EFFECT OF NANO-ENCAPSULATED BIO-STIMULANT ON THE GROWTH, YIELD, AND QUALITY OF CHILLI (*CAPSICUM ANNUUM* L.)

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A thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

University of Nottingham

School of Biosciences

2023

Abstract

Bio-stimulants have demonstrated significant positive outcomes in enhancing plant health, boosting soil fertility, mitigating the adverse effects of environmental stress on plant growth, improving tolerance to heavy metal contamination in soil, and augmenting crop quality and yield. Limited studies have reported the usage of bio-stimulants in soilless medium to enhance plant growth, yield and quality. There are lack of innovative microbial and plant-based bio-stimulants for soilless cultivation systems. Only scarce information is available on the combined effect of bio-stimulants of various origins. Furthermore, microbial-based bio-stimulants have short shelf-life, which makes them not suitable for soilless systems. The hypothesis of the study is co-application of nano-encapsulated bio-stimulants, specifically endophytic plant growth-promoting bacteria and moringa leaf extract, may positively impact the growth, yield, and quality of chilli plants cultivated in soilless media. The main aim of this study is to evaluate the synergistic effect of nano-encapsulated bio-stimulants consisting of endophytic bacteria and moringa leaf extract in soilless medium.

To achieve this objective, this research was conducted using encapsulated nano-bio-stimulant to overcome the rapid degradation and increase the absorption efficiency by plants. Seventy endophytic bacteria were screened for various biochemical assays and plant growth-promoting traits in chilli seedlings. Fresh and dry moringa leaf was extracted using aqueous and polar solvents via maceration, decoction, and infusion methods to determine the phytochemical contents and plant growth-promoting efficiency of the extracts. Later, the selected endophytic bacteria was incorporated with nano-alginate whereas moringa leaf extract was encapsulated into nano-emulsion. The population of bacteria in nano-gel was determined after 6 months of shelf-life, and nano-emulsion was characterized using zetasizer. A completely randomized block design (RCBD) was adopted for this

experiment. There were two treatments, T1 (Liquid fertilizer) and T2 (Liquid fertilizer and nano-encapsulated bio-stimulant) with five replications of each treatment. Each replication consists of eight plants. The efficiency of encapsulated nano-biostimulant consisting of endophytic bacteria and moringa leaf extract applied via drenching method were evaluated on the seed germination, vegetative growth, yield, and post-harvest quality of chilli. Post-harvest analyses include chroma value, phenolic, flavonoid, ascorbic acid, and capsaicin content of chilli fruits. Finally, chilli root microbiome analysis was done using next-generation sequencing (NGS) to determine the impact of nano-encapsulated bio-stimulant on bacterial population in chilli roots.

Bacillus subtilis and *Streptomyces panaciradicis* were selected from seventy endophytic bacteria isolated from the leaf and root of neem, sesbania, moringa, and chilli plants. The selection was based on their potential to exhibit beneficial traits in biochemical assays, reduce mean seed germination time, increase germination energy, enhance Chilli's root colonization, and improve chilli seedlings' vegetative growth. Aqueous extraction of dry moringa leaf produced more flavonoids (74.89 quercetin equivalent mg/ml) compared to polar solvent extraction methods. A combined application of moringa leaf extract and endophytic bacteria demonstrated a positive response on the root and aboveground biomass and yield of chilli. Aqueous extraction of dry moringa leaf could be formulated into a stable oil-in-water nano-emulsion with particle size below 230 nm, polydispersity below 0.4, and a zeta potential value of more than -30 mV. Endophytic bacteria were formulated into a nano-alginate gel and could maintain a bacterial population of 1×10^8 cfu/ml throughout the storage period of six months. Nano-encapsulated bio-stimulants, when applied together on a soilless medium, increased the yield, flavonoid, phenolic, and ascorbic acid content of chilli. The total yield of chilli plants treated with nano-encapsulated bio-stimulant was 575.41g, whereas chilli plants not treated with bio-stimulants recorded a 522.16g yield. Chilli plants treated with bio-stimulant showed a higher L value of -15.38±11.9 and chroma valuef of 8.77±5.56 in chilli fruits

compared to -8.29±7.97 and 5.30±6 in chillies not treated with bio-stimulants. This finding is in agreement with the enhanced photosynthesis activity of chilli plants treated with bio-stimulants and the metabolites produced by endophytic bacteria such as rhodopsin, rhodoxanthan, lycoxanthan, rubixanthin, echinenone, carotene, zeaxanthin, and astaxanthin. Post-harvest quality analysis revealed the potential of nano-encapsulated bio-stimulants in increasing flavonoid content in red chillies to 15.7 mg/L, phenolic contents in green chillies to 0.908 mg/L and ascorbic acids in red chillies to 144.29 mg/L compared to chillies that were not treated with bio-stimulants. Chillies without bio-stimulant treatment recorded 13.0 mg/L of flavonoids, 0.82 mg/L of phenolic contents and 124 mg/L of ascorbic acid respectively. Root microbiome analysis revealed that nano-encapsulated bio-stimulant treatment has the majority of unique groups of bacteria that did not exist in other treatments. The principal variation of bacteria between treatments was assessed based on Weighted UniFrac and Bray-Curtis distances, which explained 48.11% and 44.96 % of the total observed variation.

This study has proved the potential of applying nano-encapsulated bio-stimulants to positively promote the growth, yield, and quality of chilli cultivated in a soilless medium. It is a pioneer research on the effect of nano-formulated bio-stimulants in a soilless medium. However, further research on the bio-stimulant dosage, application time, and method could maximize the benefit of bio-stimulant on crops.

Acknowledgment

I would like to express my deepest gratitude to my principal supervisor, Professor Asgar Ali, and my co-supervisors, Dr. Yasmeen Siddiqui and Dr. Christina Vimala Subramaniam, for their unwavering support and guidance throughout the research process. Their expertise, valuable insights, and constructive feedback have been instrumental in shaping this thesis.

I also express my sincere appreciation to the examiners for their valuable input and suggestions that significantly improved the quality of this work. I am grateful to the staff in the Faculty of Science and Engineering (FOSE) and the Graduate School of the University of Nottingham, Malaysia, who provided me with a nurturing academic environment and access to resources necessary for this research.

I want to acknowledge the support and encouragement of my postgraduate colleagues for their companionship, brainstorming sessions, and motivation.

My heartfelt thanks go to my parents, Mr. Matthews @ Machap and Mrs. Agnes @ Nagaratmah, my husband, Mr. Victor Paul, and my sons Joshua Abhivaanan and Jadon Pavithran for their love, support, and belief in my abilities. Their constant encouragement and sacrifices allowed me to pursue higher education and complete this thesis successfully. I sincerely thank my sisters, brothers-in-law, nieces, and nephews for their support.

Lastly, I am indebted to Malaysian Agriculture Research and Development Institute (MARDI) for sponsoring a full-time scholarship to pursue my studies.

TABLE OF CONTENTS **Page**

Abstract		i
Acknowledgment		iv
List of Figures		xv
List of Tables		xxiii
List of Appendices		xxvi
List of Abbreviations		xxix
CHAPTER 1.0: INTRODUCTION		1
1.0	Introduction	1
1.1	Hypothesis	5
1.2	Objectives	5
1.3	Impact of research	6
CHAPTER 2.0: LITERATURE REVIEW		7
2.1	Sustainable Crop Production	7
2.2	Major Challenges in Crop Production	8
2.3	Strategies for Sustainable Crop Cultivation	9
2.4	Botany and taxonomy of chilli	10
2.4.1	Chilli Cultivation in Malaysia	11
2.5	Application of Organic and Microbial Bio-stimulants in Chilli Cultivation	13
2.6	Bio-stimulant for Crop Cultivation	14
2.6.1	Classification of Bio-stimulants	16
2.6.2	Potential of Endophytic Bacteria as Bio-stimulant	17
2.6.2.1	Phosphorus Solubilization	19
2.6.2.2	Potassium Solubilization	19
2.6.2.3	Siderophore Production	20
2.6.2.4	Nitrogen Fixation	20

2.6.2.5 ACC Deaminase Production 21
2.6.2.6 Indole acetic-acid Production 21
2.6.2.7 Bio-control Activities of Endophytes 22
2.7 Potential of Moringa Leaf Extract as Bio-stimulant 23
2.8 Limitations of Bio-stimulant 24
2.9 Nanotechnology in Crop Cultivation 26
2.10 Nanotechnology in Bio-stimulant Development 27
2.10.1 Nano-encapsulation 28
2.10.2 Nano-emulsion 28
2.11 Advantages of Nanotechnology in Crop Cultivation 29
2.12 Bio-stimulant Performance Indicators 31
2.12.1 Seed Germination and Vigour 32
2.12.2 Vegetative Growth Parameters 33
2.12.3 Photosynthesis and Chlorophyll Content 35
2.12.4 Yield and Post-harvest Quality 36
2.12.5 Rhizobiome Assessment 38
2.13 Conclusion 41

CHAPTER 3: ISOLATION AND SCREENING OF PLANT GROWTH-PROMOTING ENDOPHYTES 42

3.0 Introduction 42
3.1 Materials and Methods 43
3.1.1 Plant Sample Collection 43
3.1.2 Isolation of Endophytic Bacteria from Roots and Leaves 45
3.1.3 Biochemical Screening of Plant Growth-promoting Traits in Endophytic Bacteria 45
3.1.4 Qualitative Screening of Plant Growth-promoting Traits in Endophytic Bacteria 46
3.1.5 *In vitro* Bio-control Activities of Endophytic Bacteria 47
3.1.6 Quantitative Screening of Plant Growth-promoting Traits 48
3.1.6.1 ACC Deaminase Production by Endophytic Bacteria 48
3.1.6.2 Indole-acetic Acid Production by Endophytic Bacteria 49
3.1.6.3 Phosphorus Solubilization by Endophytic Bacteria 50

3.1.6.4 Quantitative Screening of Biofilm Production by Endophytic Bacteria 51
3.1.7 Statistical Analysis 51
3.2 Results and Discussion 52
3.2.1 Endophytic Bacteria from the Roots and Leaves of Moringa, Sesbania, Neem, and Chilli 52
3.2.2 Plant Growth-promoting Biochemical Traits in Endophytic Bacteria 56
3.2.3 Phosphorus Solubilization by Endophytic Bacteria 67
3.2.4 Potassium Solubilization by Endophytic Bacteria 69
3.2.5 Nitrogen Fixation by Endophytic Bacteria 70
3.2.6 Chitinase Production by Endophytic Bacteria 72
3.2.7 ACC Deaminase Production by Endophytic Bacteria 72
3.2.8 Indole acetic-acid (IAA) Production by Endophytic Bacteria 74
3.2.9 Siderophore Production by Endophytic Bacteria 76
3.2.10 Bio-control Activity of Endophytic Bacteria Against *Colletotrichum* sp. 77
3.2.11 Bio-control Activity of Endophytic Bacteria against *Fusarium oxysporum* 78
3.2.12 Biofilm Production by Endophytic Bacteria 79
3.3 Conclusion 80

CHAPTER 4: CHARACTERIZATION OF PLANT GROWTH-PROMOTING ENDOPHYTES AND THE EFFICIENCY IN PROMOTING THE GROWTH OF CHILLI SEEDLINGS 82

4.0 Introduction 82
4.1 Materials and Methods 83
4.1.1 Preparation of Endophytic Bacterial Culture 83
4.1.2 Assessment of the Compatibility between Selected Endophytic Bacteria 84
4.1.3 Priming of Chilli Seeds with Endophytic Bacteria 84
4.1.4 Effect of Endophytic Bacterial Treatment on Chilli Seed

Germination 84
4.1.5 Effect of Selected Endophytic Bacterial Treatment from Seed Germination Test on the Vegetative Growth of Chilli Seedling 87
4.1.5.1 Effect of Endophytic Bacteria on the Relative Chlorophyll Content of Chilli Seedlings 88
4.1.5.2 Effect of Endophytic Bacteria on the Length and the Surface Area of Chilli Root 88
4.1.5.3 Effect of Endophytic Bacteria on the Height of Chilli Seedlings 88
4.1.5.4 Effect of Endophytic Bacteria on the Leaf Area Meter of Chilli Seedlings 88
4.1.5.5 Effect of Endophytic Bacteria on the Root-to-Shoot Ratio of Chilli Seedlings 89
4.1.6 Molecular Identification of the Selected Potential Endophytes based on Seedlings' Vegetative Growth and Bioinformatics Analysis 89
4.1.7 Metabolites Production by the Identified Endophytic Bacteria 89
4.1.8 Root Colonization Analysis by the Selected and Identified Endophytic Bacteria 90
4.1.8.1 Total Bacterial Population in Chilli Roots Inoculated with the Selected Endophytic Bacteria 91
4.1.8.2 Root Colonization by Single and Mixed Cultures of the Selected Endophytic Bacteria 91
4.1.9 Effect of Single and Mixed Cultures of the Identified Endophytic Bacteria on Chilli Growth and Yield 93
4.1.10 Effect of Single and Mixed Cultures of Identified Endophytic Bacteria on the Vegetative Growth of Chilli Plants 94
4.1.11 Effect of Single and Mixed Cultures of Identified Endophytic Bacteria on the Yield of Chilli Plants 94
4.1.12 Statistical Analysis 94
4.2 Results and Discussion 95
4.2.1 Assessment of the Compatibility between the Selected

Endophytic Bacteria 95
4.2.2 Effect of Endophytic Bacterial Treatment on Chilli Seed Germination 96
4.2.3 Effect of the Selected Endophytic Bacterial Treatment on the Vegetative Growth of Chilli Seedlings 99
4.2.4 Molecular Identification of the Selected Endophytic Bacteria and Phylogenetic Analysis 104
4.2.5 Metabolites Production by the Identified Endophytic Bacteria 107
4.2.6 Chilli Root Colonization by Single and Mixed Cultures of the Selected Endophytic Bacteria 110
4.2.7 Effect of Single and Mixed Cultures of Identified Endophytic Bacteria on the Vegetative Growth of Chilli Plants 113
4.2.8 Effect of Single and Mixed Cultures of Identified Endophytic Bacteria on the Yield of Chilli Plants 115
4.2.9 Conclusion 120

CHAPTER 5: CO-APPLICATION OF THE SELECTED ENDOPHYTIC BACTERIA AND MORINGA LEAF EXTRACT ON THE VEGETATIVE GROWTH AND YIELD OF CHILLI 122

5.0 Introduction 122
5.1 Materials and Methods 123
5.1.1 Preparation of Moringa Leaf Extract (MLE) 123
5.1.2 Phytochemical Analysis of Moringa Leaf Extract 126
5.1.2.1 Phenolic Content in Moringa Leaf Extract 126
5.1.2.2 Total Flavonoids in Moringa Leaf Extract 126
5.1.2.3 Free Radical Scavenging Activity of 2, 2-diphenyl-1-picryl -1-phenyl hydrazyl-hydrate (DPPH) of Moringa Leaf Extract 127
5.1.3 Effect of Moringa Leaf Extracts on the Vegetative Growth of Chilli Seedlings 127
5.1.3.1 Relative Chlorophyll Content of Chilli Seedlings 127
5.1.3.2 Root Length and Root Surface Area of Chilli Seedlings 128
5.1.3.3 Plant Height and Root: Shoot Ratio of Chilli Seedlings 128

5.1.4 Assessment of the Compatibility between Moringa Leaf Extract and Endophytic Bacteria 128
5.1.5 Synergistic Effect of Moringa Leaf Extract and Endophytic Bacteria on the Vegetative Growth of Chilli 129
5.1.6 Synergistic Effect of Moringa Leaf Extract and Endophytic Bacteria on the Yield of Chilli 133
5.1.7 Statistical Analysis 134
5.2 Results and Discussion 134
5.2.1 Phytochemical Contents in Moringa Leaf Extract 134
5.2.2 Effect of Moringa Leaf Extracts on the Vegetative Growth of Chilli Seedlings 139
5.2.3 Assessment of the Compatibility between Moringa Leaf Extract and Endophytic Bacteria 145
5.2.4 Synegistic Effect of Moringa Leaf Extract and Endophytic Bacteria on the Vegetative Growth of Chilli 150
5.2.5 Synegistic Effect of Moringa Leaf Extract and Endophytic Bacteria on the Yield of Chilli 153
5.3 Conclusion 154

CHAPTER 6: CHARACTERIZATION OF NANO-ENCAPSULATED ENDOPHYTIC BACTERIA AND MORINGA LEAF EXTRACT 156

6.0 Introduction 156
6.1 Materials and Methods 157
6.1.1 Encapsulation of Endophytic Bacteria and Moringa Leaf Extract 157
6.1.2 Effect of Homogenization on Bulking Material 158
6.1.2.1 Effect of the Homogenization Process on the Size and Polydispersity Index of Bulking Materials 158
6.1.2.2 Evaluation of the Bulking Materials and Plant Extract using Field Emission Scanning Electron Microscopy (FESEM) Analysis 159
6.1.3 Nano-encapsulation Formulations of Bio-stimulants 159
6.1.3.1 Nano-gel Formulation of Endophytic Bacteria 159

6.1.3.2 Nano-emulsion Formulation of Moringa Leaf Extract 160
6.1.4 Characterization of Nano-formulations 161
6.1.4.1 Physical Appearance of Nano-gel and Nano-emulsion 161
6.1.4.2 Physico-chemical Properties of Nano-emulsion 162
6.1.4.3 Structural Image of Nano-gel and Nano-emulsion using Field Emission Scanning Electron Microscopy (FESEM) Analysis 162
6.1.5 Assessment of the Stability of Nano-gel and Nano-emulsion Formulation during Storage 162
6.1.5.1 Physical Appearance of Nano-emulsion during the Storage Period 162
6.1.5.2 Physico-chemical Properties of Nano-emulsion and Nano-gel during the Storage Period 163
6.1.5.3 Functional Groups Detection in Raw Moringa Leaf Extract and Nano-emulsion of Moringa Leaf during the Storage Period 163
6.1.5.4 Shelf life of Encapsulated Endophytic Bacteria in Nano-gel 163
6.1.6 Effect of Nano-encapsulation Formulation on Chilli Seed Germination and Root Length 164
6.2 Results and Discussion 164
6.2.1 Effect of the Sonication Process on the Size and Polydispersity Index and Image of Bulking Materials 164
6.2.2 Characterization of Nano-gel and Nano-emulsion Formulation 169
6.2.2.1 Physical Appearance of Nano-emulsion and Nano-gel 169
6.2.2.2 Physico-chemical Properties of Nano-emulsion Formulations 171
6.2.2.3 Structural Image of Nano-gel and Nano-emulsion using Field Emission Scanning Electron Microscopy (FESEM) Analysis 175
6.2.2.4 Functional Compounds Detection in Nano-emulsion 177
6.2.3 Assessment of the Stability of Nano-gel and Nano-emulsion Formulation during Storage 183
6.2.3.1 Physico-chemical Properties of Nano-encapsulation Formulations during Storage 183
6.2.3.2 Functional Groups Determination in Nano-emulsion after Six Months of Storage 186
6.2.3.3 Shelf life of Encapsulated Endophytic Bacteria in Nano-gel 187
6.2.3.4 Effect of Nano-encapsulation Formulation on Chilli Seed Germination and the Length of Root 188

6.3 Conclusion 191

CHAPTER 7: EFFECT OF NANO-ENCAPSULATED BIO-STIMULANTS ON YIELD AND POST-HARVEST OF CHILLI 192

7.0 Introduction 192
7.1 Material and Methods 194
7.1.1 Nano-encapsulated Bio-stimulant Preparation 194
7.1.2 The Effect of Co-application of Nano-encapsulated Endophytic Bacteria and Moringa Leaf Extract in Soilless Cultivation System 195
7.1.3 The Effect of Nano-encapsulated Bio-stimulants on the Photosynthesis Activity of Chilli Plants 197
7.1.4 The Effect of Co-application of Nano-encapsulated Bio-stimulants on the Vegetative Growth of Chilli 198
7.1.5 The Effect of Nano-encapsulated Bio-stimulants on Chilli Length, Color, and Yield 199
7.1.6 The Effect of Nano-encapsulated Bio-stimulants on Phenolic and Flavonoid Contents in Chilli Fruits 201
7.1.7 The Effect of Nano-encapsulated Bio-stimulant on the Capsaicin Content in Chilli Fruits 202
7.1.8 The Effect of Nano-encapsulated Bio-stimulants on Ascorbic Acid of Chilli Fruits 203
7.1.9 The Impact of Nano-encapsulated Bio-stimulants on the Nutrient Content in Soilless Planting Material and in Different Parts of Chilli Plant 205
7.1.10 The Impact of Nano-encapsulated Treatments on Chilli Root Microbial Profiling Using Next-generation Sequencing Analysis (NGS) 206
7.1.10.1 Collection of Chilli Rhizosphere Samples and Metagenomics DNA Extraction 206
7.1.10.2 Library Preparation and Sequencing 207
7.1.10.3 Microbiome Data Analysis 207
7.1.11 Statistical Analysis 208

7.2	Results and Discussion	208
7.2.1	The Effect of Nano-encapsulated Bio-stimulants on the Photosynthesis Activity of Chilli Plants	208
7.2.2	The Effect of Nano-encapsulated Bio-stimulants on the Vegetative Growth of Chilli Plants	210
7.2.3	The Effect of Nano-encapsulated Bio-stimulants on the Yield and Post-harvest Qualities of Chilli such as Chilli Length, and Color	213
7.2.4	The Effect of Nano-encapsulated Bio-stimulants on Phenolic and Flavonoid Contents in Chilli Fruits	216
7.2.5	The Effect of Nano-encapsulated Bio-stimulant on the Capsaicin and Ascorbic Acid Content in Chilli Fruits	218
7.2.6	The Effect of Encapsulated Bio-stimulant Application via Drenching Method on the Nutrient Content of Different Parts of the Chilli Plant and the Soilless Medium	220
7.2.7	The Effect of Nano-encapsulated Treatments on Chilli Root Microbial Profiling Using Next-generation Sequencing Analysis (NGS)	224
7.2.8	Rarefaction	226
7.2.9	Shared and Unique ASVs	228
7.2.10	Alpha- and Beta- diversity	229
7.2.11	Phylogenetic Tree	234
7.2.12	Composition of the Microbial Community in Chilli Root	235
7.2.13	Correlation of the Chilli Root Microbiome	240
7.2.14	Linear Discriminant Analysis Effect Size (Biomarker Identifier)	243
7.2.15	Association among Chilli Root Microbial Profiling, Bio-stimulant Treatment and Nutrient Content in Soilless Medium	246
7.2.16	Association among Chilli Root Microbial Profiling, Bio-stimulant Treatment, and Nutrient Content in Different Parts of the Chilli Plant	249
7.3	Conclusion	253
CHAPTER 8: GENERAL CONCLUSION		254

