhanced antioxidant capacity is crucial for maintaining cellular redox homeostasis and protecting plant cells from oxidative damage. Additionally, the PGPB-inoculated plants showed lower electrolyte leakage, a marker of membrane damage (Sairam et al., 2005), suggesting improved membrane stability and reduced cellular stress due to the presence of PGPB strains. In the greenhouse study, the EB3+RB3 treatment led to enhanced lignin content in banana plants. Lignin is known to strengthen cell walls and provides increased protection against pathogenic attacks (Samain et al., 2022). The reinforcement of cell walls through lignification is an essential defense mechanism in plants, and the enhanced lignin content observed in the consortium-inoculated plants signifies the priming effect induced by the PGPB treatment. Importantly, the positive effects of the strains EB3 and RB3, observed individually in hydroponic studies on salt-stressed banana plants, were further validated in the current study when the strains were applied as a consortium.

The selection of *Bacillus* sp. RB3 and *Pseudomonas* sp. EB3 for the greenhouse study was based on their demonstrated effectiveness in protecting banana plants against salinity stress in the hydroponic study. Furthermore, both strains exhibited antifungal activity against Foc-TR4 in a dual culture assay (Fig. 4.21). In the controlled greenhouse experiment, the consortium of RB3 and EB3 strains showed a plant-protective effect against Foc-TR4 when applied to banana plants, which was reflected in the lower disease severity index values. Previous studies have highlighted the antagonistic activities of various *Bacillus* sp. and *Pseudomonas* sp. against against phytopathogenic fungi, including *Gaeumannomyces graminis* and *Rhizoctonia cerealis* (Castro Tapia et al., 2020), *Alternaria* sp. (Müller et al., 2018), as well as *Fusarium* sp. (Khan et al., 2018). In the context of the RB3 and EB3 consortium, the levels of chitinase and β-1,3-glucanase and were significantly higher in pre-treated banana plants with the consortium and subsequent inoculation with Foc-TR4 (Fig. 4.28 and Table 4.3). This increase in enzyme activity coincided with a reduction in the symptoms of fungal infection in the banana plants pre-treated with consortium. Furthermore, elevated levels of PAL known for inducing the synthesis of salicylic acid to elevate systemic resistance in banana plants in response to infection (Kim &

Hwang, 2014); PPO, facilitating oxidation of phenols into quinone toxic compounds that could restrict the pathogen at infected sites (Abo-Elyousr et al., 2022); and LOX, a component of the pathogen-induced response limiting the pathogen spread in plant tissues (Kolomiets et al., 2000), collectively underscore overall reinforced protection against Foc-TR4 infection in banana plants inoculated with the consortium. The findings of this study highlight the potential of the RB3 and EB3 consortium as a bioprotective agent for banana plants under salinity stress and Foc-TR4 infection. The consortium treatment effectively enhanced antioxidant capacity, reduced oxidative damage, improved membrane integrity, strengthened cell walls, and induced defense-related enzyme activity. These beneficial effects contribute to the overall resilience and protection of banana plants.

5.7 Priming Banana Through Consortium Inoculation Provides Enhanced Resistance against Salinity and Foc-TR4 stresses.

The general mechanisms underlying the plant health enhancement by PGPB involve the ability of consortia, such as the EB3 and RB3, to prime plants for increased resilience against subsequent stresses, even when the stresses differ in nature, such as Foc-TR4 infection and salinity stress. While specific investigations addressing the comprehensive stress protection provided by a PGPB consortium in banana are lacking, analogous instances are documented for other crops. For instance, Essalimi et al. (2022) demonstrated the bioprotective effects of a consortium comprising *Bacillus toyonensis* Pr8 and *Pseudomonas stutzeri* Pr7 in tomatoes, conferring resilience against infection by pathogenic fungi *Fusarium oxysporum* f.sp. *melonis* and *Verticillium dalhiae* and salt stress. Another study by Samain et al. (2022) reported that a PGPB microbial consortium, consisting of *Arthrobacter* sp. strain AA and *Paenibacillus* sp. strain B2 enhanced the protection of wheat plants against fungus *Zymoseptoria tritici* infection and drought. Although the specific mechanisms for stress protection were not studied in detail, these authors suggested that the enhanced protection against multiple stresses is likely attributed to the positive effects of PGPB on improving plant traits, such as root and shoot growth, increased photosynthetic pigments, elevated proline content, and induction of antioxidant and defense-related enzymes (Essalimi et al., 2022).