RERERENCES 261

APPENDIX 290

LIST OF FIGURES **Page**

Fig. 3.1	Examples of endophytes isolated from the plant leaf and root	55
Fig. 3.2	Qualitative beneficial traits of endophytic Bacteria	66
Fig. 3.3	Orthophosphate solubilized from insoluble rock phosphates by endophytic bacteria after eight days of incubation	69
Fig. 3.4	Quantitative screening of ACC deaminase-producing endophytes based on standard α-ketoglutaric graph	74
Fig. 3.5	Quantitative analysis of indole acetic-acid production by potential endophytic bacteria	75
Fig. 3.6	Hydroxamate-siderophore production by potential endophytic bacteria	75
Fig. 3.7.	Inhibition percentage of *Colletotrichum* sp. by antagonistic endophytes in dual culture agar plate assay	78
Fig. 3.8	Inhibition percentage of *Fusarium* sp. by the antagonistic activity of endophytes in a dual culture plate assay	79
Fig. 3.9	Exopolysaccharides (Biofilm) production by endophytic bacteria	80

Fig. 4.1	Compatibility test between three isolates of endophytic bacteria	95
Fig. 4.2	Graph shows the mean germination time (MGT) of chilli seeds treated with potential endophytic bacteria	98
Fig. 4.3	Graph depicts the germination energy (%) of chilli seeds treated with potential endophytic bacteria	99
Fig. 4.4	Root images of chilli seedlings inoculated with endophytic bacteria captured using WinRhizo Root Scanner	103
Fig. 4.5	Phylogenetic tree of the identified endophytic bacteria using the Neighbour-Joining method	105
Fig. 4.6	Morphology of *Bacillus subtilis* in potato dextrose agar	106
Fig. 4.7	Morphology of *Streptomyces panaciradicis* in potato dextrose agar	106
Fig. 4.8	Field Emission Scanning Electron Microscopy (FESEM) of chilli roots colonized with endophytic bacteria	112
Fig. 4.9	Efficiency of *Bacillus* and *Streptomyces* endophytic bacteria to increase the height of chilli plants	115
Fig. 4.10	Efficiency of *Bacillus* and *Streptomyces* bio-stimulant to increase the length of chilli	

fruits 116

Fig. 4.11 Efficiency of *Bacillus* and *Streptomyces* bio-stimulant to increase the length of chilli roots 116

Fig. 4.12 Efficiency of *Bacillus* and *Streptomyces* bio-stimulant to increase the total area of chilli root surface 117

Fig. 4.13 Efficiency of *Bacillus* and *Streptomyces* bio-stimulant to increase the chilli root diameter 117

Fig. 4.14 Efficiency of *Bacillus* and *Streptomyces* bio-stimulant to increase the chilli root volume 118

Fig. 4.15 Efficiency of *Bacillus* and *Streptomyces* bio-stimulant to increase the aboveground biomass of chilli plants 118

Fig. 4.16 Efficiency of *Bacillus* and *Streptomyces* bio-stimulant to increase the root biomass of chilli plants 119

Fig. 4.17 Yield of five chilli plants 40 days after transplanting 119

Fig. 5.1 Moringa leaf extraction using aqueous decoction and maceration methods 129

Fig. 5.2 Relative chlorophyll content of chilli seedlings treated with moringa leaf extract of various extraction methods 139

Fig. 5.3 The height of chilli seedlings treated with moringa

leaf extracts of boiling, infusion, and maceration methods 140

Fig. 5.4 Total root surface area of chilli seedlings treated with moringa leaf extract of various extraction methods 141

Fig. 5.5 Root length of chilli seedlings treated with moringa leaf extract of various extraction methods 141

Fig. 5.6 Root-to-shoot ratio of chilli seedlings treated with moringa leaf extract of various extraction methods 143

Fig. 5.7 Root biomass of chilli seedlings treated with moringa leaf extract of various extraction methods 143

Fig. 5.8 Shoot biomass of chilli seedlings treated with moringa leaf extract of various extraction methods 144

Fig. 5.9 Morphology of endophytic bacteria tested with moringa leaf extracted using boiling and maceration methods 144

Fig. 5.10 Pure cultures of *Streptomyces* and *Bacillus* show no growth inhibition when mixed with diluted (1:100) moringa leaf extract 149

Fig. 5.11 Root length of chilli plants treated with moringa leaf extract and endophytic bacteria 150

Fig. 5.12 Root surface area of chilli plants treated with moringa leaf extract and endophytic bacteria 151

Fig. 5.13 Aboveground biomass of chilli plants treated with

	moringa leaf extract and endophytic bacteria	152
Fig. 5.14	Root biomass of chilli plants treated with moringa leaf extract and endophytic bacteria	152
Fig. 5.15	Yield per chilli plant treated with combined application of moringa leaf extract and endophytic bacteria	153
Fig. 6.1	Encapsulation methods for plant extract and bacteria	158
Fig. 6.2	Nano-gel containing *Bacillus* sp. culture	170
Fig. 6.3	Physical appearances that show the stability of the nano-emulsion system	171
Fig. 6.4	Characterization of moringa leaf nano-emulsion	174
Fig. 6.5	Distribution of *Bacillus* cells on nano-gel using FESEM imaging	176
Fig. 6.6	A well-dispersed oil-in-water nano-emulsion of moringa leaf extract (5,000x magnification)	177
Fig. 6.7	Oil-in-water nano-emulsion of moringa leaf extract formed clusters (5,000 x magnification)	178
Fig. 6.8	FTIR spectra show the difference in the functional groups of raw moringa leaf extract and nano-emulsion of moringa leaf extract	181-182
Fig. 6.9	FTIR spectra show the stability of moringa leaf nano-emulsion after two months of storage	187

Fig. 7.1	Dark adaptation method for quantum yield (QY) measurement to determine the photosynthesis activity of chilli plants	198
Fig. 7.2	Phytochemical analysis in chilli fruits. (a) Phenolic content; (b) Flavonoid content	202
Fig 7.3	Chilli pericarps and the chilli extracts in vials for capsaicin and ascorbic acid analysis using HPLC	205
Fig.7.4	Effect of nano-encapsulated bio-stimulants on the quantum yield (QY) of chilli plants at one-month interval	210
Fig. 7.5.	Effect of nano-encapsulated bio-stimulants on the height of chilli plants	212
Fig. 7.6	Effect of nano-encapsulated bio-stimulants on aboveground biomass of chilli plants	212
Fig. 7.7	Effect of nano-encapsulated bio-stimulants on the biomass of chilli roots	213
Fig. 7.8	Effect of nano-encapsulated bio-stimulants on the capsaicin content in chilli fruits	219
Fig.7.9	Effect of nano-encapsulated bio-stimulants on the ascorbic acid content in chilli fruits	220
Fig. 7.10	Rarefaction curves comparing the alpha diversity of bacteria in chilli root rhizosphere (a) Shannon; (b) Goods' coverage, and (c) Simpson	227
Fig. 7.11	Venn diagram of the shared and unique amplicon	

	sequence variants of chilli root microbiome	228
Fig. 7.12	Alpha diversity was calculated based on Bray-Curtis index using PERMANOVA statistics	232
Fig. 7.13	Beta diversity represented by the Principal Coordinate Analysis (PCA) plot with distances between subjects determined by the weighted UniFrac metric	233
Fig. 7.14	Beta diversity represented by the Principal Coordinate Analysis (PCA) plot with distances between subjects determined by the Bray Curtis metric	234
Fig. 7.15	Dendogram shows linkage between bacterial populations in chilli roots of different treatments	235
Fig. 7.16	Pie chart shows the main phyla commonly found in chilli roots of all three treatments	237
Fig. 7.17	Species-level identification of each chilli root sample in all the treatments	239
Fig. 7.18	Percentage of *Bacillus* and *Streptomyces* genus in the root microbiome of all three treatments	240
Fig. 7.19	Correlation between bacterial genus in chilli roots using Sparse Partial Correlation Coefficient (SPARCC)	242
Fig. 7.20	Linear Discriminant Analysis (LDA) scores of differentially abundant species among individuals in each treatment. The top 15 LDA scores represent the effect size of each abundant phylum	244

Fig. 7.21 Linear Discriminant Analysis (LDA) scores of differentially abundant species among individuals in each treatment. The top 15 LDA scores represent the effect size of each abundant class 245

Fig. 7.22 Heatmap shows the correlation between Actinobacteriota and Firmicutes groups in different treatments and the nutrient content in the soilless medium 248

Fig. 7.23 Heatmap shows the correlation between Actinobacteriota and Firmicutes groups in different treatments and the nutrient content in different parts of chilli 252

LIST OF TABLES **PAGE**

Table 3.1	Details of plant sampling for the isolation of endophytes	44
Table 3.2	Endophyte isolates from medicinal plants and local chilli varieties	54
Table 3.3	Screening of beneficial activities in endophytic bacteria isolated from moringa, neem, sesbania, and chilli plants	59
Table 3.4	Classification of plant growth-promoting endophytes according to beneficial traits and host plant	64
Table 4.1	Seed germination treatments using the selected endophytic bacteria based on their superior *in vitro* beneficial traits	86
Table 4.2	The details of the individual and mixed culture treatments of four endophytic bacterial inoculants selected from seed germination test on Chilli seedlings' vegetative growth	87
Table 4.3	Logistic regression predicting the likelihood of seed germination based on treatments	98
Table 4.4	Vegetative growth analysis of chilli seedlings with beneficial endophytic bacteria	102
Table 4.5	Molecular identification of endophytic bacteria based on National Centre for Biotechnology Information (NCBI) GenBank database BLAST search	104

Table 5.1	Moringa leaf extraction using decoction, maceration and infusion methods	125
Table 5.2	The details of the treatments containing various combination of aqueous extract of moringa leaf and endophytic bacteria	130
Table 5.3	Phytochemical analysis of moringa leaf extract	137
Table 5.4	Compatibility of endophytic bacteria with moringa leaf extracted using boiling and maceration methods	148
Table 6. 1	The effect of sonication on moringa leaf extract and bulking materials based on zetasizer and FESEM analyses	166
Table 6.2	Physico-chemical properties of moringa leaf extract nano-emulsion	173
Table 6.3	Fourier-Transform Infrared Spectroscopy (FTIR) analysis on the changes in the functional groups of moringa leaf extract and nano-emulsion of moringa leaf extract	179
Table 6.4	Physico-chemical stability of nano-emulsion formulations	185
Table 6.5	Shelf life of the nano-formulation and its efficiency in stimulating seed germination and the root length of chilli	190
Table 7.1	Nano-encapsulated bio-stimulant treatments for the cultivation of chilli in soilless growth medium	196

Table 7.2	Maturity index of chilli fruits	199
Table 7.3	The effect of nano-encapsulated bio-stimulants on the length, color, and yield of chilli	215
Table 7.4	The effect of nano-encapsulated bio-stimulants on the phenolics and flavonoid content in green and red chillies	217
Table 7.5	Nutrient content in soilless medium (cocopeat) after uprooting chilli plants	222
Table 7.6	Nutrient contents in different parts of the chilli plants using ICP-MS	223
Table 7.7	Amplicon sequence unit (ASV) details of each group of treatment applied to chilli plants	224
Table 7.8	Individual alpha diversity metrics in chilli roots for every group of treatment	231
Table 7.9	The composition of microbial community between different treatments of chilli roots	236

LIST OF APPENDIX **PAGE**

Fig. A1	Standard graph for soluble phosphorus (orthophosphate)	290
Fig. A2	Qualitative screening of ACC deaminase-producing endophytes	291
Fig. A3	Alpha ketoglutaric standard calibration curve	292
Fig. A4	Pre-liminary screening of indole acetic acid-producing endophytes	293
Fig. A5	Indole-acetic acid standard calibration curve	294
Fig. A6	Screening of siderophore-producing endophytes	295
Table A4	Logistic regression predicting the likelihood of seed germination based on treatments	296
Fig. A7	The DNA sequences of *Bacillus subtilis* (MR3) in the NCBI database under the accession numbers OM714810	297
Fig. A8	The DNA sequences of *Streptomyces panaciradicis* (MR13) deposited in the NCBI database under the accession numbers OM001090	298
Fig. A9	GC-MS chromatogram of *Bacillus* sp. (MR3)	299
Fig. A10	GC-MS chromatogram of *Streptomyces* sp. (MR13)	300
Fig. A11	The highest peak produced by *Bacillus* sp. at RT 7.167 min	301

Fig.A12	The highest peak produced by *Streptomyces* sp. at RT 7.202 min	302
Table A5	Metabolites produced by *Bacillus* sp. and *Streptomyces* sp. based on largest peak area	303
Table A6	The functional metabolites of *Bacillus* (MR3)	305
Table A7	The functional metabolites of *Streptomyces* (MR13)	308
Table A8	Unidentified compounds of *Bacillus* sp. (MR3)	313
Fig. A9	Gallic acid standard graph	320
Fig. A10	Quercetin standard graph	320
Table A11	Physico-chemical properties of moringa leaf nano-emulsion	321
Fig. A12	Experimental layout for chilli cultivation using a fertigation system	324
Table A13	Fertigation schedule according to the Department of Agriculture, Malaysia	325
Fig. A14	Standard graph of capsaicin	326
Fig. A15	Standard graph of ascorbic acid	326
Fig. A16	Chilli root microbiome at order level	327
Fig. A17	Chilli root microbiome at class level	328

Fig. A18 Chilli root microbiome at family level 329

Fig. A19 Chilli root microbiome at genus level 330

LIST OF ABBREVIATIONS

AB	Liquid fertilizer AB
ACC	1-aminocyclopropane-1-carboxylate
ASV	Amplicon Sequence Unit
Chl	Chlorophyll
CIRP	Christmas Island Rock Phosphate
DPPH	2,2-diphenyl-1-picrylhydrazyl
EC	Electrical conductivity
GAE	Gallic acid equivalent
GE	Germination Energy
GI	Germination Index
HCN	Hydrogen Cyanide
HMDS	Hexamethyldisiloxane
IAA	Indole acetic-acid
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
IPA	Indole-3-pyruvic acid
IPM	Integrated Pest Management
LDA	Linear Discriminant Analysis
LEfSe	Linear Discriminant Analysis Effect Size
L-Trp	L-Trptophan
MGT	Mean Germination Time
NCBI	National Centre for Biotechnology Information
OTU	Operation Taxonomic Unit
PCA	Principal Component Analysis
PCoA	Principal Coordinate Analysis
PDA	Potato Dextrose Agar
PGPB	Plant growth-promoting bacteria
PGPEB	Plant growth-promoting endophytic bacteria
PGP	Plant growth promoting
PGPR	Plant growth-promoting rhizobacteria
PiCRUSt2	Phylogenetic Investigation of Communities by Reconstruction of Unobserved Stats (Software)
PSB	Phosphorus-solubilizing bacteria

PVK	Pikovaskaya’s agar
QILME	Quantitative Insights into Microbial Ecology (software)
QY	Quantum Yield
ROS	Reactive Oxygen Species
RT	Room Temperature
SparCC	Sparse Correlations for Compositional Data
SPRI	Solid Phase Reversible ImmobilizationMethodology
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
VI	Vigor Index

CHAPTER 1.0
Introduction

In recent years, the global population growth and rising demand for nutritious food have been identified as pressing issues, particularly in the post-pandemic and climate change era. Innovative and improvised technologies have been introduced in modern crop cultivation to enhance crops' growth, resilience, productivity, and quality. Promoting sustainable agriculture practices has become increasingly important to ensure that food supplies meet the increasing demand while maintaining the quality of products.

In Malaysia, the National Agro-Food Policy 2021-2030 (NAP) aims to promote sustainable and efficient agriculture practices and encourage modern technologies to improve agricultural productivity. Additionally, the Malaysian government's 'MyOrganic' initiative, launched in 2018, promotes adopting organic farming practices, including bio-stimulants and other natural inputs, to improve soil health and plant growth. In Malaysia, the bio-stimulant market is still nascent and relatively small compared to other countries in the region. However, the market is expected to grow in the coming years due to increasing demand for sustainable agricultural practices and the government's initiatives to promote organic farming. According to a report by Mordor Intelligence, the bio-stimulant market in the Asia-Pacific region, including Malaysia, is expected to grow at a compound annual growth rate of around 11.2% from 2020 to 2025, while the global bio-stimulant market was valued at USD 2.4 billion in 2020 and is projected to reach USD 4.9 billion by 2026, growing at a CAGR of 11.3% during the forecast period (2021-2026).

Eco-friendly soil amendments and conditioners have been explored extensively to revitalize soil health from the adverse impacts caused by chemical fertilizers. Soil degradation, nutrient pollution, greenhouse gas emission, eutrophication, and pesticides leaching into the surrounding soil were among the significant effects of chemical fertilizers and pesticides that have led to low soil organic carbon and reduced soil microbial biodiversity (I. Arif, Batool, & Schenk, 2020).

The conventional methods using soil as the planting medium have caused many soil-borne diseases and declined soil fertility due to heavy metals toxicity, drought, salinity, and acidity. The problems caused by soil as a planting medium have allowed the soilless medium to invade the agricultural systems with many benefits. In recent years, soilless agriculture has grown in popularity among commercial farmers, where hydroponics involves the direct provision of nutrient solutions in a liquid medium. In contrast, the solid medium includes growing plants in peat, perlite, vermiculite, coco peat, rock wool, sand, and sawdust enriched with nutrient solutions (Azizoglu et al., 2021). However, soilless growing mediums lack the complex microbial communities found in soil, so incorporating beneficial microorganisms into soilless growing systems can be essential for achieving optimal plant growth and crop productivity. By manipulating the microbial community in the rhizosphere of the soilless systems, plant nutrition could be increased in addition to reducing the plant stress response and pathogen attack (Edmonds, Sackett, Lomprey, Hudson, & Moser, 2020). One standard method is using plant-growth-promoting rhizobacteria. Root colonizing PGPR has been used in soil and soilless crop cultivation systems due to their positive effects on nutrient uptake, plant stress control, and antagonism with pathogens (Balliu et al., 2021).

Bio-stimulants are substances of natural origin that can improve the physiological functions of plants, resulting in increased uptake of nutrients, higher crop yield, and improved crop quality (Agliassa et al., 2021). They offer unique benefits that can complement bio-fertilizers and organic amendments in crop production. PGPR acts as phytostimulators by synthesizing various phytohormones that could influence cell division, cell enlargement, root initiation, phototropism, growth rate, apical dominance, and plant geotropism (Bhat et al., 2022). In addition, it could increase abiotic stress tolerance, fix nutrients for easy absorption, activate plant growth regulator synthesis, producing siderophores, volatile organic compounds, and protective enzymes (Bhat et al., 2022). Applying beneficial microorganisms could enhance plant growth and yield via various direct and indirect methods. Direct methods such as nutrient solubilization and mobilization, phytohormone production, chelating compounds production for nutrient transportation, and plant stress alleviating

compound production have been discussed extensively in past research. Indirect methods such as antibiotic production enzymes related to fungal wall lysis and antifungal and antibacterial compounds production were also necessary to protect plants from pests and disease, promoting their growth and yield (Kumari, Bhatnagar, Deepali, Mehla, & Vashistha, 2022). *Trichoderma*, *Pseudomonas*, *Bacillus*, Arbuscular Mycorrhizal Fungi (AMF), Phosphobacteria, *Azosprillum*, *Azotobacter*, *Rhizobium*, etc., were among the beneficial microorganisms that were introduced as bio-fertilizer or bio-stimulant for crop growth and yield (Kumari et al., 2022). Beneficial microorganisms could act as bio-stimulants, enhancing plant growth and crop production in response to abiotic stress in extreme environments (Romano et al., 2020). The inadequate development of microbial inoculants due to issues with formulating them has resulted in a lack of successful inoculants capable of performing their intended functions; hence, immobilization has been recommended to create effective formulations for PGPR inoculants (Vassilev et al., 2017).

Likewise, the application of bio-stimulants made from plant extracts is also deemed to boost the growth and yield of crops. Plant extracts that have been added as bio-stimulants are also reported to bring benefits to plant growth and production. Moringa leaf extract was regarded as plant-based and a cheap source of natural plant growth enhancers (I. Afzal, Akram, Rehman, Rashid, & Basra, 2020; D. S. Arora & Kaur, 2019; Refai et al., 2023). It contains various beneficial vitamins, nutrients, flavonoids, phenols, and phytohormones, which can positively impact crop growth and development (Mashamaite, Ngcobo, Manyevere, Bertling, & Fawole, 2022). There has been growing interest among researchers in exploring the potential of moringa leaf extract to enhance crop yield and quality. A study has suggested that bio-stimulants derived from moringa leaf extracts are easy to prepare, cheap, effective, and environmentally safe (Zulfiqar, Casadesús, Brockman, & Munné-Bosch, 2019). Another study has suggested that moringa leaf extract can serve as a natural bio-stimulant due to cytokinin (zeatin), gibberellic acid, ascorbic acid, phenolic compounds, and other minerals in significant quantities. These compounds can enhance plants' antioxidant and nutritional profile, thereby, increasing their growth and yield (M. S. Rana, Hoque, & Abedin, 2019). Additionally, it is proven that moringa leaves

are rich in various active compounds that could enhance crop growth (Ahmad, Tanveer, Liaqat, & Dole, 2019; Alkuwayti, El-Sherif, Yap, & Khattab, 2020; Hussain et al., 2021; Rady, Kuşvuran, Alharby, Alzahrani, & Kuşvuran, 2019). However, to date, there has been limited research into developing an optimal formulation for using moringa leaf extract as a bio-stimulant.

While bio-stimulants can provide many benefits to plants, some limitations and challenges are associated with their use. Bio-stimulants' limitations include lack of standardization, variable effectiveness, limited research, cost, and compatibility with other bio-products. Due to being a relatively new area of study, regulations and standardization have yet to be firmly established for bio-stimulant production. The limited application of bio-stimulants, particularly in developing countries such as Malaysia, can be attributed to several factors, including a lack of research on their effectiveness on specific crops and soilless media, limited knowledge about their compatibility with other bio-stimulants, unclear understanding of their mode of action; as well as challenges related to storage and high production cost.