In a consortium, individual bacterial species may excel in specific growth-promoting activities, such as nitrogen fixation or phosphate solubilization. The additive effects of these activities can lead to improved stress protection compared to monocultures (Tsoi et al., 2019). Furthermore, it is worth noting that EB3 strain is an endophytic bacterium. While both RB3 and EB3 strains produced similar levels of IAA, RB3 exhibited higher ACC deaminase activity (Fig. 4.5). These differences, along with the distinct adaptability of the strains, may enhance their capacity to prime banana plants against Foc-TR4 infection and salinity stress when applied as a consortium. Further research is needed to elucidate the precise mechanisms underlying the stress protection provided by the strains RB3 and EB3 consortium in banana plants. Understanding the mechanisms of stress priming by PGPB consortia in banana plants can contribute to the development of sustainable and eco-friendly strategies for improving banana productivity and resilience in the face of salinity stress and Foc-TR4 infection. By harnessing the beneficial interactions between beneficial bacteria and plants, it is possible to enhance crop protection and mitigate the negative impacts of various stresses, ultimately contributing to food security and sustainable agricultural practices.

5.8 PGPB Consortium Increases MaWRKY Gene Expression in Banana Root under Salinity and Foc-TR4 Stresses

The phenotypic and physiological changes observed in plants undergoing stress are often regulated by the activation of stress-responsive genes and the involvement of transcription factors (TFs), including those from the WRKY family (Diao et al., 2021). In this study, nine WRKY genes were chosen for gene expression analysis under stress conditions, based on their known association with Foc-TR4 infection in bananas. Specifically *MaWRKY145, MaWRKY45, MaWRKY24, MaWRKY89, and MaWRKY65* reported to be highly expressed in banana roots at

3, 27, 51 hours post-infection (Goel et al., 2016); and *MaWRKY28, MaWRKY23, MaWRKY4,* and *MaWRKY25* found to be expressed in the corm at 1, 7 and 14 days post-infection (Zhang et al., 2019). The current study confirmed the elevated expression of these nine WRKY genes in banana plants following Foc-TR4 inoculation and under salt stress conditions (Fig. 4.30-31). Interestingly, most of these genes showed higher expression levels even in the absence of stresses when plants were inoculated with the consortium strains. However, it is noteworthy that *MaWRKY4* and *MaWRKY23* exhibited relatively lower expression in plants without consortium inoculation. While these two genes were not activated in the presence of consortium, along with the other seven *MaWRKY* genes studied, they demonstrated higher expression in plants primed with PGPB when subjected to stress compared to plants exposed to stress alone. These observations suggest that all nine genes may play a role in protecting plants from stress, with seven of them involved in priming, while the effects of *MaWRKY23* and *MaWRKY4* are evident only in conjunction with stress. Similar findings were reported in a study by Samain et al. (2022), where induced overexpression of WRKY TFs was observed during consortium priming of wheat plants against biotic and abiotic stresses. Among the cis elements identified in the *MaWRKY* gene promoters, MYC cis-elements were previously identified by in-silico analysis of induced systemic resistance as important regulators of priming (Pieterse et al., 2014). Eight of the nine stress-responsive *MaWRKYs* in this study (except for *MaWRKY145*) contain MYC cis-responsive elements, suggesting their potential association with stress priming in banana. As gene expression changes were measured relative to the non-inoculated plants, and data were available for both stresses at the 4-week time point, it was observed that expression under Foc-TR4 stress was higher compared to expression under salt stress for six of the assayed WRKY genes, but not for *MaWRKY24*, *MaWRKY65* and *MaWRKY89*. Based on this, it is likely that *MaWRKY89, MaWRKY65* and *MaWRKY24*. Based on this, it is likely that *MaWRKY89, MaWRKY65* and *MaWRKY24* are more closely associated with salt stress responses, while *MaWRKY25,* *MaWRKY4, MaWRKY65, MaWRKY45, MaWRKY23,* and *MaWRKY145* are more strongly associated with the biotic stress caused by Foc-TR4.