Chilli is a high-value and high-demand vegetable crop essential to Malaysian cuisine (Yusoff et al., 2022). Besides their economic importance, chilli fruits rich in vitamins, phenolics, and flavonoids are vital for human health (Cui et al., 2022). The self-sufficiency level of chilli production in Malaysia is only 51.4% (Zakaria, Ismail, Awang, Megat Wahab, & Berahim, 2020). Thus, Malaysia imports 39% chillies from Thailand and Vietnam to fulfill the demand (Yusoff et al., 2022). Pest, disease, and high temperature have been identified as the major contributors to a decline in Malaysia's chilli production (Cui et al., 2022; Yusoff et al., 2022). Thus, soilless chilli cultivation is becoming increasingly popular in Malaysia as farmers seek to optimize the yield and reduce the environmental impact (Zakaria et al., 2020).

Nanoparticles have a direct effect, such as the availability of nutrients in the rhizosphere, and an indirect impact, such as the stimulating effects on bacterial community in a plant-microbe interaction (S. Rai et al., 2023). Thus, the plant growth-promoting endophytes and moringa leaf extracts were encapsulated in

this study as bio-stimulant for chilli plants grown in a soilless medium. This research assessed the combined application of nano-encapsulated moringa leaf extract and PGP endophytes bio-stimulant on chilli crops grown using the soilless medium. Nano-encapsulation method was used to prepare the bio-stimulants to extend the shelf-life and to increase their penetration to plant cells during application to achieve optimum growth and yield.

1.1 Hypothesis

This study hypothesizes that combining bio-stimulants, specifically endophytic plant growth-promoting bacteria and moringa leaf extract, may positively impact the growth, quality, and yield of chilli plants cultivated in soilless media.

1.2 Objectives

The main objective of this research is to assess the effectiveness of the encapsulated bio-stimulant consisting of endophytic bacteria and moringa leaf extract on chilli growth, yield, and quality in the soilless medium by the drenching method. The main objective was achieved by accomplishing five sub-objectives representing five experimental chapters.

a) Chapter three demonstrated the isolation and screening of beneficial endophytic bacteria using biochemical assays.
b) Chapter four described the effect of endophytic bacteria on the vegetative growth and yield of chilli and the selection of potential bacterial bio-stimulant candidates.
c) Chapter five has shown the impact of moringa leaf extract on the vegetative growth and yield of chilli, and the extraction method and solvent selection.
d) Chapter 6 demonstrated nano-formulation preparation for endophytic bacterial and moringa leaf extract.
e) Chapter 7 determined the effect of the combined application of nano-encapsulated bio-stimulant on chilli growth, yield, and quality, and their impact on the root microbiome of chilli grown in a soilless medium.

1.3 Impact of research

This research can be regarded as the pioneering research on developing an environmentally friendly nano-formulation using endophytes and moringa leaf extract for sustainable crop cultivation. It could significantly impact farmers' economic growth if the bio-stimulant amendment can increase the yield and quality of chilli in a soilless medium that merely relies on hydroponic liquid fertilizer (AB fertilizer). Integrating nanotechnology in this research is expected to stabilize the endophytic bacteria and biological compounds of moringa leaf extract during storage. This technology could be explored further to be applied along with liquid fertilizers in fertigation and hydroponic systems for various crop cultivation.

CHAPTER 2.0
LITERATURE REVIEW

2.1 Sustainable Crop Production

The United Nation's Food and Agriculture Organization has set a goal for 2030 that focuses on sustainable technologies for producing essential goods, implementing adaptable farming systems that boost productivity and production while preserving the ecosystem, enhancing resilience to climate change, extreme weather, droughts, and other disasters, and gradually improving soil quality. Agriculture has been the backbone of the economic development of a nation. Sustainable crop production is crucial for ensuring food security, reducing environmental impact, and fostering long-term financial stability in a country. It refers to the methods and practices used to produce crops that meet the demands of the increasing population without compromising on the quality of the produce. Farming techniques that are environmentally friendly and economically viable are essential to improve livelihood and productivity without harming the environment.

In Malaysia, the government has taken several measures to encourage sustainable crop production, promote eco-friendly practices, mitigate the harmful impact of agriculture on the environment, and foster sustainable economic development in the agricultural sector. These policies and initiatives encompass a range of areas, including reducing pesticide use, adopting organic farming practices, and promoting bio-fertilizers and organic amendments in crop cultivation. The National Agro-Food Policy 2021-2030 aims to transform the agriculture and agro-food sector towards sustainable, inclusive, and competitive growth. The policy promotes sustainable crop production, resource-efficient agriculture, and environmentally friendly practices. Aside from this, the National Key Economic Areas (NKEAs) identified the agriculture sector as one of the key economic sectors to increase productivity, create employment opportunities, and promote sustainable practices in the agriculture sector. Apart from that, the Integrated Pest Management (IPM) Program encourages using environmentally friendly pest control methods to reduce harmful pesticides in crop production by

reducing the negative impact of pesticides on the environment and human health. In addition to all these efforts, the government of Malaysia provides financial incentives and technical support to farmers who adopt organic farming practices. It aims to promote sustainable crop production by reducing synthetic inputs and promoting natural resource management.

2.2 Major Challenges in Crop Production

Extreme weather change, soil degradation, water scarcity, lack of modern farming technologies and pest diseases that damage crops, declining soil fertility and increasing soil contamination, and environmental stresses have been the significant limitations in crop cultivation in the last few years that have contributed to the reduction of yields and quality. In Malaysia, limited arable land availability, extreme climate change, soil degradation, limited water availability, pest and disease outbreaks, and labor shortages are a few factors that negatively affect agriculture sustainability in the long term.

Food safety and security are essential to nurturing a healthy and productive society. Unfortunately, most food producers use excessive chemicals such as fertilizers, pesticides, insecticides, and fungicides that can deteriorate human health and harm biodiversity (Meena et al., 2017). Apart from causing environmental pollution and posing a health risk, frequent and over-application of chemical fertilizers could reduce soil fertility.

Soil fertility is one of the primary threats to agricultural productivity that can be improved by biological nutrient cycling (M Kaleem Abbasi & Anwar, 2015). The fertility of Malaysian tropical soil, such as peat, sandy, and acid sulfate, is mostly low. In addition, soil richness has further deteriorated tremendously due to excessive and imbalanced use of agrochemicals such as fertilizers (Adarsh Kumar, Usmani, & Kumar, 2017), pesticides, herbicides (Sun et al., 2019), and insecticides.

Global warming has been regarded as the primary cause of biotic and abiotic stresses to plants, such as extreme temperature, salinity, drought, and pathogen

attack. These biotic and environmental stresses could hamper plants' absorption and utilization of nutrients. A plant's nutrient use efficiency determines its health and quality. Consequently, crops do not get enough specific nutrients from the soil (Meena et al., 2017). Nutrient imbalance in a plant makes it more vulnerable to pathogenic attacks (Marchiol, Iafisco, Fellet, & Adamiano, 2020). It may cause a reduction in productivity, low quality of yield, stunted growth, and low immunity to plant diseases. New strategies have been explored for the sustainable cultivation of crops.

2.3 Strategies for Sustainable Crop Cultivation

The Green Revolution succeeded in providing food security to meet the growing global population. However, its heavy reliance on fertilizers and pesticides resulted in adverse environmental consequences. Alternative strategies for using the chemicals for plant disease control and plant growth stimulation are crucial to ensure reduced negative impacts on human health and the environment. Promising strategies such as implementing sustainable farming practices, developing drought-resistant crop varieties, using integrated pest management, improving soil health, and investing in modern farming technologies are vital to overcoming the challenges for enhanced food security. Previous studies have reported that productivity can increase to 50-60% when applied plant-growth-promoting rhizobacteria (Nwachukwu & Babalola, 2022). New biocontrol and bio-stimulant products based on the consortia of microorganisms or natural substances could be a better strategy to improve their efficiency. (Besset-Manzoni, Rieusset, Joly, Comte, & Prigent-Combaret, 2018). (H. Li et al., 2021)suggested seed bio-priming with beneficial microorganisms as an innovative crop protection tool for sustainable crop yield improvement. Bio-stimulants can induce the end of dormancy and boost the germination of numerous plant species, even in abiotic stress, by modifying the physiological processes within the seeds (Shubhpriya Gupta, Doležal, Kulkarni, Balázs, & Van Staden, 2022). Aside from this, bio-stimulants in organic farming can improve plant resilience to nutrient limitations (Karabulut et al., 2021).

The lack of agricultural land has been a critical issue worldwide due to modernization and urbanization. Soilless production systems over conventional systems give higher yields, better pest control management, and more efficient labor use (Adak, Tozlu, & Gubbuk, 2018). Incorporating beneficial microorganisms such as cyanobacteria in the rooting medium should be emphasized as an economical and sustainable strategy to increase the availability of primary and micronutrients in the soilless medium (Bharti et al., 2020). Applying bio-stimulants can help address nutrient deficiencies in soilless growing media. Bio-stimulants' effect in improving nutrient content was proven by cultivating strawberry plants in a soilless medium that exhibited an 18-20% increase in fruit yield, skin firmness, and high nutritional value (Soppelsa et al., 2019). Furthermore, applying bio-stimulant treatments in existing farming systems resulted in a 70% and 50% increase in cassava and okra yields, respectively. Bio-stimulant application allowed a reduction in nitrogen fertilizer use while still achieving optimal yields at lower rates of N fertilization (Canellas et al., 2022).

2.4 Taxonomy and Botany of chilli

Chilli belongs to the *Capsicum* genus and Solanaceae family. Twenty-five species within the genus have been grown and cultivated globally. Out of these, *C. annuum, C. chinense, C. frutescence, C. pubescence,* and *C. baccatum* are recognized as domesticated plants (Lob, Aris, Sidique, Ibrahim, & Jin, 2017). The genus comprises a diverse group of sweet and hot chilli peppers. Among all, *C. annuum* is the most widely cultivated and economically important species and includes both sweet- and pungent-fruited cultivars of various shapes and sizes. *C. annuum* L. are perennial woody plants grown as herbaceous annuals. It is said to be the first-ever domesticated crop in America. The species are grown in temperate climates, but they are especially productive in warm and dry climates. It is also known as bell peppers.

Chillies can be eaten raw, cooked, pickled, roasted, or dehydrated. Chilli extracts contain phytochemicals including vitamin C, A, flavonoids, anthrocyanins, capsaicinoids, and carotenoids. It is also a good source of vitamin C, vitamin A,

vitamin E, vitamin B5, potassium, magnesium, iron, calcium, phosphorus, and carotenoids.

The *Capsicum annuum* plant is a tender perennial subshrub, with a densely branched stem. The plant reaches 0.5-1.5 m tall depending on the species. Typically leaves are smooth, simple, entire, glabrous, and flat. The flowers are usually solitary, and creamy white, and the seeds are straw-colored. Single white flowers develop into the fruit, typically green when unripe. Ripening fruits usually change to red, yellow, orange, or red. Taproots are strong, and lateral roots are numerous. Stem irregularly angular to subterete, up to 1 cm in diameter, much branched, often tomentose near branchings, green to brown-green, often with purplish spots near nodes. Leaves alternate, simple, variable; petiole up to 10 cm long; leaf-blade ovate. Flowers, usually borne singly, terminal; pedicel up to 3 cm long in flower, up to 8 cm long in fruit; calyx cup-shaped, persistent, and enlarging in fruit (K. R. . Swamy, 2023).

2.4.1 Chilli Cultivation in Malaysia

Chilli is widely grown worldwide, particularly in Asian and South American countries. They are the mature fruits of different species of the *capsicum* genus and serve as a popular condiment and vegetable in cooking. (J. Jiang et al., 2018). It is also an important agricultural crop known for its nutritional value as an excellent source of phytochemicals with antioxidant properties (Ertani et al., 2015).

Global chilli production is reported to increase by nearly 30% due to its increasing demand and wide utilization in cuisines, pharmaceuticals, cosmetics, and natural coloring agents. In Malaysia, chilli varieties such as kulai 469, kulai 151, kulai 223, kulai 461, and kulai 568 are preferred by chilli growers. The chilli varieties grown in Malaysia include local and imported varieties, with some of the most popular being ‘bird's eye’ and ‘village’ chilli. According to the crop statistical report 2019 by the Department of Agriculture Malaysia, chilli plantations in Malaysia cover 2,664 ha of plantation area and 26,354 metric tons of production yearly. However, about 39% of the country's demand for chilli is

met by importation from Thailand and Vietnam, especially during festive seasons (Yusoff et al., 2022). Some of the significant chilli-growing regions in Malaysia include Kelantan, Johor, and Pahang.

Chilli can be grown in various soil types, although well-drained soils with good fertility and organic matter are generally preferred. The crop can be produced using conventional and organic farming methods, using a range of inputs, including fertilizers, pesticides, and other agrochemicals to manage pests and diseases.

Soilless culture under a protected structure has been used in Malaysia for commercial vegetable production to improve plant growth, yield, and farmers' income (Zakaria et al., 2020). Soilless chilli cultivation in Malaysia typically uses a substrate or growing medium, such as rock wool, coco coir, or peat moss, to support the plant roots. The substrate is then irrigated with nutrient-rich water to give the plants essential nutrients for growth. Soilless cultivation has several advantages over traditional soil-based cultivation methods. With soilless cultivation, the grower has precise control over the nutrient levels in the growing medium, which can result in better plant growth and higher yields. Soilless cultivation uses less water than traditional soil-based methods. Furthermore, it can be done in a smaller area than conventional soil-based methods, as the plants are grown in a vertical system or another space-efficient setup. It also can reduce the incidence of pest and disease problems from soil pathogens. In short, the advantages of soilless production systems over conventional systems are higher yield, better pest control management, and more efficient labor use (Adak et al., 2018). Plant bio-stimulant provide an excellent fit with the soilless system due to their ability to improve nutrient use efficiency, tolerance to abiotic stress, and improved quality of produce (Admane et al., 2023).

Chilli is primarily grown in lowlands using conventional and fertigation systems. Fertigation systems are more popular among entrepreneurs as the growth and yield of chilli have been reported to increase by 3-4 times greater than conventional cultivation systems (Y. Mohd, Arshad, Muhammad, & Sidek, 2014). Despite the high cost and initial capital required to implement the

fertigation system, higher production levels offset these expenses. By utilizing this method, it is possible to achieve a significant increase in yield, up to two or three times higher than the conventional approach, which provides substantial net income for entrepreneurs (Y. S. Mohd, Arshad, Farah, Muhamad, & Sidek, 2016). Nonetheless, a viability analysis demonstrated that this technology has a strong competitive advantage due to its ability to generate higher returns than the conventional method. It is aligned with the current agricultural practices of sustainable agricultural development.

2.5 Application of Organic and Microbial Bio-stimulants in Chilli Cultivation

Chilli cultivation can significantly benefit from incorporating organic amendments into the soil, as it can lead to a range of advantages, such as increased soil fertility, improved nutrient availability, and accelerated plant growth. Organic amendments are substances added to soil to increase its organic matter content, which can improve soil structure, water-holding capacity, and nutrient availability.

Plant growth-promoting bacteria (PGPB) have been widely reported to increase crop yield and show enhanced growth on various plants. However, reports on successful cases of bio-formulation on chilli growth and yield are still limited. A study on the impacts of bio-stimulant during an entire cycle of chilli cultivation showed positive effects on plant growth, fruit quality, and chemical composition at flowering and maturity growth stages (Ertani et al., 2015). PGPBs were reported to revitalize soil characteristics and play an essential role in converting poor-quality land into arable land (Gouda, George, Das, Paramithiotis, & Shin, 2018). Green seaweed extracts have been reported to induce the germination of pepper seeds up to 68% (Muthu-Pandian Chanthini et al., 2019). The seaweed extracts were able to augment the soil in primed chilli seeds. When goat manure and urea were applied in conjunction with EM4, they synergized the growth parameters and yield of the chilli plant. In addition, the use of urea was positively correlated with the leaf's nitrogen content and chlorophyll index, while increasing amounts of goat manure showed a positive

correlation with stomatal density (Setiawan, Maghfoer, & Nihayati, 2016). The inoculation of mineral solubilizing and nitrogen-fixing bacterial strains resulted in increased growth and physiological parameters in chilli (Devi, Kaur, Kour, Yadav, & Suman, 2022). However, a contradicting report indicated that applying bio-fertilizer in various concentrations did not increase the average total chlorophyll content of curly red chilli plants (Siswanti, Utaminingsih, & Pangestuti, 2019). Likewise, it was found that phosphate-solubilizing microorganisms and rock phosphate alone did not significantly influence chilli growth (M. K. Abbasi, Musa, & Manzoor, 2015). Most studies have reported the effect of PGPB on chilii grown on soil. Still, minimal studies have reported the impact of bio-stimulant application for chilli growth in a soilless medium. Thus, it is crucial to investigate the response of the chilli plant when bio-stimulant is applied to the soilless medium.

2.6 Bio-stimulant for Crop Cultivation

When applied in small quantities, bio-stimulants are natural or synthetic substances that trigger plants' metabolic and physiological processes. In addition, it could increase abiotic stress tolerance, fix nutrients for easy absorption; activate plant growth regulator synthesis, producing siderophores, volatile organic compounds, and protective enzymes (Bhat et al., 2022). Plant bio-stimulant studies have gained the recent attention of the scientific community and farmers due to their multi-functions, especially during this period of drastic climate change. It has greater scope to be developed into a more refined product to assist the plant in combating climate change while boosting growth and productivity. It plays both the roles of bio-fertilizer and bio-control, where it can improve plants' physiological responses towards nutrient acquisition, increase nutrient absorption efficiency and tolerance to biotic and abiotic stresses, and enhance plant growth, yield and health while reducing mineral fertilizer consumption. Besides, bio-stimulant also could augment soil microbial function, nutrients, and pH in the rhizosphere (Shanmugam & Seth, 2018). Bio-stimulants could improve the biodiversity and nutrient composition of the soil.

Plant bio-stimulant is not dependent on their nutrient content but on the stimulation of the plant's natural physiological processes such as the plant growth or the plant's defense system (R. Bulgari, Cocetta, Trivellini, Vernieri, & Ferrante, 2015; Chanda, Merghoub, & EL Arroussi, 2019). They may contain various active substances, including beneficial microorganisms that work together to trigger the plant's physiological system and stress tolerance, and improve production quality. (Chanda et al., 2019) emphasized that the mode of action of the plant bio-stimulants needs to be investigated to understand the plant's metabolic pathway that the bioactive compounds in the bio-stimulant might stimulate. The composition of plant bio-stimulants usually is unknown due to the complexity of the chemical compounds and the wide range of substances (R. Bulgari et al., 2015) used in their formulation. It contains various bioactive compounds that might work in synergism with plants. However, the mechanisms of each bio-stimulant component need to be investigated to get a holistic view of the physiological changes caused by bio-stimulant on soil and plant (García-Gaytán et al., 2018). In general, bio-stimulants have been reported to work with numerous mechanisms, such as increased cation exchange capacity, stimulation of plasma membrane H+-ATPase, and activation of phenylpropanoid metabolism, which involves stress responses of plants (Jardin, 2015). The different components in the bio-stimulant could be the precursors for the biosynthesis of various compounds and diverse metabolic pathways. Therefore, the mechanisms are pretty complicated and vague. However, the phenotypic characteristics show that bio-stimulants positively affect crops' growth and yield. In addition, bio-stimulant prepared using the same raw materials might have a different mode of action that depends on the processing method (Rodrigues, Baptistella, Horz, Bortolato, & Mazzafera, 2020). The effect and way of action of the bio-stimulant might vary, although the origin of the bio-stimulant is the same. Most studies characterize the bio-stimulating impact on the raw material but fail to evaluate the presence and stability of the compounds in the formulated products. The mechanisms of bio-stimulant might differ according to their core function. For example, humic acids and protein hydrolysates can enhance nutrient availability by modifying soil physicochemical properties and forming complexes with micronutrients to increase their availability for plant uptake (Karabulut et al., 2021).

Specific elicitors that influence plant growth or defense mechanisms would be very helpful in addressing the significant challenges due to climate change. Studies have indicated that bio-stimulants applied with and without fertilizers could increase seed germination, seedlings growth, plant physiological growth, plant yield, and quality of fruits and vegetables (Abourayya et al., 2020; M. Ali et al., 2019; Dutta et al., 2019; Kapur, Sarıdaş, Çeliktopuz, Kafkas, & Paydaş Kargı, 2018). Numerous laboratory and field-scaled experiments have corroborated the benefits and efficiency of bio-stimulants. It can tolerate environmental stresses such as drought, salinity, and heavy metal pollution (Forouzi, Ghasemnezhad, & Nasrabad, 2020; M. H. Hussein, Eltanahy, Al Bakry, Elsafty, & Elshamy, 2021; A. Noman et al., 2018). These are special features not commonly found in other bio-based or chemical-based agricultural inputs.

2.6.1 Classification of Bio-stimulants

At the earlier stage, The European Bio-stimulant Industry introduced eight categories of bio-stimulants (Calvo, Nelson, & Kloepper, 2014). However, after a few revisions to the definition of bio-stimulant, the European Union (EU) Fertilizing Products Regulation made a claim-based definition proposal in 2019 (Ricci, Tilbury, Daridon, & Sukalac, 2019). Here, the manufacturer might have to justify the claims on the product's label whether the bio-stimulant increases nutrient use efficiency and tolerance against abiotic stresses, enhances the quality of crops, or solubilizes the fixed nutrients in the soil before placing it in the EU market. Generally, the classification has been done based on the source of the bio-stimulant, application method, active ingredients, target crop, and physical form of bio-stimulant. Natural bio-stimulants were made by enhancing or modifying natural substances. In contrast, synthetic bio-stimulants were artificial growth-regulating compounds to simulate the natural compounds with growth-promoting and disease-preventing effects on plants. For example, Cyanobacterium-based silver nanoparticles are considered synthetic bio-stimulants, as the silver nanoparticles were biosynthesized from an aqueous extract of the Cyanobacterium (Hamed, Hagag, & El-raouf, 2019). The majority

of carbon nanomaterials are synthesized, while some exist naturally. Organic bio-stimulants can be extracted from microorganisms, plants, organic materials, and agricultural and industrial waste materials. In contrast, inorganic bio-stimulants can be made from mesoporous silica, hydroxyapatite, zeolites, metals, and metal oxide for better efficiency. Therefore, there has yet to be a clear-cut classification for bio-stimulant.

2.6.2 Potential of Endophytic Bacteria as Bio-stimulant

PGPR acts as phytostimulators via the synthesis of various phytohormones that could influence cell division, cell enlargement, root initiation, phototropism, growth rate, apical dominance, and geotropism in plants (Bhat et al., 2022). *Trichoderma*, *Pseudomonas*, *Bacillus*, Arbuscular Mycorrhizal Fungi (AMF), Phosphobacteria, *Azosprillum*, *Azotobacter*, *Rhizobium*, etc., were among the beneficial microorganisms that were introduced as bio-fertilizer or bio-stimulant for crop growth and yield (Kumari et al., 2022). Beneficial microorganisms could act as bio-stimulants and enhance plant growth and crop production in response to abiotic stress in extreme environments (Romano et al., 2020). Direct methods such as nutrient solubilization and mobilization, phytohormone production, chelating compounds production for nutrient transportation, and plant stress alleviating compound production have been discussed extensively in past research. Indirect methods such as antibiotic production enzymes related to fungal wall lysis and antifungal and antibacterial compounds production were also necessary to protect plants from pests and disease, promoting their growth and yield (Kumari et al., 2022).

Beneficial microorganisms originating from the soil rhizosphere have been found to have the ability to promote plant growth and increase soil fertility. They are the most environmentally friendly and a better alternative to conventional agricultural practices in attaining sustainable agriculture (Shikha Gupta & Pandey, 2019). PGPR is used to produce microbial stimulants and enhancers to increase soil fertility, promote plant growth and prevent plant diseases. They may avoid or limit the spread of disease via various mechanisms such as the production of antibiotics, cell-wall degrading enzymes, lowering ethylene levels,

induced systemic resistance of the host (ISR), decreasing the amount of iron available to pathogens by siderophore production and the synthesis of pathogen-inhibiting volatile and non-volatile compounds (Santoyo, Moreno-Hagelsieb, del Carmen Orozco-Mosqueda, & Glick, 2016).

Endophytes are microorganisms that colonize plant tissues without inducing any disease symptoms. It is a specialized group of rhizobacteria that have acquired the ability to invade their plant host. Some unique features of the endophytes should have facilitated the colonization in the host plant. It is indicated that root colonization by bacteria is considered a requirement for a successful biological control activity (Weng, Wang, Li, Shen, & Zhang, 2013). Plants and microorganisms communicate via chemical interaction, especially by manipulating rhizodeposits from roots. Colonization by endophytes and the adaptation to a niche require genomic specialization as different plants attract microbial populations to their rhizosphere (Farrar, Bryant, & Cope-Selby, 2014). The majority of the endophytes exist both as free-living and endophytic microorganisms because bacteria must demonstrate rhizosphere competence, attachment to the root, and establishment in the host plant in the process of transition from soil to the plant tissues (Farrar et al., 2014).

Due to the broad host range and successful colonization ability, endophytes are proposed as a powerful tool in bio-organic stimulant production. Furthermore, the beneficial effects of the endophytic bacteria on host plants are usually more significant than those of many rhizospheric bacteria (Imran Afzal, Shinwari, Sikandar, & Shahzad, 2019). It was also reported that plant growth-promoting bacterial endophytes might demonstrate superior beneficial activity more directly in the host than rhizobacteria (Orozco-Mosqueda, Glick, & Santoyo, 2020). Endophytic bacteria belonging to one genus were reported to show a wide range of host diversity (Audipudi, Chakicherla, & Bhore, 2017). The relationship between endophytes and plants is unique as plants can select a microbiome to live within their tissue that can facilitate plant development by dissolving insoluble nutrients in the soil, controlling phytopathogens, delivering plant hormones, promoting plant growth, mitigating biotic and abiotic stress, enhancing plant tolerance and bio-remediating contaminated soil. Endophytes

encourage plant growth and protect the plant from pathogens; they can communicate and interact with the plant more efficiently than rhizospheric bacteria (Santoyo et al., 2016). It has been reported that endophytes isolated from moringa barks showed a significant increase in the shoot and root biomass in tomato plants after treating tomato seeds with the endophytes (Khan et al., 2016). Plants deprived of endophytes would be more vulnerable to environmental stress and diseases. Therefore, it is speculated that endophytes possess unique characteristics as bio-stimulant or bio-fertilizer candidates.

2.6.2.1 Phosphorus Solubilization

Phosphorus (P) is a major limiting nutrient in arable soils. Many mineral fertilizers applied to soil become unavailable rapidly due to precipitation and fixation with ferric and aluminum ions in acidic soils and calcium ions in alkaline soils. Only 0.1% of the total P in soil is estimated to be available for plant assimilation (Rezakhani, Motesharezadeh, Tehrani, Etesami, & Mirseyed Hosseini, 2019). Thus, most of the arable soils are rich with fixed and unavailable P that could be solubilized or released with the help of soil microorganisms. Phosphate-solubilizing endophytic bacteria are of great potential to be bio-stimulants due to their ability to increase phosphorus bioavailability, soil fertility, and crop productivity. Microbial phosphate solubilization is an important bio-stimulant trait via various mechanisms such as phytase, phosphatase, organic acids, and siderophore production (De Zutter et al., 2021). They were also suggested as a substitute for mineral phosphate fertilizers to meet plants' P demand (Kalayu, 2019).

2.6.2.2 Potassium Solubilization

Potassium is an essential nutrient for plant growth and development. It is highly mobile as a positively charged cation and can be easily transported from the roots to other plant parts. Its presence is crucial for synthesizing chlorophyll, which is necessary for the plant's ability to do photosynthesis. Furthermore, potassium helps to regulate the opening and closing of stomata cells, which allows the plant to control water loss through transpiration. It also promotes water uptake by the

roots, ensuring the plant has access to enough water to support its growth. When a plant experiences a potassium deficiency, it can result in decreased production and yield.

Potassium solubilization refers to the process of making K available to plants by converting insoluble forms of K in the soil into soluble forms. This process occurs through various mechanisms, including weathering of minerals, synthesis of capsular polysaccharides, and production of organic ligands. Capsular polysaccharides are complex carbohydrates that some microorganisms can synthesize. These polysaccharides can act as chelators, which bind to K and make them more available to plants.

2.6.2.3 Siderophore Production

The iron limitation is also another common problem in agricultural soils. Iron deficiency in plants could disrupt chlorophyll production in plants, which will affect photosynthesis. Some beneficial microorganisms can secrete iron-chelating compounds or siderophores, which bind with iron to form a siderophore-iron complex. Siderophores with a great affinity towards Fe^{3+} could make it available for their growth and plants (Shikha Gupta & Pandey, 2019). It is typically used to control root pathogens by iron competition process (Fgaier & Eberl, 2011). The siderophores produced by microorganisms can be classified into four main classes such as carboxylate, hydroxamates, catecholate, and mixed type. These microorganisms can bind toxic metals such as Cr^{3+}, Al^{3+}, Pb^{2+}, Cd^{2+}, and Hg^{2+} and are thus used to detoxify heavy metal contamination in soil (N. K. Arora & Verma, 2017). The ability to produce siderophore is one trait that makes microbes successful competitors in the soil environment (Loaces, Ferrando, & Scavino, 2011).

2.6.2.4 Nitrogen Fixation

Biological nitrogen fixation is a classic example of a symbiotic relationship between nitrogen-fixing bacteria and plant root nodules. Rhizosphere diazotrophs face different challenges compared to symbiotic nitrogen fixers

because they are not provided with a stable environment like pH, energy, nutrients, and oxygen, which are optimized for symbiotic nitrogen fixers by their plant host (Smercina, Evans, Friesen, & Tiemann, 2019). However, some free-living nitrogen-fixing bacteria can also fix the atmospheric nitrogen into ammonia, making it accessible for plant uptake. The nitrogenase enzyme system mediates this conversion. Nitrogenase is comprised of two metalloproteins: the iron (Fe) protein, which synthesizes ATP, and the molybdenum-iron (Mo-Fe) protein, which reduces substrates such as N_2 and protons (Smercina et al., 2019).

2.6.2.5 ACC Deaminase Production

Ethylene is an essential modulator of plant growth and development and an important feature in the response of plants to a wide range of stresses, including nutrient deficiency, heavy metals, organic and inorganic chemicals, extreme temperatures, drought, flooding, salinity, ultraviolet light, insect, nematode and phytopathogen damages and mechanical wounds (Glick, 2014). ACC is ethylene's immediate precursor, inhibiting seedling growth under various abiotic and biotic stresses. Stress induces ACC oxidase that prompts the plant's synthesis of ethylene and defensive genes (Glick, 2014). ACC deaminase is an enzyme that catalyzes the cleavage of ACC, the immediate precursor of ethylene in plants, into α-ketobutyrate and ammonia. It presents in the cytoplasm of bacteria at a deficient level. ACC deaminase-producing bacteria reduce plant ACC concentration and the extent of ethylene inhibition of plant growth. ACC deaminase must function before any significant amount of ACC oxidase is induced (Glick, 2014). Seeds coated with ACC deaminase-producing bacteria act as a mechanism to regulate ethylene level in plants so that it does not elevate and harm root growth (Z. Li, Chang, Lin, Li, & An, 2011). It benefits ethylene-sensitive plants such as canola, pepper, and tomatoes (Penrose & Glick, 2003).

2.6.2.6 Indole Acetic-acid Production

The production of Indole-3-acetic acid (IAA) is a crucial aspect of plant activity as it is the primary member of the auxin family responsible for regulating various plant functions. IAA plays an essential role in activities such as leaf formation,

embryo development, root initiation and development, abscission (the natural falling of leaves), phototropism (the growth response to light), geotropism (the growth response to gravity), fruit development, and more. IAA significantly influences root growth, increasing the length and number of root branches, hairs, and laterals (Chandra, Askari, & Kumari, 2018). This increased root growth, in turn, aids in the uptake of nutrients from the surrounding soil, ultimately leading to better plant health and development.

A major pathway for the biosynthesis of Indole-3-acetic acid (IAA) involves a combination of transamination and decarboxylation reactions of L-tryptophan (L-Trp), carried out by microorganisms and plants. The process begins with converting L-Trp into indole-3-pyruvic acid (IPA) by aminotransferases, which is then followed by the decarboxylation of IPA into IAA by decarboxylases. This pathway is widely observed in various microorganisms and plants and is considered one of the primary mechanisms for IAA synthesis (Aswathy, Jasim, Jyothis, & Radhakrishnan, 2013).

2.6.2.7 Bio-control Activities of Endophytes

The bio-control activity of endophytic bacteria could be mediated by various mechanisms, such as the production of hydrogen cyanide, enzymes (chitinases, cellulases, proteases), and antibiotics. Hydrogen cyanide is a secondary metabolite that could inhibit cytochrome C oxidase and other metalloenzymes in microorganisms. Hence, it is considered toxic to most microorganisms. Chitin is a natural polymer of N-acetyl-D-glucosamine monomers linked by β-1-4-covalent bonds. Chitinases found in some microorganisms can decompose chitin, a component of the cell wall of fungi (Brzezinska, Jankiewicz, & Walczak, 2013). Chitinase production is an essential criterion of a defense method against fungal pathogens. Biological control of some soil-borne fungal diseases has been correlated with chitinase production by microorganisms.

Anthracnose caused by *Colletotrichum truncatum*, formerly known as *C. capsici*, is a significant disease of chilli that appears as ripe fruit-rot and dieback that causes the infected plants to bear fewer fruits with low quality

(Chanchaichaovivat, Ruenwongsa, & Panijpan, 2007). It causes a yield loss of up to 50% worldwide. It was reported that PGP- inoculated chilli plants could induce resistance up to 71% against the pathogen *Colletotrichum truncatum* by utilizing the innate immunity of the chilli plant (Gowtham, Murali, Singh, Lakshmeesha, & Murthy, 2018). *Trichoderma* has been reported to stimulate induced systemic resistance against the pathogen in chilli (Saxena, Mishra, Ray, Raghuwanshi, & Singh, 2019). (Ertani et al., 2015) indicated that bio-stimulant application on chilli positively affected plant growth and yield without compromising food quality.

Fusarium oxysporum is a soil-borne fungus that causes chilli wilt disease. This pathogen is distributed worldwide with a great diversity of hosts that causes damage to various crops. Endophytic bacteria were previously reported to show high antifungal activity against *F. oxysporum* (G. F. Wang et al., 2019).

2.7 Potential of Moringa Leaf Extract as Bio-stimulant

When plant extracts that are rich in bioactive compounds applied as foliar or spray, they can stimulate the plant defense system and growth. *Moringa oleifera* is regarded as one of the promising alternatives for inorganic fertilizers (M. Ali et al., 2019). It is considered a natural bio-stimulant due to its high amount of cytokinin (zeatin), gibberellic acid, ascorbates, phenolics, and minerals (Refai et al., 2023). It has gained research priority in recent years as it could show promising results on seed germination, nutrient use efficiency, plant growth and yield under abiotic stress, and good post-harvest quality of produce (Abd El-Mageed, Semida, & Rady, 2017; Alkuwayti et al., 2020; Batool, Khan, & Basra, 2020; Desoky, Elrys, & Rady, 2019; El-Serafy & El-Sheshtawy, 2020; Elrys, Merwad, Abdo, Abdel-Fatah, & Desoky, 2018; Howladar, 2014; Nasir, Khan, Basra, & Malik, 2016; Rady et al., 2019; Shaukat et al., 2017; ShM, NE, MS, & AM, 2017; Waqas, Khan, Akhter, Noor, & Ashraf, 2017; Zulfiqar, Casadesús, et al., 2019). A study has suggested that bio-stimulants derived from moringa leaf extracts are easy to prepare, cheap, effective, and environmentally safe (Zulfiqar, Casadesús, et al., 2019). Another study has suggested that moringa leaf extract can serve as a natural bio-stimulant due to cytokinin (zeatin),

gibberellic acid, ascorbic acid, phenolic compounds, and other minerals in significant quantities. These compounds can enhance plants' antioxidant system and nutritional profile, thereby increasing their growth and yield (M. S. Rana et al., 2019). Additionally, it is proven that moringa leaves are rich in various active compounds that could enhance crop growth (Ahmad et al., 2019; Alkuwayti et al., 2020; Hussain et al., 2021; Rady et al., 2019).

The liquid extracted from moringa leaf has been used to improve the growth and productivity of several crops (Mashamaite et al., 2022). Moringa leaf extract sprayed onto the leaves of onions, bell pepper, soya beans, sorghum, coffee, tea, chili, melon, and maize has also shown an increase in yields (M. Ali et al., 2019). Seed priming using moringa leaf extract could effectively increase the biomass and yield of wheat plants under salt stress (Rady et al., 2019). Plants can produce defense-related oxide-reductive enzymes such as peroxidase and polyphenol oxidase to form a physical barrier upon pathogen attack (Gowtham et al., 2018). Besides, it was recommended as a bio-organic fertilizer due to its ability to enhance the physiological growth of Eruca plants (rocket) with only 2% of moringa leaf extract and 3% of moringa twig extract (Abdalla, 2013). Despite environmental stresses, moringa leaves were proven to enhance crop harvest (Shah, Razaq, Ali, Han, & Chen, 2017). Moringa leaf extracts were mainly used for promoting growth and alleviating abiotic stresses that cause impediments to plant growth. Moringa leaf and twig extracts exhibited a robust scavenging effect on 2,2-diphenyl-2-picrylhydrazyl (DPPH) free radical superoxide, nitric oxide extreme, and inhibition of lipid peroxidation which can prevent oxidative damage to plant tissues (Abdalla, 2013).

2.8 Limitations of Bio-stimulant

Although bio-stimulants could tackle agricultural issues such as abiotic stress, pest and disease, loss of nutrients, and heavy metal pollution, it has not been used widely on conventional farms for various reasons. For instance, drastic climate change caused rapid degradation of the bio-stimulant components in soil. It was reported that many plant-based bio-pesticides, such as Azadirachtin and rotenone, were degraded during field application due to high temperature and

microbial processes (Singh, Handa, & Manchanda, 2021). Unlike other agricultural inputs, bio-stimulant is more cost-effective as it could be efficient in low concentrations. However, fast degradation could lead to frequent application and increase crop production costs. Apart from the active ingredients from plants, bio-stimulant products containing PGPR are often reported to have low cell density and reduced longevity during the storage of the product and upon application to soil, leading to reduced efficiency. (Bhatia, Gulati, & Sethi, 2021) elucidated that maintaining stable colonization of applied PGPR strains in the rhizosphere has been a major challenge. Consequently, PGPR-based bio-stimulants are considered unstable and inefficient, producing inconsistent laboratory and field experiment results. Shelf life is the utmost important feature of a product that determines its sustainability in the commercial market. No matter how efficient a product is, it will not reach a large group of consumers if the shelf life is short. Short shelf life has been the greatest challenge in developing bio-fertilizers, bio-pesticides, and bio-stimulants using microorganisms and other natural compounds that may degrade rapidly. For example, the bioactive compounds of microalgae bio-stimulant were susceptible to degradation over time (Stirk et al., 2021). The secondary metabolites, such as carotenoids in microalgal biomass (*Chlorella vulgaris* and *Haemotococcus Pluvialis*), are unstable as the degradation can be triggered by light, high temperature, and the presence of oxygen (Gouveia & Empis, 2003). Long-term storage of liquid microbial formulations under ambient temperature conditions is crucial for commercialization (Elsakhawy, Ghazi, & Abdel-Rahman, 2021). Predominantly, stability is vital for liquid bio-stimulants such as vermicompost leachate, seaweed extract, and microbial bio-stimulant that are very sensitive to the storage condition (Shubhpriya Gupta et al., 2021). The inadequate development of microbial inoculants due to issues with formulating them has resulted in a lack of successful inoculants capable of performing their intended functions hence, immobilization has been recommended to create effective formulations for PGPR inoculants (Vassilev et al., 2017).

2.9 Nanotechnology in Crop Cultivation

According to the European Commission, "Nanomaterial means a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate, where 50% or more of the particles are in the number size distribution of one or external dimension is in size range of 1-100 nm (P. K. Rai et al., 2018).

In agriculture, nano-technology plays a vital role in increasing the nutrient use efficiency of crops. It also helps to reduce the wastage of nutrients as the predicted amount of fertilizers can be supplied to produce in a slow-release manner for optimum utilization. Nanoparticles have a high surface tension that helps to hold material more robustly than standard surfaces. Nanocoatings protect fertilizers and aid in the slow release of nutrients to plants (Wilson et al., 2008). A controlled delivery system will be helpful for the application of fertilizers and pesticides. Nanoparticles can act as efficient delivery vehicles due to their large surface area and easy absorption via various mechanisms such as adsorption, attachment, encapsulation, or entrapment of active ingredients into the nano-matrix (Ghormade, Deshpande, & Paknikar, 2011). Nanoparticles combined with bio-fertilizers have shown good plant growth promotion under field conditions. It is also cost-effective as one liter of nano-biofertilizers can be used in several hectares of crops (Duhan et al., 2017). The nano size of the particles aids in their solubility and capacity to absorb water and ions (Wilson et al., 2008). Excessive fertilizers are leached out, become fixed in soil or contribute to water pollution as crop usage is less than half of the fertilizer applied. Nanofertilizers are known as 'smart fertilizers' due to their potential to control and slow the delivery of one or more nutrients to supply to crops (Zulfiqar, Navarro, Ashraf, Akram, & Munné-Bosch, 2019).

Nanomaterial application in agriculture has paved the way for commercializing many agricultural products with enhanced efficiencies, such as fertilizers, pesticides, and bio-priming agents (Bhatia et al., 2021; Xiao, Boada, Llugany, & Valiente, 2021). It could increase the nutrient use efficiency by crops and helps to reduce the wastage of nutrients as the predicted amount of fertilizers can be

supplied to produce in a slow-release manner for optimum utilization. Nanoparticles can act as efficient delivery vehicles due to their large surface area and easy absorption via various mechanisms such as adsorption, attachment, encapsulation, or entrapment of active ingredients into the nano-matrix (Ghormade et al., 2011). Nanoparticles combined with bio-fertilizers have shown improved plant growth promotion under field conditions. It was reported to be cost-effective as a reduced quantity of nano-bio-fertilizers was used in several hectares of crops (Duhan et al., 2017). The nano size of the particles also aids in their solubility and capacity to adsorb water and ions (Wilson et al., 2008). Likewise, nano-sized bio-stimulants ares an upcoming technology in plant sciences. When prepared in nano-size, the akin components that show positive effects on the plant are expected to exhibit enhanced quality, traits, and performance. Improved nano-formulations of fertilizers with high nutrient use efficiency ensure minimal loss of nutrients (Chugh, Siddique, & Solaiman, 2021). Similar effects could be observed in nano-encapsulated bio-stimulants where nano-sized active ingredients could boost the plant's physiological processes with superior efficiency. The reduced size of active components or elicitors could easily penetrate plant cells and directly interact with the plant's metabolic pathways, immune system, stress alleviating processes and improve the nutrient use efficiency.

2.10 Nanotechnology in Bio-stimulant Development

New formulations are essential in producing bio-organic fertilizers/stimulants to enhance the efficiency and longevity of the microorganisms in the product and soil. Nano-encapsulation is an emerging technology based on nano-formulations. Nanoparticles such as carbon nanotubes and silica nanoparticles were found to stimulate plant disease resistance and increase plant growth (Pour, Saberi-Riseh, Mohammadinejad, & Hosseini, 2019). Likewise, it was reported that a formulation containing PGPR and endophyte bacteria with graphite and silica nanoparticles was used as a biocontrol delivery system (Djaya, Hersanti, Istifadah, Hartati, & Joni, 2019). Iron oxide nanoparticles were used to immobilize bacterial cells for applications in different areas of biological research, including medicine, food technology, biotechnology, and

environmental sustainability (Ranmadugala, Ebrahiminezhad, Manley-Harris, Ghasemi, & Berenjian, 2018).