Among the analyzed *MaWRKY* genes, *MaWRKY4* showed the highest elevation upon inoculation with the consortium under Foc-TR4 stress conditions at both the 4th and 6th weeks. This finding aligns with the role of its Arabidopsis ortholog, which acts as a crucial MAMP-triggered immunity (MTI) regulator (Birkenbihl et al., 2018). This suggests the potential involvement of *MaWRKY4* as a mediator in biotic stress responses, evident in its greater increase in expression in banana under Foc-TR4 stress compared to the increase observed under salinity stress. The promoter region of *MaWRKY4* contains cis-regulatory elements including MYB, As-1, and W-box, suggesting that several interacting proteins linked to biotic stress are responsible for regulating this transcription factor. These elements are conserved across plant species and are also found in the promoters of PR genes in other plants, including rice and Arabidopsis (Kaur et al., 2017). The heightened expression of *MaWRKY4* that results from the consortium inoculation provides insights into how EB3+RB3 primes banana plants against Foc-TR4 stress. In addition to *MaWRKY4*, the two genes with the highest expression levels among the nine examined genes in consortium-primed and Foc-TR4-challenged banana plants were *MaWRKY45* and *MaWRKY65* at the six-week mark. *WRKY51*, the rice ortholog of *MaWRKY45* is known to positively regulate resistance against the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (Hwang et al., 2016). Interestingly, the promoter regions of *MaWRKY45* and its rice ortholog share common cis-elements such as TC-rich repeats, MYB, As-1, and W-box, which are responsive to the presence of bacteria. While rice responds to pathogen infection, the presence of these elements in *MaWRKY45* suggests their involvement in biotic stress priming mediated by the EB3+RB3 consortium in banana, shedding light on potential interacting proteins that activate the banana biotic stress response. *MaWRKY65*, an ortholog of *WRKY42* in Arabidopsis, plays a crucial role in regulating phosphate homeostasis and plant growth by facilitating phosphate acquisition and translocation (Su et al., 2015). Promoter analysis of *MaWRKY65* and its ortholog revealed the presence of biotic stress-responsive cis-elements, including TGACG, As-1, MYB, and ABRE, indicating potential interacting factors and shared pathways between these plants. Considering the proficiency of EB3+RB3 strains in phosphate solubilization (Fig. 4.1), their inclusion in the consortium may promote increased expression of *MaWRKY65*, which would improve phosphate acquisition and translocation in banana plants and increase their resistance to Foc-TR4 stress.

Under salt stress conditions, EB3+RB3 consortium inoculation resulted in the highest elevation of three transcription factors (TFs): *MaWRKY89* at 4 weeks, *MaWRKY145* and *MaWRKY23* at both 2 weeks and 4 weeks. This indicates temporal differences in the plant's response to stress. The *MaWRKY23* ortholog in Arabidopsis acts as a positive regulator of auxin signaling, fostering the growth of roots and shoots as well as flavonol biosynthesis (Grunewald et al., 2012). The presence of cis-element auxin-responsive (AuxRR-core) in the promoter of *MaWRKY23* and its Arabidopsis ortholog suggests its involvement in auxin production. Assuming that the *MaWRKY23* plays a role analogous its ortholog, this is consistent with both the EB3 and RB3 producing IAA (Fig. 4.5). The elevated expression of *MaWRKY23* in consortium-primed plants aligns with the enhanced root growth and plant height observed under salinity stress conditions. These findings indicate a potential role for *MaWRKY23* in mediating auxin-related responses in consortium-primed banana plants. The *MaWRKY89* ortholog in Arabidopsis functions as an abiotic stress regulator (Van Aken et al., 2013). Notably, abiotic stress-responsive cis-elements including ERE and W-box are present in both the Arabidopsis and *MaWRKY89* promoter regions. MaWRKY89's substantial increase in expression, induced by the EB3+RB3 consortium, indicates that it plays a role in priming banana plants for salt tolerance. The presence of common cis elements in the promoters of *MaWRKY89* and its Arabidopsis ortholog provides avenues for future investigations into the underlying mechanisms. *MaWRKY145,* another highly expressed TF upon consortium inoculation under salinity stress, is implicated in the regulation of H_2O_2 levels in its Arabidopsis ortholog by stimulating high catalase activity during leaf senescence (Guo et al., 2017). The promoter regions of *MaWRKY145* and its Arabidopsis counterpart share cis-elements such as CAT-box and MYB. The substantial upregulation of *MaWRKY145* expression suggests its potential role in consortium-mediated salt tolerance priming in banana plants. The presence of shared cis elements in the promoters of *MaWRKY145* and its Arabidopsis ortholog highlights potential mechanisms worth exploring in future studies.