2.10.1 Nano-encapsulation

Encapsulation is a technique in which homogenous or heterogenous functional compounds are entrapped by a polymeric material, which confines them from the external environment. For example, probiotic bacteria were successfully formulated into nano-encapsulation of poly (vinyl alcohol) and sodium alginate nanofibers to delay the microbial growth in the fish fillets to avoid contamination by mesophilic bacteria (Ceylan, Meral, Karakaş, Dertli, & Yilmaz, 2018). Encapsulation techniques in agriculture have been widely researched in pest and disease management, innovative fertilizers, bio-sensors, environmental remediation, and water management (Ur Rahim et al., 2021).

The common materials to produce encapsulation layers are carbohydrates (polymers), proteins, and lipids. Among all these, biopolymers such as chitosan and alginate have been utilized successfully to encapsulate active ingredients such as nano-fertilizers, nano-pesticides, nano-insecticides, nano-fungicides, nano-herbicides, bio-stimulant and bio-fertilizer. Alginate has been used widely for microencapsulation due to its environmental compatibility and biodegradability. Researchers (Perez et al., 2018) also reported the potential of low-cost chitosan-starch macro beads for the delivery of plant growth-promoting bacteria. Bentonite was suggested as a potential filler material in sodium alginate immobilization of PGPR (Y. He et al., 2015). Several reports were found on the feasibility of calcium-alginate microcapsules being used as a delivery method for microbial bio-fertilizers or plant growth-promoting bacteria (Y. He et al., 2015; Wu, Zhao, Kaleem, & Li, 2011).

2.10.2 Nano-emulsion

Nano-emulsion is a stable, transparent, or translucent liquid formed by the dispersion of two immiscible liquids, typically oil, and water, with the aid of an emulsifying agent. In other words, nano-emulsions are fine oil-in-water

dispersions with droplet sizes ranging from 100-500 nm. It exhibits better penetration efficacy of the active ingredients due to the emulsion's large surface area and low surface tension (Haziqah, Marzuki, Wahab, & Hamid, 2019). It also protects the encapsulated compound from hydrolysis and oxidation. Nano-emulsions of bacteria have been proposed as a promising approach for delivering beneficial bacteria as bio-stimulants. Using nano-emulsions can protect bacteria from environmental stresses, such as high temperatures, pH, and ultraviolet radiation, hence can improve their survival and ability to colonize plant tissues, resulting in improved plant growth and health. It also can improve the bioavailability of the bacteria by increasing their surface area, which enhances their interaction with plant roots and tissues. Better root development can improve nutrient uptake, stress tolerance, and plant growth and yield. Besides, nano-emulsions enable the controlled release of bacteria over an extended period, which may improve their efficiency and persistence in the plant rhizosphere. Finally, it facilitates the co-delivery of bacteria with other bio-stimulants or agricultural inputs, which may lead to synergistic effects and improved plant growth and health.

2.11 Advantages of Nano-technology in Crop Cultivation

Encapsulation techniques can efficiently deliver beneficial microorganisms to the target site with minimal cell loss (Schoebitz, López, & Roldán, 2013). The viability of microorganisms could be maintained high and stable in the bio-organic fertilizer and soil when encapsulation technology is used. Meanwhile, microencapsulation involves tiny particles of liquid or solid material coated with a continuous film of polymeric material (Suganya & Anuradha, 2017).

Past studies have stated that the colonization of PGPR was improved by immobilizing it to biodegradable microcapsules (Wu et al., 2011). The microcapsules provide temporary protection from various soil factors such as temperature, humidity, pH, competitors, and predators. The microcapsules gradually release the bacteria to the surrounding environment in a controlled mode, effectively colonizing the targeted site. It was reported that alginate-chitosan-alginate microcapsules (degree of chitosan deacetylation of 96-98 %)

reached higher bacterial cell density than non-encapsulated free culture (Qi et al., 2006). In addition, a study has highlighted the ability of alginate-chitosan-alginate microcapsules as an immobilized carrier for fermentation to increase maximum biomass concentration compared to free culture (Qi et al., 2006). Studies have also demonstrated that chitosan nanomaterials enhance nutrient uptake, improve the photosynthetic rate and increase crop yield (Adisa et al., 2019).

Smart fertilizers based on slow or controlled release systems have improved crop yields, soil productivity, and lower environmental nutrient loss (Calabi-Floody et al., 2018). Mineral nutrients or bacterial inoculants can be encapsulated in nanomaterials, coated with a thin protective film, or encapsulated in nanoscale particles using zeolites, which act as a nutrient reservoir (Saleh & Webster, 2020). Nutrient-augmented –zeolite fertilizers are the only nanomaterials tested and proven effective through many laboratory and field experiments (R. Liu & Lal, 2021).

The chemical decomposition of secondary metabolites in non-microbial bio-stimulants could cause hydrolysis, oxidation, polymerization, and isomerization reactions that may occur over time (Antil & Gupta, 2021). Encapsulation provides the solution for liquid and solid formulation problems in maintaining the viability of microbial agents by extending the shelf life while upholding their biological traits (Saberi-Riseh & Moradi-Pour, 2021; Saberi-Riseh, Moradi-Pour, Mohammadinejad, & Thakur, 2021). The advantages of the encapsulation method include protecting the core material from extreme weather conditions, allowing safe handling of pesticides and herbicides, regulating the release of active components into the target site, preventing early degradation of active ingredients, enhancing efficiency, and facilitating precision targeting of the functional compound. Hence, the efficiency of active compounds could be improved by encapsulating them in biopolymers (Sharma et al., 2021).

Encapsulation of agricultural nanomaterials is an up-coming technology that could lessen the negative impacts on human health, plants, animals, and living things in the soil and develop environmentally friendly products. This technique

ensures the safety of nanomaterials and minimizes the risks before it is widespread in the environment. Polymeric nanomaterials with biodegradable and biocompatible characteristics were proven beneficial in delivering the active compounds to the target site with minimal loss (Ramachandraiah & Hong, 2021). The so-called wall material in the polymeric structure controls the release of bioactive components, thus leading to extended shelf life and the optimal uptake of nutrients by plants (Pateiro et al., 2021). A study conducted on encapsulated PGPR, salicylic acid, and zinc oxide nanoparticles revealed that the shelf life of the bacteria extended with enhanced plant growth and effective plant disease control characteristics (Panichikkal, Prathap, Nair, & Krishnankutty, 2021; Panichikkal, Puthiyattil, Raveendran, Nair, & Krishnankutty, 2021). These findings corroborated the successful integration of encapsulation technology and nano-technology. The encapsulation is a powerful tool to deliver the active compounds directly to the target area with slow-release features. A study has reported that nano-encapsulation of savory essential oil in natural biopolymers such as Arabic gum, gelatin, and Persian gum showed efficient herbicidal activities compared to non-encapsulated essential oil (Taban, Saharkhiz, & Khorram, 2020). Similarly, the encapsulation of two crucial oil, thyme and dill, in silver nanoparticles increased the fungicidal activity against crop pathogens (Weisany et al., 2019).

Apart from these, encapsulation improved plants' responses to abiotic stresses. Nitric oxide-releasing nanoparticles that were encapsulated into chitosan nanoparticles alleviated the detrimental effects of water deficit in sugarcane plants and increased physiological parameters of the plant, such as photosynthetic rate, root-shoot ratio, and plant biomass (Silveira et al., 2019).

2.12 Bio-stimulant Performance Indicators

A critical component of all bio-products, including plant bio-stimulants, is assessing the impact on their target crop or soil. Specific bio-stimulating indicators or activities identified based on the active ingredients are vital to give a holistic view of the primary function of the respective bio-stimulant products.

There are numerous plant performance indicators to determine the competence of each treatment or agricultural input.

2.12.1 Seed Germination and Vigor

Seed treatments, including seed priming, seed coating, and seed soaking, are essential methods that can enhance seed germination, increase seedling uniformity and vigor, and break seed dormancy. These techniques can improve the effectiveness of crop production and yield, even under unfavorable conditions. The germination patterns of seeds can be influenced by the timing of seed production and environmental conditions experienced by the mother plant during seed maturation. These factors play essential roles in determining the success of seed germination (Sobarzo-Bernal, Gómez-Merino, Alcántar-González, Saucedo-Veloz, & Trejo-Téllez, 2021). The seed germination index (GI) is commonly used to assess the potential phytotoxic effects of various substances in growth media, substrates, or soils. The index is calculated by measuring the seed germination percentage and root elongation length. It is worth noting that the GI variable can exceed a mean value of 100, which means that GI values above 80% and below 100% indicate no phytotoxicity.

In comparison, values between 50% and 80% suggest moderate phytotoxicity and values below 50% indicate high phytotoxicity. If the GI value exceeds 100%, the substance or treatment is considered as a phytostimulant or phytonutrient (Sobarzo-Bernal et al., 2021). A quick and effective establishment of seedlings after planting is crucial to achieve efficient production. Additionally, the seedlings' quality and ability to grow and survive after transplanting is a critical factor in crop cultivation. The phytochrome system controls the germination of seeds in the dark development stage, where it involves processes such as glycolysis, beta-oxidation, tricarboxylic acid cycle, and attenuation of root growth.

Bio-stimulant priming is a method used to initiate vital metabolic processes, including repairing damaged proteins and synthesizing new ones. The researchers have shown that using extracts from seaweed could lower the

potential compounds that hinder the action of abscisic acid in seeds, leading to a significant enhancement in the germination indices (Jafarlou, Pilehvar, Modaresi, & Mohammadi, 2022). However, seaweed extract priming at a high concentration (1.5%) showed a significant adverse effect on the germination of milkweed seeds due to the inhibition of seeds to absorb water. Evaluating plant performance treated with bio-stimulant under saline conditions also revealed their potential to tolerate abiotic stresses. An experiment under saline conditions exhibited salt tolerance of seeds in terms of improvement in seed germination, relative water content, chlorophyll content, proline content, soluble sugar content, root activity, and activities of superoxide dismutase, catalase-peroxidase and ascorbate peroxidase (H. Li et al., 2021). A study has demonstrated that bio-stimulant priming of seaweed extracts and polysaccharides, had positive effects on tomato seed germination at a specific concentration by increasing germination percentage and germination speed and reducing mean germination time (Mzibra et al., 2021). Soil application of the same bio-stimulant on tomato seedlings demonstrated a significant impact on growth parameters such as the number of leaf; shoot length, fresh and dry weight of stems and roots, and chlorophyll content due to the chelating properties of polysaccharides (Mzibra et al., 2021).

2.12.2 Vegetative Growth Parameters

Vegetative growth is a stage in a plant's life cycle that focuses on developing non-reproductive structures such as roots, stems, and leaves. During this phase, the plant dedicates its resources to establishing a strong foundation for future growth and reproduction via cell division and expansion, which causes the plant to grow taller and broader. Additionally, new leaves produced are essential for photosynthesis, and roots grow deeper and wider to access more water and nutrients from the soil. Several factors can influence vegetative growth, including genetics and environmental conditions such as light, temperature, humidity, and water and nutrient availability.

Using bio-stimulants can positively influence the vegetative growth of plants by promoting physiological processes that enhance growth and development. These

substances can improve nutrient uptake and utilization, stimulate root growth and development, and increase plants' ability to tolerate environmental stresses. The effectiveness of bio-stimulants can be influenced by several factors, including the plant species, the specific bio-stimulant used, and the ecological conditions in which the plant is grown.

One of the primary ways that bio-stimulants promote vegetative growth is by improving nutrient uptake and utilization. Bio-stimulants contain various substances, such as amino acids, enzymes, and vitamins, which can improve the plant's availability and uptake of essential nutrients, leading to increased biomass accumulation, improved leaf area development, and higher photosynthetic rates.

Furthermore, bio-stimulants can play a crucial role in enhancing the development and growth of roots, which is vital for vegetative growth. Certain bio-stimulants comprise substances, like auxins and cytokinins, which can promote the growth and development of roots. An extensive root system can enhance the plant's capacity to absorb water and nutrients and improve its resilience to withstand stressful conditions. Thus, the emphasis should be given to the details such as the changes in root structure and root colonization by microorganisms; the differences in root biomass; the differences in the secretion of amino acids, organic acids, volatile acids, and other root exudates; the nutrient availability in soil, roots and leaves and nutrient use efficiency by plants and shift in the soil microbiome (Adeleke, Ayangbenro, & Babalola, 2022; Khatoon et al., 2020; Mataranyika, Chimwamurombe, Venturi, & Uzabakiriho, 2022). These are regarded as the direct changes caused by bio-stimulants containing beneficial microorganisms. The dry weight of the shoot: root ratio represents one of the critical indicators of plant survival in challenging environments (Sobarzo-Bernal et al., 2021). Aside from this, growth parameters such as yield, vegetative growth, fruit quality, and others could be the indirect effects of microbial bio-stimulants (Fiuza et al., 2022). For example, foliar application of bio-stimulant containing moringa leaves extract, seaweed extract, and fulvic acid could positively improve the vegetative growth, fruit set percentage, fruit yield, and fruit physical and chemical characteristics as well as leaf nutritional value (Mosa et al., 2023).

Bio-stimulants can also enhance plants' ability to endure biotic and abiotic stresses. Specific bio-stimulants comprise substances that can help to increase the plant's resilience to environmental stresses, like drought, heat, and cold, as well as enhance its resistance to diseases and pests.

2.12.3 Photosynthesis and Chlorophyll Content

The application of bio-stimulants in plants can have a favorable impact on the process of photosynthesis. This is attributed to bio-stimulants' ability to boost the development and growth of leaves and chloroplasts, which are crucial for photosynthesis, as well as improve the overall efficiency of the process. Some bio-stimulants contain substances that help to increase the plant's capacity to absorb and use light energy, which is necessary for photosynthesis (A. Noman et al., 2018). This leads to an increase in the rate of photosynthesis and, ultimately, higher biomass production. Furthermore, bio-stimulants can enhance the plant's efficiency in the production of chlorophyll, which is essential for capturing light energy during photosynthesis and tolerating environmental stresses, such as heat and drought, which can positively affect the process of photosynthesis (Liatile, Potgieter, & Moloi, 2022). Bio-stimulants' effectiveness on photosynthesis may vary based on various factors, such as the specific type of bio-stimulant, the type of plant, and the prevailing environmental conditions. It was proven that seed priming with moringa leaf extract could improve the photosynthetic efficiency, growth, and yield of wheat plants (Rady et al., 2019). Sugar beet extract was revealed as an effective bio-stimulant due to its potential to increase the photosynthetic pigments after the seed treatment (A. Noman et al., 2018).

Chlorophyll, the green pigment, is essential for converting light into chemical energy. It determines the photosynthetic rate and primary food production in plants as a response to environmental stress and fertilizer application (Yongbin Li, Shi, Zhang, & Chen, 2019). Chlorophyll pigments consist of two main types: a (Chl a) and b (Chl b). The ratio of these Chla: Chl b increases linearly at low light intensity but gradually and linearly at higher light intensity. Thus, Chla:

Chl b is an effective measurement in predicting the abundance of chlorophyll associated with photosystems 1 and 11 (Sonobe, Yamashita, Mihara, Morita, & Ikka, 2020) and has been used to evaluate the response of crops to changing environmental conditions as well as to disease nutritional and environmental stresses. Thus, chlorophyll content could be an important diagnostic indicator for plant growth studies. The variation in the total chlorophyll: carotenoid ratio has been used to evaluate plants' environmental stress.

2.12.4 Yield and Post-harvest Quality

Bio-stimulants could enhance the quantity and quality of plant yield by facilitating the absorption and utilization of essential nutrients like nitrogen and phosphorus, which are crucial for plant growth and development, which results in increased biomass production and crop yield (Layek et al., 2018). Additionally, bio-stimulants can improve the quality of crops by promoting the accumulation of essential nutrients, such as vitamins and minerals, and enhancing the flavor and aroma of fruits and vegetables. Some bio-stimulants contain compounds that can increase the production of secondary metabolites responsible for fruit and vegetables' taste, smell, and color (Kapur et al., 2018). Bio-stimulants can improve the overall quality of crops, making them more desirable to consumers.

Associating bio-stimulant application with crop productivity has been a common way of evaluating the performance of bio-stimulant on various crops under different management practices. It was anticipated that primary plant metabolism is triggered by signaling molecules such as peptides, oligopeptides, and free amino acids, especially under stress conditions (Karabulut et al., 2021).

Fruit quality is one of the most important criteria that determine the market value of the produce. Farmers can enjoy handsome revenue if the quality of the fruit remains good in appearance, fresh, firm, and nutritious during storage. The quality of agricultural produce, especially fruits, depends on its physical (weight, firmness, color), chemical (soluble solids content, acidity, pH), and nutritional (phenolic contents and antioxidant capacity attributes) aspects. Color is a notable

trait associated with crop quality by customer-choice behavioral mechanisms. Very few studies have investigated bio-stimulant efficiency on fruit quality and the influence of bio-stimulants on the post-harvest quality of fruits (Rodrigues et al., 2020). Therefore, the post-harvest quality of fruits is vital to determine profit and customer satisfaction. Several quality traits could be tested to assess the effect of bio-stimulant application (Admane et al., 2023; Garai et al., 2021; Layek et al., 2018; Liatile et al., 2022). Reactive oxygen substance production and related enzyme activities to control oxidative damage are vital to investigate the ability of bio-stimulants to attenuate stress in fruits (Rodrigues et al., 2020). Shriveling and stem browning are indicators of reduced quality of chilli that are associated with loss of water. Water loss happens through the cuticle. Thus, the cuticle's thickness will determine the fruit's susceptibility to damage (Glowacz & Rees, 2016).

Bio-stimulants are active in promoting final crop quality traits and the concentration of secondary metabolites such as phenols, flavonoids, and ascorbic acids (Karabulut et al., 2021). Chilli has high phytochemicals, including carotenoids, capsaicinoids, polyphenols, and ascorbic acid, at the red ripening stage (Pola, Sugaya, & Photchanachai, 2020). The carotenoid content in chilli could reveal the effectiveness of bio-stimulant as carotenoids have excellent scavenging activity for reactive oxygen species (ROS). In chilli fruit, ROS can accumulate due to different stresses, such as high temperature, drought, and pathogen infestation. The accumulation of ROS can lead to the oxidation of lipids, proteins, and DNA, ultimately resulting in cell death and fruit deterioration. One of the main consequences of ROS accumulation in chilli fruit is reduced shelf life due to the onset of oxidative stress. The increased levels of ROS in chilli fruit can lead to a decrease in ascorbic acid content and an increase in polyphenol oxidase activity, which can cause browning and the breakdown of cell walls. ROS can also affect the flavor and aroma of the fruit by modifying volatile organic compounds, resulting in a loss of quality. Fully ripe chilli exhibits higher antioxidant activity than green chillies due to the increased amount of carotenoids, phenolic, flavonoids, and ascorbic acid that may be affected by genotype, plant growth conditions, and post-harvest management (Gómez-García & Ochoa-Alejo, 2013).

2.12.5 Rhizobiome Assessment

The interaction between microbial community help in maintaining the soil ecosystem, nutrient recycling, balance of the food chain, and essential physiological activities in the plant (Nwachukwu & Babalola, 2022). The rhizosphere is a zone of high biological activity with many positive and negative interactions between plants and rhizobacteria. It can be classified into three zones: the rhizoplane, ecto-rhizosphere, and endo-rhizosphere (Akinola, Ayangbenro, & Babalola, 2021). The rhizosphere is the root surface where the host plants directly interact with rhizosphere soils. It might be a selective barrier in establishing plant root microbiome and nutrient exchange in the soil-plant system (Ding et al., 2019). In addition, rhizoplane microbiota strongly correlated with the yield of foxtail millet (Jin et al., 2017). The endosphere is an exclusive microhabitat that creates a distinct niche requiring numerous adaptations for successful colonization and harbors a microbiome that differs from the adjacent communities (Jha et al., 2020).

Metagenomics is a valuable tool to trace the shift in rhizomes' taxonomic and functional components. Sequencing of 16S rRNA using Polymerase Chain Reaction (PCR) is the most popular technique to evaluate various environments' microbial diversity and richness, including soilless medium (Chiarello et al., 2022). A study on the diversity and composition of microbial communities revealed that the rhizospheric soil of the *Capsicum annuum* plant was significantly changed by wilt disease (Guigón-López, Avila-Quezada, González-Escobedo, Muñoz-Castellanos, & Muñoz-Ramirez, 2023). Rhizosphere soils applied with organic fertilizers showed a more extraordinary microbial richness and diversity than the conventional farming samples (Guigón-López et al., 2023; Luo et al., 2023). Likewise, bio-fertilizer application contributed to the accumulation of beneficial bacteria in the sugarcane rhizosphere and the decline of pathogenic bacteria (Q. Liu et al., 2022). Different fertilizer treatments were also reported to select other rhizosphere microbial communities by modifying the rhizosphere soil microenvironment and releasing chemical secretions. Rhizosphere microbes utilize the carbon metabolites secreted by plant roots for their growth and biological activities. In return, the

rhizospheric microbes benefit the host plant by providing nutrients, growth-stimulating hormones, inhibiting phytopathogens, and enhancing tolerance to environmental stress (Ding et al., 2019). Root exudates such as photosynthates (carbon compounds, electrons, protons, water, and inorganic ions), phytosiderophores, and polysaccharides form rhizospheric deposits. Photosynthates aid in nutrient acquisition, while phytosiderophores and polysaccharides include a mucigel that provides protection and the free flow movement of microbes in the rhizosphere (Olanrewaju, Ayangbenro, Glick, & Babalola, 2019). The root exudates correlate with a plant's mode of photosynthesis, where C4 plants exude more organic acids and amino acids than C3 plants which exude more carbohydrates and organic carbons (Olanrewaju et al., 2019). The change in soil microbiome diversity after treating the chickpea seed and soil with microbial bio-stimulant and its potential to increase plant growth, yield, nutritional content, plant health, and immunity against plant diseases were revealed in a recent study (Mukherjee et al., 2022). Therefore, the richness and the change in microbial community as a response to bio-stimulant could be a reliable indicator of its efficiency. The response of microbial communities varies under bio-stimulant treatment and could affect the soil microbiome differently.

The response of microbial communities varies under different agricultural inputs. Chemical fertilizers, organic fertilizers, bio-stimulants, and nanoparticles could affect the soil microbiome differently. The microbial community may show positive, negative, or neutral effects toward these substances. Soil microorganisms naturally could actively participate in degrading the mineral or biological compounds added to the soil. The mineral or biological compounds could be harmful to a specific group of microorganisms but inert to other microorganisms. The effect of silver nanoparticles in changing the abundance of specific microbial species was investigated using the pyrosequencing technique (Y. Yang et al., 2014). It is also noted that the rhizosphere harbors a different microbial community than bulk soil, and its community is expected to respond differently to nanoparticles due to the modifications in roots and their exudates which could alter the microenvironment both chemically and physically (Sillen et al., 2015). Soil microbiome analysis could indicate the dominant bacterial

group and its functional properties correlated with plant growth, nutrient content, soil nutrient, and enzyme activity. The change in soil microbiome diversity after treating the chickpea seed and soil with microbial bio-stimulant and its potential to increase plant growth, yield, nutritional content, plant health, and immunity against plant diseases were revealed in a recent study (Mukherjee et al., 2022). Thus, soil microbiome analysis could be used as an indicator to study the potential role of soil bacteria and the association between crop productivity and soil fertility with microbial bio-stimulant (Mukherjee et al., 2022). The effect of single and combined plant bio-stimulants such as microbial inoculants, seaweed extracts, and fulvic acids to improve root health and crop production in citrus trees affected with Huanglongbing (HLB) disease that causes fibrous root loss was investigated (Castellano-Hinojosa, Meyering, Nuzzo, Strauss, & Albrecht, 2021) . The study determined the effect of the various bio-stimulant treatments and management practices in different soil and environmental conditions on the rhizosphere's microbial community. In addition, the correlation with micro and macronutrients in roots was also studied. Longer-term bio-stimulant application and higher application rates may be necessary to translate the relative abundance of specific bacterial and fungal taxa in the citrus rhizosphere into measurable effect on tree health and productivity (Castellano-Hinojosa et al., 2021). Furthermore, the rhizosphere microbiome have a greater influence on the growth and health of host plants than the soil microbiome. It also could elucidate the interaction between added microorganisms or bio-inoculants and native microbial communities. It was emphasized that validating soil microbial inoculants using soil microbiome analysis is vital before commercialization to indicate a clear plant-soil-microbe interaction. Furthermore, soil microbial community analysis could prevent adverse effects such as invasive pathogenic microorganisms and loss of diversity in indigenous soil microbial ecosystems (Nuzzo, Satpute, Albrecht, & Strauss, 2020).

2. 13 Conclusion

The literature review underlined the importance of adapting modern technologies in crop cultivation. Chilli planting in Malaysia including the taxonomy, botany, nutritional quality, economic value, demand, and cultivation using fertigation system was elaborated. The benefits of using microbial and plant-based bio-stimulants for crops were reviewed thoroughly. The major limitations of bio-stimulant production were emphasized and the corrective actions and strategies that could be used to improve bio-stimulant development were suggested. The advantages and disadvantages of nanotechnology in the past research were reviewed. The benefits of the encapsulation technique to extend the shelf-life of microbial inoculants were also discussed in the literature review. Finally, the important criteria for evaluating the efficiency of bio-stimulants products were recommended based on past research.

CHAPTER 3
ISOLATION AND SCREENING OF PLANT GROWTH-PROMOTING ENDOPHYTES

3.0 Introduction

Plant growth-promoting rhizobacteria has proven to be a promising agricultural approach that plays a vital role in crop protection, growth, or biological disease control (Laslo et al., 2012). An efficient screening method is essential for isolating PGPR (Shi et al., 2022). Screening rhizobacteria with one or a few PGPR traits is a fundamental criterion for selecting bacteria (Raheem & Ali, 2022). Endophytic bacteria form mutualistic relationships with host plants in the long term, and they can promote the growth and accumulation of effective components in host plants (Tao et al., 2022). Most endophytes play crucial roles in plant growth-promotion and resistance through their ability to participate in essential processes such as nutrient mobilization, phytohormone production, and pathogen suppression (Husseiny, Dishisha, Soliman, Adeleke, & Raslan, 2021). Different endophytic isolates with various plant growth-promoting characteristics can integrate and improve plant growth and health by employing diverse mechanisms within different periods of the plant's life cycle. However, minimal studies have investigated the plant growth-promoting activities of microbial endophytes from medicinal plants. Thus, this study is aimed to isolate plant growth-promoing endophytic bacteria (PGPEB) from medicinal plants for bio-stimulant development.

Chapter 3 is the first experimental chapter that describes the isolation and screening of plant growth-promoting endophytes to be incorporated into bio-stimulant for chilli. Endophytic bacteria were isolated from the roots and leaves of medicinal plants such as moringa, sesbania, neem, and two local varieties of chilli plants. Common beneficial traits for plant growth-promoting endophytes like the dissolution of insoluble phosphorus and potassium; production of siderophores, indole acetic-acid, ACC deaminase, hydrogen cyanide, chitinase, and exopolysaccharides; and nitrogen fixation were screened for all the isolated

endophytes. *In vitro,* bio-control activities of endophytes against *Colletotrichum* sp. and *Fusarium* sp. were also evaluated using dual culture agar assay. Quantification analysis was done when the majority of isolates showed positive results. The remaining beneficial traits were characterized using qualitative assays and phenotypical changes, mainly when only a few isolates exhibited positive results. The primary purpose of the isolation is to discover the most potential endophytic bacteria that can be incorporated in the nano-formulation of bio-stimulants for chilli. A quantitative analysis was carried out on specific beneficial traits such as indole acetic acid, ACC deaminase, and phosphorus solubilization to scrutinize the selection of bacteria as most of the bacteria showed positive results for all these three beneficial traits in qualitative analysis.

3.1 Materials and Methods

3.1.1 Plant Sample Collection

Three medicinal plants, *Moringa oleifera*, *Sesbania grandiflora*, and *Azadirachta indica,* and two local varieties of *Capsicum annuum*, namely Chilli Kulai (MC 11) and L5 were chosen for the sample collection of roots and leaves. The sampling was done from three matured plants/trees of each species/variety with three biological replications of leaves and roots. Leaves from three different branches and roots from three different root branches of a plant were pooled to make it one biological replication. The samples were collected in sterile plastic bags and brought to the lab for immediate analysis or kept at 4℃ overnight before analysis. The details of the sampling are described in **Table 3.**

Table 3.1. Details of plant sampling for the isolation of endophytes

Species	Plant parts	Age of plant	Geographical location (coordinate	Cultivation method
Moringa oleifera	Fruit, Tap root & Young leaves	*17 years*	Senawang, Negeri Sembilan 2°42'42.5"N 102°00'23.3"E	Conventional
Sesbania grandiflora	Tap root & Young leaves	*3 years*	Broga Ballo Estate *2°56'16.3"N 101°52'59.0"E*	Conventional
Azadirachta indica	Tap root & Young leaves	*17 years*	Senawang, Negeri Sembilan 2°42'42.5"N 102°00'23.3"E	Conventional
Capsicum anuum MC 11	Fibrous roots & Young leaves	*2-3 months*	Malaysian Agriculture Research and Development Institute 2°59'27.4"N 101°42'05.2"E	Organic
Capsicum anuum L5	Fibrous roots & Young leaves	*4-5 months*	Malaysian Agriculture Research and Development Institute 2°59'27.4"N 101°42'05.2"E	Conventional

3.1.2 Isolation of Endophytic Bacteria from Roots and Leaves

Each plant species/variety consisted of three pooled leaf and root samples. The plant samples were washed in distilled water repeatedly to remove soil debris and air-dried. One gram of plant sample (leaf/root) was weighed in sterile conical flasks separately. The plant samples were washed in sterile distilled water several times using a shaker until the water became clear. Later, it was washed with 70% ethanol for 5 minutes, followed by 1% sodium hypochlorite for 2 minutes, and finally rinsed with sterile distilled water for 5 min x 4 times. A hundred microliters of the final rinse were inoculated onto triplicate plates of potato dextrose agar (PDA, Oxoid, UK) and tryptic soy agar (TSA, Oxoid, UK) to check the sterilization efficiency. Sterile mortar and pestle were cleaned with 70% ethanol before macerating plant samples with sterile phosphate buffer. Before macerating the plant sample, a small part of the plant tissue was grown on PDA and TSA in triplicates. The macerated plant sample was serially diluted in phosphate buffer until dilution 10^4. A hundred microliters of each diluent were inoculated onto duplicate plates of PDA and TSA. All the plates were incubated at room temperature for one week. Colonies with different morphologies were selected, purified, and stored at 4°C. Colonies of identical morphologies from the same plant were discarded to reduce unnecessary repetition of work. Fungal colonies were disregarded in the isolation process.

3.1.3 Biochemical Screening of Plant Growth-promoting Traits in Endophytic Bacteria

The bacterial isolates were purified and pre-cultivated in Tryptic Soy Agar (TSA, Oxoid, UK) for 48h at 28°C. All isolates were assessed for plant growth-promoting biochemical assays such as solubilization of phosphorus and potassium; nitrogen fixation; production of indole acetic-acid, ACC deaminase, siderophores, and exopolysaccharides (biofilm). Qualitative assays were done to

narrow down the number of bacterial isolates with potential plant growth-promoting traits, while quantitative assays were done to select the most efficient isolate. *In vitro* antagonistic activities were assessed using dual cultures in agar medium for common chilli pathogens such as *Fusarium oxysporum* and *Colletotrichum* sp.

3.1.4 Qualitative Screening of Beneficial Traits in Endophytic Bacteria

Phosphorus solubilization was tested according to the method described by (Shikha Gupta & Pandey, 2019; Ringuet, Sassano, & Johnson, 2011). Potassium solubilization was done following the process described by (Zhang & Kong, 2014) and nitrogen fixation was tested according to (Baldani, Reis, Videira, Boddey, & Baldani, 2014). Endophytic bacteria were grown overnight in nutrient broth in falcon tubes at 28°C. Fifty microliters of the culture were inoculated onto Pikovaskaya's agar (PVK) (HiMedia Laboratories, India), Aleksandrov's agar (HiMedia Laboratories, India), and Asby's glucose agar (HiMedia Laboratories, India). PVK contained calcium phosphate as the sole insoluble phosphorus source. In contrast, Aleksandrov's agar contained potassium aluminum silicate as the sole insoluble potassium source and no nitrogen source was included in Asby's glucose agar. The plates were incubated at 28°C for one week. Colonies that produced clear zone were selected as potential endophytes with positive activity. Chitinase activity was detected on 1% (w/v) colloidal chitin agar plates. The test was performed according to the method described by (Saima, Kuddus, Roohi, & Ahmad, 2013). The hydrogen cyanide test was conducted according to the method described by (Passari, Mishra, Leo, Gupta, & Singh, 2016). The siderophore production test was done according to the method described by (Ferreira, Vilas-Boas, Sousa, Soares, & Soares, 2019). In 96 well microplate, 100 µl of bacterial culture supernatant was mixed with 100 µl of CAS reagent, and 10 µl of shuttle solution was added. The plate was incubated for 20 min, and absorbance was read at 630 nm (UV-VIS

Spectrophotometer, Model: Variosken Flash Multimode Reader, ThermoFisher Scientific, USA).

3.1.5 *In vitro* Bio-control Activities of Endophytic Bacteria

Antagonistic activities against *Fusarium oxysporum* and *Colletotrichum* sp. were screened using agar plate assays (Yasmin et al., 2020). Chilli pathogens such as *Colletotrichum* sp. (Anthracnose) and *Fusarium oxysporum* (*Fusarium* wilt) were collected from the pathology unit of the Horticultural Department, Malaysian Agriculture Research and Development Institute (MARDI). The pathogens were sent for molecular identification at Apical Scientific Sdn. Bhd. Malaysia to check the purity of the culture.

Fungal pathogens (*Colletotrichum* sp. and *Fusarium oxysporum*) were grown on PDA for 7-14 days at 28 °C. One centimeter of agar was plugged out from the edge of fungal mycelium using a cork borer and placed at the center of a new PDA medium. Endophyte bacteria were inoculated in a straight line 1 cm away from the pathogen agar plug. The agar plates were sealed with parafilm and incubated for 7-14 days at 28 °C. Control plates were grown with the pathogen. The average value was calculated and presented. The inhibition percentage was calculated as described below:

$$Inhibition\ percentage\ (\%) = \frac{(C - B)}{C} x\ 100$$

C= Average diameter of fungal mycelium grown in the control plate

B= Average diameter of fungal mycelium grown in the presence of antagonistic bacteria.

3.1.6 Quantitative Screening of Plant Growth-promoting Traits

Plant growth-promoting traits such as ACCdeaminase, indole-acetic acid production, and phosphorus solubilization by endophytic bacteria were quantified, as most of them showed positive results in the qualitative assays.

3.1.6.1 ACC Deaminase Production by Endophytic Bacteria

ACC-deaminase production analysis was performed according to the method described by (S. Z. Ali, Sandhya, & Rao, 2014; Penrose & Glick, 2003). The assay was based on the hydrolysis of ACC, which produced α-ketobutyrate. This assay was done using a 96-well microplate reader. Endophytes were grown in 15 ml Tryptic Soy Broth (TSB) at 200 rpm overnight in a shaking incubator at 30 °C. The cell was harvested by centrifugation at 4000 rpm for 10 min at 4 °C. Supernatant was removed, and the cells were washed with 5 ml of DF salts minimal medium. The washing procedure was repeated twice after centrifugation. Then, cells were suspended in 5 ml DF salts minimal medium added with 45 µl of 0.5 M ACC solution. The tubes were incubated for 24 h at 200 rpm and 30 °C followed by centrifugation at 4000 rpm for 10 minutes at 4 °C. Supernatant was removed, and cells were washed with 0.1 M Tris-HCL, pH 7.6. The washing procedure was repeated twice, and the pelleted cells were kept at -20 °C until use. For ACC deaminase assay, bacterial cell pellets were mixed with 1 ml of 0.1 M Tris-HCI, pH 7.6, and transferred to a 1.5 ml microcentrifuge tube. The mixture was centrifuged at 16,000 g for 5 minutes. After removing the supernatant, cells were re-suspended in 600 µl of 0.1M Tris-HCI, pH 8.5. Thirty microliters of toluene were added and vortexed for 30 seconds at the highest setting. Two hundred microliters of toluenized cells were placed in a fresh 1.5 ml microcentrifuge tube and added with 20 µl of 0.5 M ACC followed by a brief vortex. The mixture was incubated at 30°C for 15 minutes. One milliliter of 0.56 M HCl was added, vortexed, and centrifuged for 5 min at 16,000 g at RT. One

milliliter of supernatant was mixed with 800 µl of 0.56 M HCl and vortexed before adding 300 µl of 2,4-dinitrophenylhydrazine reagent to the glass tube. It was vortexed and incubated at 30 °C for 30 minutes before mixing with 2 ml of 2N NaOH. Finally, 300 µl of the sample was used to read absorbance at 540 nm in a 96-well microplate reader. The stock solution of α-ketobutyrate was prepared and diluted with Tris-HCl, 0.1 mol/L, to make a 10 mmol/L solution just before use. The concentration of standard α-ketobutyrate ranged between 0.1 and 1 µmol with a total volume of 200 µl of each standard. Three hundred microliters of the 2,4-dinitrophenylhydrazine reagent were added, and the contents were vortexed and incubated at 30°C for 30 min. At this time, the α-ketobutyrate is derivatized as phenylhydrazine. The color of phenylhydrazine is developed by adding 2 mL of 2M NaOH. The solution is mixed before measuring the absorbance at 540 nm. A standard curve of absorbance was plotted against standard concentration. The standard curve was used to calculate the concentration of α-ketobutyrate produced by endophytic bacteria.

3.1.6.2 Indole-acetic acid Production by Endophytic Bacteria

The indole-acetic acid assay was conducted based on the established colorimetric method by (Chandra et al., 2018; Naveed et al., 2013; Passari et al., 2016). Nutrient broth (15 ml) was prepared with 0.1 % tryptophan. Potential endophyte isolate was cultured separately into the growth medium and incubated in a shaking incubator at 28°C, 100 rpm for 48 h. After incubation, indole-acetic acid production was determined by Salwoski's reagent. A series of standards within the range of 0 ppm to 200 ppm was prepared from a stock solution of 1000 µg/mL of pure indole-acetic acid in acetone in the same culture medium. According to the pre-determined design, a hundred microliters of samples and standards were transferred into a 96-well microplate. Then, 200 µL of Salwoski reagent was added to all the wells, and the microplate was incubated at RT for 25 minutes. The absorbance was read at 530 nm in a UV-Vis Spectrophotometer

(Model: Variosken Flash Multimode Reader, ThermoFisher Scientific, USA). A standard curve of absorbance against the concentration of indole-acetic acid was plotted. The concentration of indole-acetic acid produced by endophytes was calculated using the standard curve.

3.1.6.3 Phosphorus Solubilization by Endophytic Bacteria

The microplate colorimetric method was done to quantify orthophosphate or soluble phosphate that was solubilized from insoluble rock phosphates by endophytes (Z. He & Honeycutt, 2005; Khati, Pankaj, Nisha, Kumar, & Sharma, 2018; Ringuet et al., 2011). All glassware was acid cleaned by soaking in a 10% HCl solution and rinsed at least three times with distilled water/ultrapure water before use. The pure culture of potential endophytes was used to inoculate 50 mL of Pikovaskaya's broth supplemented with Christmas Island Rock Phosphate (CIRP) as the sole insoluble phosphorus source (Yeast extract, 0.5 g/L; dextrose, 10 g/L; CIRP, 5 g/L; ammonium sulfate, 0.5 g/L; potassium chloride, 0.2 g/L; magnesium sulfate, 0.1 g/L; manganese sulfate, 0.0001 g/L and ferrous sulfate, 0.0001 g/L) in conical flasks. The flasks were incubated in a shaking incubator for one week at room temperature. The samples were analyzed after eight days of the incubation period. Samples were filtered using 0.22 µm syringe filters to remove particulates before analysis. The reagents (R) used were R1: 51 mM ammonium molybdate; R2: 4.9 N sulfuric acid; R3: 0.9 µM ascorbic acid (freshly made solution) and R4: 1.2 mM antimony potassium tartrate. The working reagent was made fresh daily by mixing the reagents in the following amounts: R1: 1.5 mL; R2: 5 mL; R3: 3 mL; and R4: 0.5 mL. Samples were added to a 96-well microplate followed by 50 µL of mixed working reagent. The microplate was vortexed at 750 rpm on a microplate shaker during the 30 minutes of reaction time. The absorbance was quantified at 880 nm (UV-VIS Spectrophotometer, Model: Variosken Flash Multimode Reader, ThermoFisher Scientific, USA). Standards were prepared using the following reagents:

Orthophosphate/soluble P: A primary stock solution of potassium phosphate monobasic (KH_2PO_4), 10 mM, and stored at 4°C. A 100 µM of secondary stock solution was prepared from the primary stock solution by diluting with distilled water, and standard solutions with orthophosphate concentrations ranging between 0 and 25 µM were made for the calibration curve. The absorbance reading shows a linear correlation against the orthophosphate concentration in the solution solubilized by endophytes from an insoluble source of phosphate.

3.1.6.4 Quantitative Screening of Biofilm Production by Endophytic Bacteria

The ability of the potent endophytic bacteria to produce a biofilm that will help in root colonization (Naveed et al., 2013) and the compatibility of the endophytes to co-exist in a mixed culture were also determined. Biofilm formation was analyzed using a 96-well microplate by staining it with 0.1 % crystal violet in a buffer [0.15 M ammonium sulfate, 100mM potassium phosphate (pH 7), 34 mM sodium citrate, and 1 mM $MgSO_4$]. Bacterial inocula were prepared by growing strains in biofilm formation medium (BFM) at 28°C for 24 h and harvested by centrifuging at 10,000 x g at 20°C for 5 min. After centrifugation, pellets were re-suspended in BFM medium at 10^8 cells/ml density. A hundred microliter of this inoculum was used to inoculate microtiter plates containing BFM. After two days of incubation, the wells were stained with 0.1 % crystal violet at RT for 45 minutes. After removing excess crystal violet, the wells were rinsed with sterile distilled water. The well-adhering cells were then solubilized with 200 ul of a mixture of 80 % (v/v) ethanol and 20 % (v/v) acetone. The absorbance of 150 µL of the destained crystal violet, which was transferred to a new microplate, was measured at 595 nm (UV-Vis Spectrophotometer Model: Variosken Flash Multimode Reader, ThermoFisher Scientific, USA).

3.1.7 Statistical Analysis

The data were analyzed using one-way ANOVA (Microsoft Excel) and the Tukey HSD test using IBM SPSS Statistics versions 25 and 28. The mean values were significant at $p< 0.05$.

3.2 Results and Discussion

3.2.1 Endophytic Bacteria from the Roots and Leaves of Moringa, Sesbania, Neem, and Chilli

Seventy bacterial endophytes were isolated from the roots and leaves of medicinal crops such as moringa (*Moringa oleifera*), neem (*Azadirachta indica*), vegetable hummingbird (*Sesbania grandiflora*), and healthy chilli plants (*Capsicum annuum* sp. MC11 and L5 varieties). The endophytes isolated from each part of the plant are detailed in **Fig. 3.1** and **Table 3.2**. The pure isolated colonies were sub-cultured at least five times to ensure survival. The isolates that could not grow after a few sub-culturing were removed from the collection list. Most endophytes were isolated from moringa roots, followed by chilli roots and leaves. The isolation was done from medicinal plants as minimal reports have been published on the plant growth-promoting activities of microbial endophytes from medicinal plants (Hassan, 2017). Bacterial endophytes from the rhizosphere environment colonize root hairs due to the secretion of root exudates and rhizodeposits. Plant roots and plant developmental stages influence the rhizosphere microbiome. Thus, plant type is a stronger modulator of microbial richness than soil type (Igiehon & Babalola, 2018). Different plant species have various root exudates, which can attract specific types of microbes to the rhizosphere by changing the physical-chemical conditions of the rhizosphere that promote nutrient acquisition and attract specific beneficial microbial groups (Zhao et al., 2021). This can increase the microbial richness in the soil associated with that particular plant species. Furthermore, some plant species may also

release antimicrobial compounds that can inhibit the growth of certain microbes, leading to a reduction in microbial richness. Therefore, the effect of plant type on microbial richness can vary depending on the plant species and the specific microbial communities involved. Consequently, it was expected that the root exudates from medicinal plants could attract novel beneficial bacteria to enter their roots. This is obvious in this research, where different plants harbor different genera of bacteria with distinct abundance. Moringa roots, chilli roots, and chilli leaves showed diverse bacterial isolates with different morphology of colonies.