CHAPTER 6: CONCLUSIONS

The primary aims of this study were to isolate halotolerant bacteria from mangrove plant rhizosphere and to identify, characterize and select halotolerant strains of bacteria for use as plant growth promoting agents. The results highlight the presence of halotolerant rhizobacteria and endophytic bacteria that can survive in high sea salt concentrations. These bacteria, including *Bacillus* sp. rhizobacterial isolates and *Pseudomonas* sp. endophytic bacteria, possess multiple beneficial plant growth-promoting traits. The study's additional goal was to investigate the potential of selected halotolerant PGPB to enhance stress tolerance in banana plants under salinity and Foc-TR4 infection. The selected strains successfully colonized banana roots and promoted enhanced growth, increased photosynthetic pigments, and elevated antioxidant enzyme activities under hydroponic conditions. A consortium of the strains of the halotolerant PGPB strains; *Pseudomonas* sp., (EB3) and *Bacillus* sp. (RB3) was prepared into a liquid formulation. The application of the consortium proved effective in priming and protecting banana plants against salinity stress and reducing the symptoms of Fusarium wilt caused by Foc-TR4 infection in a greenhouse study. Furthermore, the study aimed to examine the physiological and biochemical changes in banana plants treated with the PGPB consortium, including nutrient uptake, ion balance, photosynthesis, water relations, and enzyme activities. The consortium inoculation resulted in numerous beneficial effects on plant growth and stress tolerance. Under both salinity and Foc-TR4 stress conditions, the consortium inoculation promoted banana plant growth and increased the accumulation of chlorophyll pigments. It also enhanced the levels of proline, which is known to confer stress tolerance, and increased the activities of various antioxidant enzymes, including SOD, CAT, GR, APX, and POD. These enzymes play crucial roles in scavenging reactive oxygen species and minimizing oxidative damage in plants. Furthermore, the consortium inoculation reduced the levels of MDA, a marker of lipid peroxidation, and electrolyte leakage, indicating enhanced membrane integrity and reduced cellular damage. Under both non-stressed and salt-stressed conditions, the consortium inoculation led to higher K^+, Mg^+ and Ca^{2+} and lower the Na^+ and Cl^- contents in the banana plants. The presence of the PGPB isolates in the consortium inhibited the development of Fusarium wilt disease symptoms by increasing the activities of key antioxidant and defense enzymes such as β-1,3-glucanase, chitinase, lipoxygenase, and polyphenol oxidase. Additionally, consortium-inoculated banana plants exhibited higher lignin content and total soluble protein, which are associated with enhanced defense responses. The final aim of this study was to investigate the molecular mechanisms involved in PGPB-mediated stress mitigation, particularly focusing on the expression of WRKY transcription factors (TFs) and their regulation of gene expression during bio-priming of banana. Molecular analysis revealed that the consortium inoculation induced the expression of several WRKY transcription factors, including *MaWRKY4, MaWRKY23, MaWRKY24, MaWRKY25, MaWRKY28, MaWRKY45, MaWRKY65, MaWRKY89,* and *MaWRKY145*. These transcription factors are known to play crucial roles in regulating biotic and abiotic stress responses in plants. The presence of common cis-elements in their promoter regions, shared with functionally characterized stress-related orthologs from other plant species, suggests potential mechanisms by which stress priming is activated in banana. These mechanisms likely contribute to changes in host physiology and metabolic responses, ultimately improving stress resilience.

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