This study noticed bacteria with similar colony morphologies among plant species or parts of the same plant species. This shows that there is a possibility that the same genus of bacteria is distributed in various crops through natural selection. Bacteria with similar morphology in different parts of the same crop species show that endophytes from roots could have traveled to the leaves via a specific colonization route. It was reported that stem and leaf surfaces produce exudates that attract microorganisms (Hardoim et al., 2015). This explains the presence of bacterial endophytes in the leaves of plants. (Hardoim et al., 2015) state that the xylem vascular system is the main transport route for the systemic colonization of internal plant compartments. In contrast, other endophytes colonize the intercellular spaces of plant cells. Endophyte colonization follow a systematic manner where it travels from roots to shoots to flowers, fruits, and seeds (Chouhan, Agrawal, & Prakash, 2022). While this explains the similar microbial genus in different parts of a plant, microbial taxonomic distinction describes the distribution of diverse microbiomes among bacteria associated with a leguminous plant's stem, leaves, and nodules (Igiehon & Babalola, 2018). The rhizosphere is where plant bacteria interact with various biological activities and the excretion of root exudates. The nature of the root exudates recruits specific endophytes to live in the host plant. Thus, the plant directly selects the type of endophytes.

Table 3.2 Endophyte isolates from medicinal plants and local chilli varieties

Name of plant	Plant parts	Abbreviation	Number of endophytes
Moringa oleifera **(Drumstick tree)**	**Leaves**	**ML**	**4**
Sesbania grandiflora **(Vegetable hummingbird)**	**Leaves**	**SL**	**3**
Azadirachta indica **(Neem Tree)**	**Leaves**	**NL**	**4**
Capsicum annuum **L. (Chilli MC 11)**	**Leaves**	**CL**	**9**
Capsicum annuum **L. (Chilli Kulai)**	**Leaves**	**CKL**	**9**
Moringa oleifera **(Drumstick tree)**	**Roots**	**MR**	**18**
Sesbania grandiflora **(Vegetable hummingbird)**	**Roots**	**SR**	**3**
Azadirachta indica **(Neem Tree)**	**Roots**	**NR**	**5**
Capsicum annuum **L. (Chilli MC 11)**	**Roots**	**CR**	**5**
Capsicum annuum **L. (Chilli Kulai)**	**Roots**	**CKR**	**10**
Total			**70**



Figure 3.1 Examples of endophytes isolated from plant leaf and root. (a) Moringa root; (b) Chilli L5 root; (c) Neem leaf; (d) Sesbania leaf; (e) Chilli MC 11 (f) Chilli root; (g) Sesbania root (h) Moringa leaf

3.2.2 Plant Growth-promoting Biochemical Traits in Endophytic Bacteria

Potential plant growth-promoting endophytes with the capability of phosphorus solubilization, potassium solubilization, nitrogen fixation, siderophore production, ACC-deaminase production, phytohormone production, chitinase production, hydrogen cyanide production, antagonistic activity against *Fusarium oxysporum* and *Colletotrichum* sp. were screened using qualitative agar medium assays. The qualitative assays were conducted to narrow down the number of potential endophytes that will be used to measure specific beneficial activities. Likewise, selecting promising plant growth-promoting bacteria was done based on qualitative and quantitative indices to reduce the number of isolates and exclude bacteria that exhibited negative results (Di Benedetto et al., 2019). In the first round of qualitative assays, endophytes showing clear zones were designated as potential bacteria for a specific activity. The second round of screening was done among the bacteria with positive activities by measuring the diameter of clear zones produced by each endophytic bacteria for the relevant activity. The beneficial activities of all the isolated endophytes were described in **Table 3.3**, and the pictures of functional traits were depicted in **Fig. 3.2**. An interesting observation was unveiled when the PGP endophytes were classified according to their host plant and beneficial characteristics (**Table 3.4**.). Endophytes isolated from sesbania and neem plants could only inhibit the growth of *Fusarium* sp. and *Colletotrichum* sp., producing siderophore, chitinase, and ACC deaminase. These beneficial traits were more associated with bio-pesticides and bio-control properties, whereas endophytes from moringa and chilli have both bio-control, bio-fertilizer, and bio-stimulating properties.

Endophytes are microorganisms that reside within the tissues of plants and can provide numerous benefits to their host plant. Endophyes could directly or indirectly benefit the plant by increasing the availability of nutrients, suppressing plant pathogens and insects, producing phytohormones, aiding in

phytoremediation and rhizoremediation, and stimulating stress resistance in host plants (Chouhan et al., 2022; Husseiny et al., 2021). They are generally deemed beneficial microorganisms for crops as they thrive within plant cells without causing damage to the plants and establish a community with other beneficial microbes by secreting various bioactive metabolites (Chouhan et al., 2022; Hassan, 2017).

Although many studies have demonstrated plant growth promotion of endophytes isolated from the same plants, some endophytes were also reported to promote growth in diverse host plants (Imran Afzal et al., 2019). Plant growth-promoting microorganisms may directly facilitate the proliferation of host plants by fixing atmospheric nitrogen, involved in the solubilization of phosphorus and potassium minerals, and supplying it to plants (Penrose & Glick, 2003). Some beneficial microorganisms are also able to produce plant hormones such as indole acetic acid, gibberellins, cytokinins, and enzymes such as ACC deaminase (1-aminocycloprapane-1-carboxylate) that promote plant growth by lowering ethylene levels of the plant (Khan et al., 2016) and by regulating genes involved in plant growth and protein production (Z. Li et al., 2011). Indole acetic-acid stimulates primary root elongation, while gibberellin influences seed germination and emergence, flower and fruit development, and stem and leaf growth.

Isolates from *Moringa oleifera* have multiple plant growth-promoting characteristics. *Moringa oleifera* is an edible, drought-tolerant wild plant famous for its medicinal and nutritional values. *M. oleifera*, known as the "miracle tree," is a rich source of beneficial nutrients such as vitamins A, C, calcium, potassium, iron, flavonoids, polyphenols, antioxidants, polysaccharides, and fibers. Its consumption has been linked to treating several health issues, including cancer, cardiovascular disease, diabetes, and obesity. Moreover, it can protect the liver against oxidation and toxicity (Refai et al., 2023). The agricultural industry can

also benefit from *M. oleifera*. The powder made from its seeds can be used as a natural coagulant to purify water, while its seed oil can serve as a plant fertilizer that helps increase crop yields (Refai et al., 2023). Due to the enormous benefits of the moringa plant, the endophytes that thrive in this plant could exhibit multiple beneficial traits for plants too. The moringa plant's abundant micronutrients, growth, and health-benefitting compounds have attracted novel and beneficial endophytes to moringa roots and leaves.

Table 3.3 Screening of beneficial activities in endophytic bacteria isolated from moringa, neem, sesbania and chilli plants

No.	Isolates	Nitrogen fixation (cm)	Phosphorus solubilization (cm)	Potassium solubilization (cm)	Indole acetic-acid production	Chitinase production	Siderophore production	ACC deaminase production	*Colletothchium* inhibition (%)	*Fusarium* inhibition (%)	Hydrogen cyanide production
1	MR1	-	-	-	+	-	-	+	-	-	-
2	MR2	-	-	-	-	+	1.1	-	-	-	-
3	MR3	-	-	-	-	+	1.1	-	51.7	-	+
4	MR4	3.2	-	2.8	+	-	-	+	-	-	-
5	MR5	1.9	1.1	1.5	-	-	2.0	-	-	-	-
6	MR6	-	-	-	-	-	1.1	-	-	-	-
7	MR7	-	-	-	-	-	1.0	-	-	38.2	-
8	MR8	-	-	-	-	-	1.1	-	-	-	-
9	MR9	1.7	-	1.3	+	-	-	-	-	-	-
10	MR10	-	-	-	++	+	1.2	+	-	-	-
11	MR11	-	-	-	-	-	-	-	-	25.5	-
12	MR12	-	-	2.2	-	-	-	-	-	-	-

13	MR13	-	1.5	2.4	++	+	1.2	+	-	28.3	-
14	MR14	2.8	-	3.3	+	++	2.1	+	-	-	-
15	MR15	-	-	-	+	-	-	-	-	-	-
16	MR16	2.7	1.2	2.4	-	-	-	-	-	44.1	-
17	MR17	-	-	-	+	-	-	+	-	-	-
18	MR18	-	-	-	-	-	0.9	-	-	-	-
19	ML1	-	-	-	++	-	1.0	+	-	63.9	-
20	ML2	-	-	-	-	-	-	-	-	44.9	-
21	ML3	-	-	-	-	-	1.1	-	52.3	-	-
22	ML4	-	-	-	-	-	1.2	-	-	-	-
23	SR1	-	-	-	-	+	0.9	-	53.5	-	-
24	SR3	-	-	-	-	-	-	-	-	-	-
25	SR4	-	-	-	-	-	-	-	-	-	-
26	SL1	-	-	-	-	+	-	-	45.8	-	-
27	SL2	-	-	-	-	-	0.8	-	-	32.2	-
28	SL3	-	-	-	-	+	-	+	-	-	-
29	NR1	-	-	-	-	-	1.0	-	44.7	41.2	-
30	NR2	-	-	-	-	+	-	-	-	33.7	-

31	NR3	-	-	-	-	-	1.2	-	-	-	-
32	NR6	-	-	-	-	-	1.1	-	-	-	-
33	NR7	-	-	-	-	-	1.0	-	-	-	-
34	NL1	-	-	-	-	-	-	+	-	-	-
35	NL2	-	-	-	-	-	-	-	47.6	-	-
36	NL3	-	-	-	-	-	-	+	-	-	-
37	NL8	-	-	-	-	-	-	+	-	-	-
38	CR1	-	-	-	-	-	-	+	-	-	-
39	CR2	-	-	-	-	+	-	+	-	-	-
40	CR3	-	1.2	-	-	-	-	-	-	-	-
41	CR4	-	-	-	+	-	-	-	-	-	-
42	CR5	-	-	1.6	-	-	1.0	-	-	27.0	-
43	CL1	2.5	1.3	3.0	-	-	-	-	48.2	-	-
44	CL2	-	-	-	-	-	1.0	-	47.6	-	-
45	CL3	-	-	-	-	-	0.7	-	-	44.1	-
46	CL4	2.7	-	1.7	+	-	-	-	-	46.4	-
47	CL5	-	-	-	-	-	0.9	-	-	18.1	-
48	CL6	-	-	-	-	-	-	-	51.1	47.9	-

49	CL7	-	1.3	-	-	-	-	-	52.3	32.2	-
50	CL8	-	-	-	-	-	-	-	-	35.2	-
51	CL10	1.9	-	2.5	-	-	-	+	-	-	-
52	CKR3	2.3	-	1.4	+	-	0.9	+	-	50.89	-
53	CKR4	2.5	1.3	2.6	+	-	1.1	+	-	44.1	-
54	CKR5	-	1.2	-	-	-	1.2	+	-	-	-
55	CKR6	3.2	1.2	3.0	-	-	0.8	-	-	23.3	-
56	CKR7	-	-	-	-	-	-	+	-	34.5	-
57	CKR8	-	2.1	-	+	-	2.0	-	-	-	-
58	CKR9	2.8	-	2.2	+	-	-	-	-	-	-
59	CKR10	-	1.4	2.1	+	-	-	-	47.6	10.7	-
60	CKR12	-	-	-	+	-	-	-	-	-	-
61	CKR13	-	-	-	-	-	-	+	-	21.8	-
62	CKL1	-	1.4	-	-	+	-	-	-	-	-
63	CKL2	-	1.3	-	-	+	-	-	-	-	-
64	CKL3	-	-	-	-	-	-	-	51.7	-	-
65	CKL4	-	-	-	+	-	-	-	-	12.9	-
66	CKL5	-	-	-	+	-	-	-	-	30.8	-

67	CKL6	3.4	1.0	3.3	+	-	1.1	-	-	34.5	-
68	CKL7	1.8	-	2.2	-	-	2.0	-	45.8	-	-
69	CKL8	-	-	-	+	-	-	-	-	-	-
70	CKL10	-	-	1.7	-	-	-	-	-	-	-

*MR: Moringa root; ML: Moringa leaf; SR: Sesbania root; SL: Sesbania leaf; NR: Neem root; NL: Neem leaf; CR: Chilli roots (L7 variety); CL: Chilli leaf (L7 variety); CKR: Chilli roots (Kulai variety); CKL: Chilli leaf (Kulai variety)

Table 3.4 Classification of plant growth-promoting endophytes according to beneficial traits and host plant

Beneficial traits	Qualitative assays	Number of endophytes isolated from moringa leaf & root	Number of endophytes isolated from chilli leaf & root	Number of endophytes isolated from neem leaf & root	Number of endophytes isolated from sesbania leaf & root
Phosphorus solubilization	Pikovaskaya's medium	3 root endophytes	6 root endophytes 5 leaf endophytes	Nil	Nil
Potassium solubilization	Aleksandrov's medium	7 root endophytes	6 root endophytes & 6 leaf endophytes	Nil	Nil
Nitrogen fixation	Ashby's medium	5 root endophytes	4 root endophytes & 5 leaf endophytes		
Siderophore production	96 well microplate assay	10 root endophytes and 3 leaf endophytes	6 root endophytes & 5 leaf endophytes	4 root endophytes	1 root endophyte & 1 leaf endophyte
Chitinase production	Chitin azure agar assay	5 root endophytes	1 root endophyte & 2 leaf endophyte	1 root endophyte	1 root endophyte & 1 leaf endophyte
ACC deaminase production	96 well microplate assay	6 root endophytes & 1 leaf endophyte	7 root endophytes & 1 leaf endophyte	3 leaf endophytes	1 leaf endophyte

Indole acetic acid production	96 well microplate assay	8 root endophytes & 1 leaf endophyte	7 root endophytes & 5 leaf endophyte	Nil	Nil
***Fusarium oxysporum* inhibition**	Dual culture assay	4 root endophytes & 2 leaf endophytes	7 root endophytes & 9 leaf endophytes	2 root endophytes	1 leaf endophyte
***Colletotrichum capsici* inhibition**	Dual culture assay	1 root endophyte & 1 leaf endophyte	1 root endophyte & 6 leaf endophytes	1 root endophyte & 1 leaf endophyte	1 root endophyte & 1 leaf endophyte
Hydrogen cyanide production	HCN agar medium	1 root endophyte	Nil	Nil	Ni

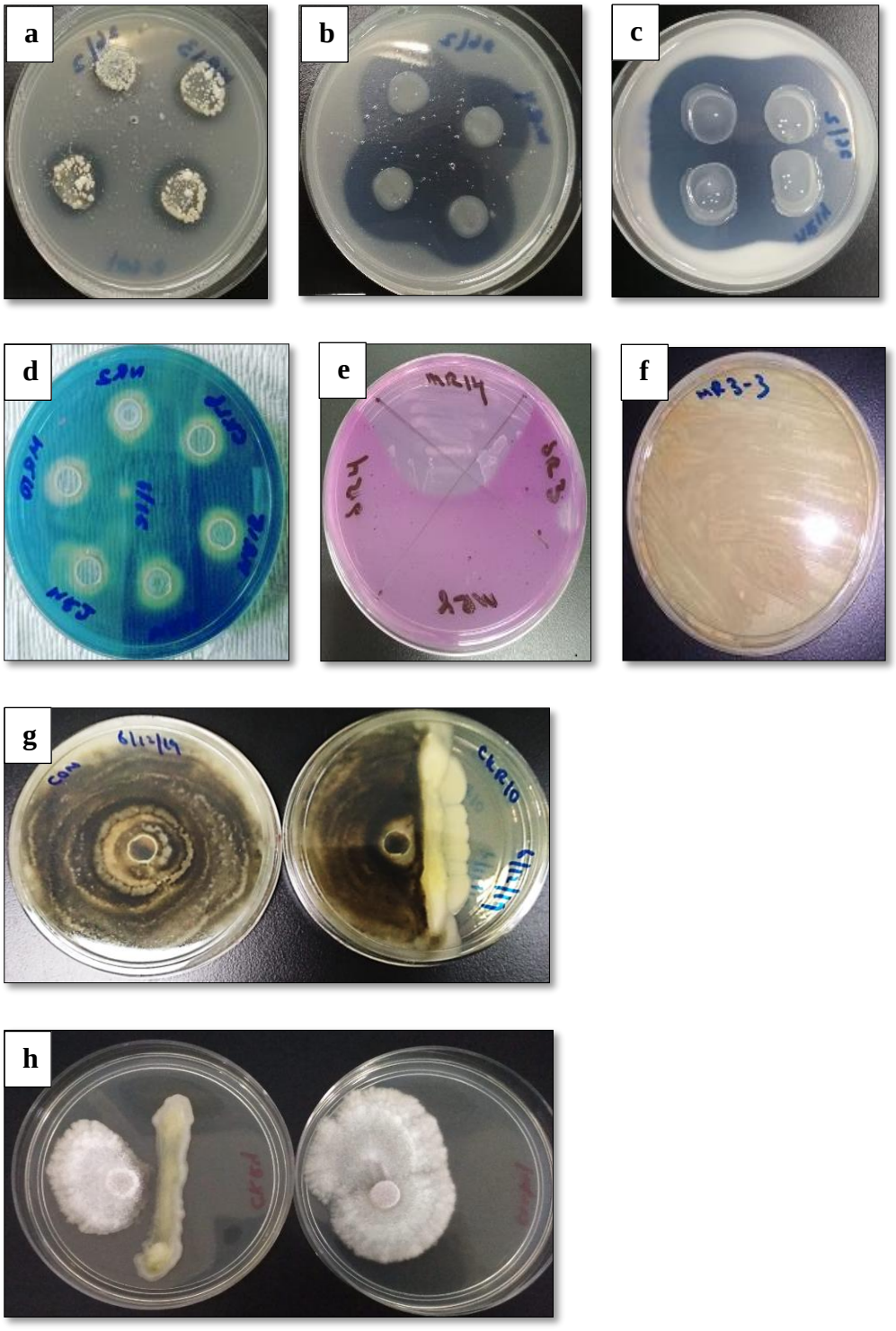


Fig. 3.2. Qualitative beneficial traits of endophytic bacteria. (a-e): Clear zone indicates phosphorus solubilization, potassium solubilization, nitrogen fixation, siderophore production and chitinase production. (f): Orange colour indicates hydrogen cyanide production. (g-h): Dual culture assay to exhibit the antagonistic activity of endophytic bacteria against *Colletotrichum* sp.and *Fusarium* sp.

3.2.3 Phosphorus Solubilization by Endophytic Bacteria

Potential beneficial endophytes were identified when a clear zone was observed around the colonies in the selective agar plate assay containing insoluble phosphorus (**Fig. 3.2 a**). The majority of the bacteria were able to show phosphorus solubilization activity in an agar medium. However, only 14 out of 70 endophytic bacteria were selected for further analysis as the size of the clear zone of all the potential phosphorus-solubilizing bacteria was similar. Among the 14 bacteria, only CKR8 and MR13 showed large clear zones, 2.1 cm, and 1.5 cm, respectively, and the rest exhibited similar sizes of clear zones. Thus, the Molybdenum blue assay measured the concentration of soluble P produced by the endophytic bacteria in a medium containing CIRP, mineral P, with low solubility. In this test, both P-releasing and mobilizing activities were determined using a microplate reader. P-solubilizing and mobilizing bacteria could liberate the P in complex form and solubilize it into an orthophosphate form, which produces a blue color. In this method, orthophosphate reacts with ammonium molybdate and potassium antimonyl tartrate in an acid medium to form phosphomolybdenum that will be reduced to molybdenum blue by ascorbic acid. A microplate-reader-based colorimetric method is reported to be sensitive, simple, and rapid for quantifying orthophosphates (Z. He & Honeycutt, 2005; Khati et al., 2018; Ringuet et al., 2011).

The standard graph of absorbance versus orthophosphate (soluble P) concentration was plotted to determine the P solubilization capability of endophytic bacterial samples (**Fig. A.1**) (**Appendix**). The concentration of P correlates with the absorbance reading. The standard P graph was used to calculate the concentration of P solubilized and liberated by the endophytic bacteria. When tested in molybdenum blue assays, MR13 could produce 4.93 ppm of soluble P followed by 3.74 ppm of soluble P by CKR8 and 0.19 ppm of soluble P in a control medium without bacteria (**Fig. 3.3**).

Phosphate-solubilizing bacteria (PSB) can mobilize mineral P in soil, increasing the bioavailability of P for plants, enhancing soil fertility, increasing crop productivity, and promoting sustainability in agriculture (Alori, Glick, &

Babalola, 2017). They improve plant growth by stimulating nitrogen fixation, enhancing trace metals' availability, and forming plant growth hormones (Shikha Gupta & Pandey, 2019).

Pikovaskaya's medium consisting of an insoluble source of phosphorus in the form of calcium phosphate is used to screen endophytes' inorganic phosphorus solubilizing ability. A clear zone around the colonies indicates P solubilization in the medium. Fourteen potential P solubilizing bacteria isolated in this screening process were from chilli roots, followed by moringa roots. These 14 endophytes were analyzed using the molybdenum blue method, an established method of measuring soluble P in a liquid medium.

Inorganic and organic phosphorus solubilization is accompanied by pH reduction of the medium due to the secretion of organic acids. Previous studies have corroborated the role of organic acids in facilitating phosphorus solubilisation (Mendes et al., 2020; Wei et al., 2018). One of the main mechanisms responsible for releasing available forms of phosphorus to plants in the soil is the production of organic acid and the activity of acid phosphatases (Saeid, Prochownik, & Dobrowolska-iwanek, 2018). Hydroxyl and carboxyl groups of organic acids chelate the cations bound to phosphate and transform them into soluble forms (Kalayu, 2019). Microorganisms could solubilize unavailable P via a ligand exchange process whereby the hydroxyl and carboxyl groups of organic acids produced by microorganisms chelate the cations bound to P and exchange with P in soil (Rashid et al., 2016). Oxaloacetic acid, succinic acid, citric acid, tartaric acid, malic acid, and maleic acid were regarded as responsible for the solubilization of rock phosphates (Reza, Singh, Datta, Purakayastha, & Singh, 2017).

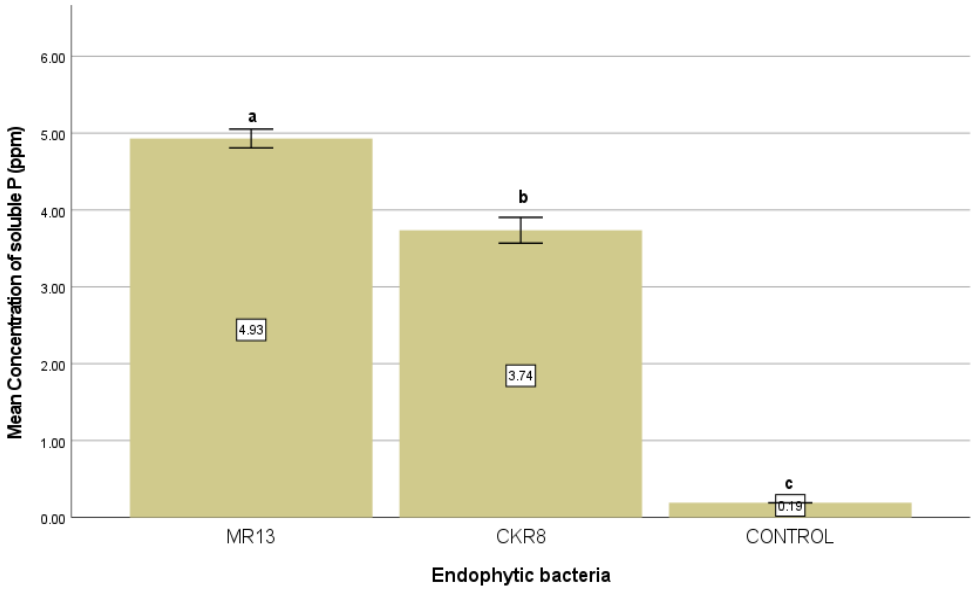


Fig. 3.3. Orthophosphate solubilized from insoluble rock phosphates by endophytic bacteria after eight days of incubation. The values are an average of 10 independent readings. According to the Tukey HSD test, means with different letters are significantly different at $p<0.05$. (IBM SPSS statistics, version 25).

3.2.4 Potassium Solubilization by Endophytic Bacteria

In the preliminary assay, Aleksandrov's medium of potassium aluminum silicate, a complex potassium source, was used to select potassium-solubilizing endophytes. A clear zone around the colonies indicates potassium solubilization due to organic acid secretion (**Fig. 3.2 b**). Only 19 isolates of 70 bacteria isolated from chilli and moringa plants could solubilize insoluble potassium in an agar medium. MR14 (3.3cm), CKL6 (3.3cm), CL1 (3.0cm), and CKR6 (3.0 cm) exhibited larger clear zones amongst the potential bacteria for potassium solubilization (**Table 3.3**).

Potassium can be found in various forms in the soil. These include exchangeable K, non-exchangeable K, dissolved K ions, and mineral K. However, plants can only utilize the soil's exchangeable and dissolved K ions (Etesami, Emami, & Alikhani, 2017). The primary forms of mineral K found in most soils are feldspar and clays, the parent materials for these soils. Additionally, secondary minerals such as illite and vermiculite, which are micaceous minerals formed from

primary minerals, are a source of mineral K (Etesami et al., 2017). Most soil K exists in silicate minerals, which account for approximately 90-98% of the total K in the soil. These minerals include muscovite, orthoclase, biotite, feldspar, illite, and mica (K. L. Rana et al., 2020). Plants absorb potassium in soluble form, whereas 90-98% of total K in the soil is in unavailable forms such as feldspar and mica. Potassium solubilizing bacteria were used as efficient bio-inoculants for enhancing K solubility in soil (Saha, Maurya, Meena, Bahadur, & Kumar, 2016). They can dissolve K-bearing minerals in soil by secreting organic acids, similar to P-solubilizing microorganisms (Bagyalakshmi, Ponmurugan, & Balamurugan, 2017).

Studies have shown that the production of carboxylic acids is one of the primary mechanisms responsible for the solubilization of potassium in the soil. Carboxylic acids are organic compounds that contain a carboxyl group (-COOH) and can chelate metal ions such as K, making them more soluble and available for plant uptake. Many microorganisms, such as bacteria and fungi, have been shown to produce carboxylic acids as part of their metabolic processes (K. L. Rana et al., 2020). These acids can be excreted into the soil, where they can chelate K and other metal ions, increasing their solubility and availability for plant uptake. Several carboxylic acids were identified as potent K-solubilizing agents, including citric acid, malic acid, and oxalic acid. These acids can help to reduce soil pH, making it more acidic and further increasing the solubility of K. Bacteria were reported to produce polysaccharides that can enhance the dissolution of minerals such as albite and quartz (K. L. Rana et al., 2020). Polysaccharides produced by microorganisms can act as a scaffold for mineral dissolution, providing a surface for metal ions to bind and increasing the efficiency of the process (K. L. Rana et al., 2020).

3.2.5 Nitrogen Fixation by Endophytic Bacteria

In the current study, Asby's glucose agar medium was used to isolate non-symbiotic nitrogen-fixing endophytes. This medium does not contain a nitrogen source. Endophytes that grow in this medium are expected to fix nitrogen in the air for its growth on the agar plate (**Fig. 3.2 c**). About 14 endophytes isolated

from chilli and moringa plants were considered potential nitrogen-fixing endophytes. Among these 14 bacteria, MR4, CKL6, and CKR6 showed larger clear zones in an N-free medium, accounting for 3.2 cm, 3.4 cm, and 3.2 cm, respectively (**Table 3.3**).

Nitrogen fixation is an energetically expensive process that requires a significant amount of carbon. Plants' root exudates are rich in carbon, consisting of various low-molecular-weight compounds such as sugars, organic acids, and mucilage. These exudates can serve as an energy source for diazotrophic microorganisms, as they require a significant amount of ATP to carry out nitrogen fixation. Root exudates in the form of flavonoids secreted by legume roots appear to participate in nitrogen fixation (Y. Jiang et al., 2022). Non-legume plant roots also may secrete flavonoid exudates to facilitate root interaction with nitrogen-fixing bacteria or suppress biological nitrification or denitrification so that more N may be retained in the soil for plant uptake (Chai & Schachtman, 2022).

The direct isolation method using carbon sources and an N-free medium resulted in significant root interactions with N-fixing bacterial diversity compared to other methods. Most bacterial isolates obtained through this method were related to diazotrophic strains reported as endophytes in various plant species (Mirza & Rodrigues, 2012). Merely observing the growth of an organism on an N-free medium is insufficient to establish it as a true diazotroph. To confirm its ability to fix nitrogen, it is imperative to gather experimental evidence through techniques such as quantifying acetylene reduction activity, investigating the expression of nitrogen fixation genes, or conducting an assimilation assay using 15N-labeled N_2 gas (Mirza & Rodrigues, 2012). The bacterial isolates that could fix nitrogen mainly produced slimy exopolysaccharides in the agar medium.

A group of researchers (Ahmadi-Rad, Gholamhoseini, Ghalavand, Asgharzadeh, & Dolatabadian, 2016) proved the capability of the nitrogen-fixing bacterial application to stimulate fruit set and fruit development of sweet cherry. Likewise, researchers have isolated endophytes from sugarcane stalks with high nitrogenase activity (Xing et al., 2016).

3.2.6 Chitinase Production by Endophytic Bacteria

Chitinolytic endophytes can be detected by the clearing zone in culture plates containing chitin. In this study, five isolates from moringa root, three isolates from sesbania (leaf and root), three isolates from chilli (leaf and root), and one isolate from neem root were found to have the ability to produce chitinases as observed by the clearing zone in chitin agar medium (**Table 3.2 e**).

The clearing zone can detect chitinolytic endophytes in a chitin culture plate. Chitin is a natural polymer of N-acetyl-D-glucosamine monomers linked by β-1-4-covalent bonds. Chitinases found in some microorganisms can decompose chitin, a component of the cell wall of fungi (Brzezinska et al., 2013). This is an essential criterion of a defense method against fungal pathogens.

Bacterial endo- and exo-chitinases work together to break down chitin, the main structural component of many insect and fungal pests that attack crops (Okongo, Puri, Wang, Singh, & Permaul, 2019; X. Wang et al., 2016). Endo-chitinases cleave the chitin polymer at internal points, generating smaller chitin oligomers. Exo-chitinases, on the other hand, cleave chitin from the non-reducing end, releasing single N-acetylglucosamine units. These two types of chitinases work cooperatively to break down chitin into its constituent monomers (Rathore & Gupta, 2015). When applied to crop fields, these bacterial chitinases can degrade the chitin in these pests, disrupting their life cycle and reducing their population, leading to improved crop yields (A. Veliz, Martínez-Hidalgo, & M. Hirsch, 2017; Rathore & Gupta, 2015).

3.2.7 ACC Deaminase Production by Endophytic Bacteria

In this study, all isolates of endophytes were screened for ACC deaminase production using 96-well microplates. The absorbance directly correlates with the concentration of ACC deaminase enzyme. Isolates that show absorbance of more than 0.15 were selected for quantitative analysis (**Fig. A2**) (**Appendix**). Nineteen isolates were able to produce ACC deaminase, where the majority were isolated from chilli roots, followed by moringa roots. The absorbance directly

correlates with the concentration of ACC deaminase enzyme. The color intensity was observed to increase in the microplates as the concentration of standard α-ketobutyrate increased. The standard graph was plotted for α-ketoglutaric (to determine the ACC deaminase activity of endophytic bacterial samples (**Fig. A3**) (**Appendix**) and used to calculate the ACC deaminase concentration produced by the endophytic bacteria. MR1, SL3, MR4, and MR14 were selected using a spectrophotometer based on yellow color production in the 96-well microplate and absorbance reading. It was found that MR1 produces the highest amount of α-ketobutyrate, 0.375 mM (**Fig. 3.4**).

Ethylene is a plant hormone that plays a crucial role in regulating various physiological processes in plants. However, the production of ethylene induced by climate change can harm plant growth and development, potentially leading to plant death if left unchecked (Shikha Gupta & Pandey, 2019). Thus, it is crucial to monitor ethylene levels in plants to mitigate its harmful effects. Plant growth-promoting bacteria employ several mechanisms to alleviate plant stress, with one primary strategy being the reduction of ethylene levels through the hydrolysis of 1-aminocyclopropane-1-carboxylic acid (ACC) by the enzyme ACC deaminase. ACC is a precursor of the plant hormone ethylene. Certain strains of PGPR have been reported to possess ACC deaminase, which can break down ACC into ammonia and α-ketobutyrate, decreasing the level of ethylene within the plant (Chandwani & Amaresan, 2022). ACC deaminase production indicates the stress tolerance traits in endophytes that could make it a potential candidate for bio-stimulant.

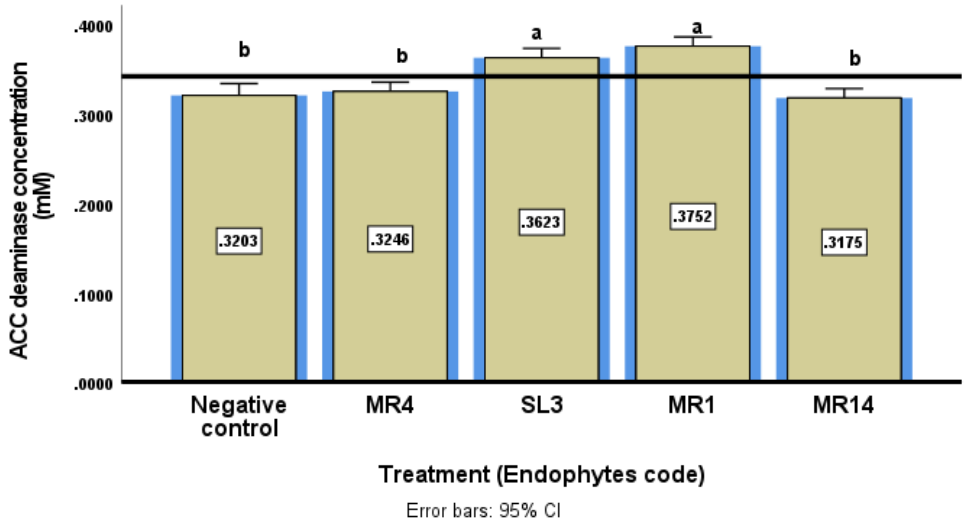


Fig. 3.4. Quantitative screening of ACC deaminase-producing endophytes based on a standard α-ketoglutaric graph. Values are means of eight independent readings. According to the Tukey HSD test, means with different letters are significantly different at $p<0.05$. (IBM SPSS statistics, version 25).

3.2.8 Indole Acetic-acid Production by Endophytic Bacteria

In the first trial, 21 out of 70 isolates, which exhibited red color formation in the 96-well microplate, were selected for quantitative analysis (**Fig. A4**) (**Appendix**). The 21 endophytic bacteria were mainly isolated from moringa root and leaf (**Table 3.4**). The standard graph was plotted for indole-acetic acid to determine the IAA concentration produced by endophytic bacterial samples (**Fig. A5**) (**Appendix**). The results revealed that ML1 and MR10 could produce 164.6±21.3 ppm and 87.6±0.29 ppm of indole-acetic acid, respectively (**Fig. 3.5**).

IAA is a naturally occurring auxin produced by PGPR through the L-tryptophan metabolism pathway. It is the first auxin group of growth stimulants identified to play a central role in plant growth (Pham et al., 2022). Salwosky's method determined the concentration of IAA produced by endophytic bacteria. Salicylic acid is known to form a violet-colored complex with ferric ions in acidic solutions. In the Salkowski method, the reaction between IAA and sulfuric acid

generates a mixture of products that includes indole, which reacts with the ferric ions in the Salicylic acid reagent to produce a pink-to-red color, indicating the presence of IAA. The intensity of the color is directly proportional to the concentration of IAA in the sample.

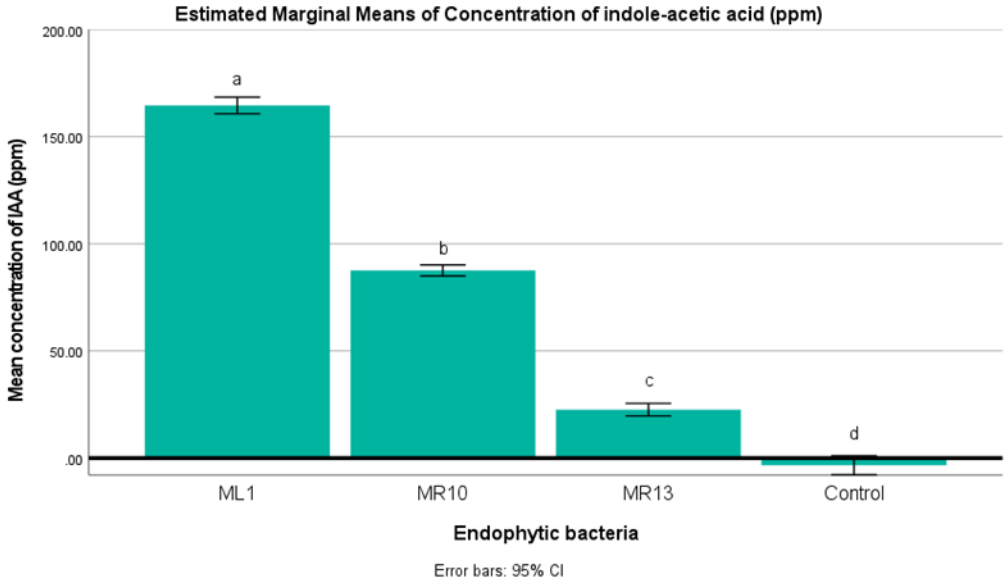


Fig. 3.5 Quantitative analysis of indole acetic-acid production by potential endophytic bacteria. The values are average of 30 independent readings. According to the Tukey HSD test, means with different letters are significantly different at $p<0.05$. (IBM SPSS statistics, version 25).

Phytohormone production by endophytes is a typical trait for root-associated endophytes (Hardoim et al., 2015). Indole-3-acetic acid plays a vital role in leaf formation, embryo development, root initiation and development, fruit development, etc. (Chandra et al., 2018). IAA facilitates root elongation and formation of lateral roots, which indirectly enhance the efficiency of water and nutrient uptake by plant root systems (Shikha Gupta & Pandey, 2019). It also affects xylem and root development and photosynthesis and can provide resistance to plants against various stresses. When present in the optimal amount, IAA promotes the secretion of root exudates and loosens the cell wall, allowing for enhanced nutrient uptake and transfer across membranes and facilitating the association between host plants and microbes (K. L. Rana et al., 2020). L-tryptophan is the direct precursor for IAA synthesis via an indole-3-acetamide pathway. In plants, root exudates secrete L-tryptophan for rhizospheric

microorganisms to enhance IAA biosynthesis in the rhizosphere (Chaiharn & Lumyong, 2011).

3.2.9 Siderophore Production by Endophytic Bacteria

Thirty endophytic bacteria were identified as hydroxamate siderophore producers (**Fig.A6**) (**Appendix**). Hydroxamate siderophore production was indicated by the formation of orange-color colonies on blue-dyed CAS agar medium because of iron removal. The siderophore produced by endophytic bacteria were classified as hydroxamates as they changed the agar plate from blue to orange (**Fig. 3.2 d**). The diameter of the orange zone produced by the potential endophytic bacteria ranged from 0.7-2.0 cm. CKL7, CKR8, MR5, and MR14 were selected for the 96-well microplate assay (**Table 3.3**). The majority of the siderophore-producing bacteria originated from moringa (12 isolates), followed by chilli (8 isolates), sesbania (3 isolates), and neem (1 isolate). CKL7 produced the highest siderophore concentration, reflected by the highest intensity of orange color production. The siderophore concentration based on absorption is shown in the graph (**Fig. 3.6**).

Iron is an essential micronutrient for plant development, and it is required by several metalloenzymes involved in photosynthesis and respiration (Ferreira et al., 2019). However, iron has low solubility in its predominant form, Fe^{3+}, in the aerobic environment and forms insoluble hydroxides and oxyhydroxides that plants and microbes do not assimilate. Microbes produce siderophores, low molecular-weight iron chelators that can bind Fe^{3+} with high affinity and are water-soluble (Sinha, 2019). Siderophores are classified as extracellular and intracellular. Extracellular siderophores are released into the environment and scavenge iron, which is then taken up by the cell. Intracellular siderophores are synthesized within the cell and function in the intracellular transport of iron (K. L. Rana et al., 2020). Siderophores are categorized based on the types of ligands they use to chelate ferric iron, which can include catecholates, hydroxamates, and carboxylates (Sinha, 2019). When siderophores bind to iron, a complex is formed, which is then absorbed by the microbial cell through specific transport systems. This criterion allows microorganisms to overcome the low solubility of

iron in their environment and acquire the necessary iron for their growth and survival (K. L. Rana et al., 2020).

Microbes that produce siderophores can scavenge iron from the surrounding environment, limiting its accessibility to phytopathogens and protecting plants from pathogenic attacks (Yu, Ai, Xin, & Zhou, 2011). Siderophores form stable complexes with iron and other heavy metals such as aluminum, cadmium, copper, gallium, indium, chromium, lead, and zinc, as well as with radionuclides like uranium and neptunium. By doing so, siderophores reduce the toxic effects of heavy metals on plants (K. L. Rana et al., 2020).

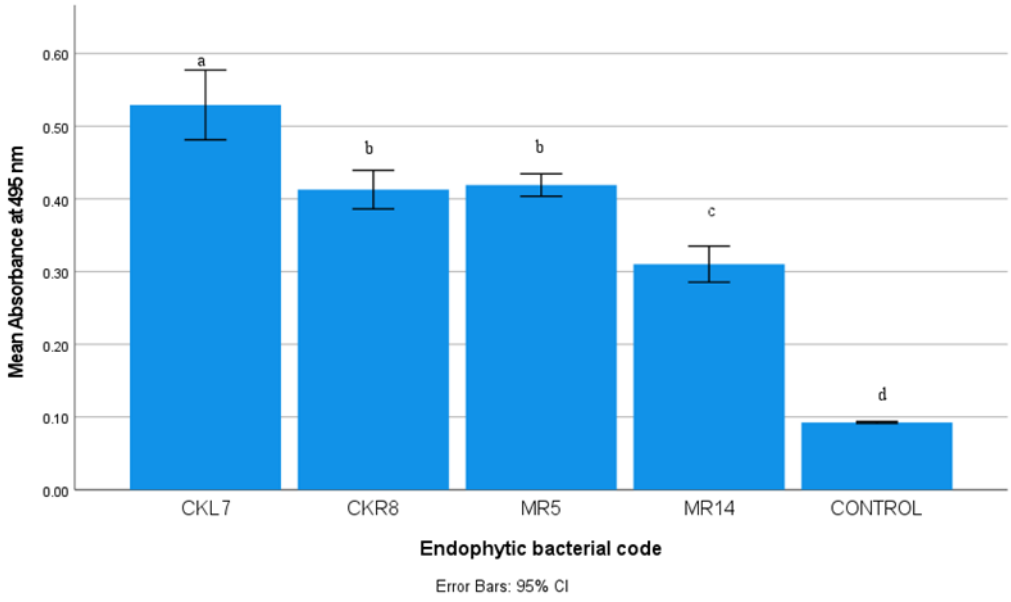


Fig. 3.6. Hydroxamate-siderophore production by potential endophytic bacteria. The values are an average of ten independent readings. According to the Tukey HSD test, means with different letters are significantly different at $p<0.05$. (IBM SPSS statistics, version 25).

3.2.10 Bio-control Activity of Endophytic Bacteria against *Colletotrichum* sp.

In this study, only one bacterial isolate, MR3, was able to change the color of filter paper from yellow to orange, which indicates the presence of hydrogen cyanide. All 70 isolates were tested for the antagonistic ability against

Colletotrichum sp. using a dual culture agar plate assay. About 13 endophytes out of 70 isolates have exhibited inhibition of *Colletotrichum* sp. in the range of 45-53% (**Fig. 3.7**). The antagonistic endophytes were able to produce metabolites that inhibit the growth of the fungal pathogen. Control potato dextrose plates that were grown with the pathogen show full growth in 5-7 days, whereas antagonistic endophytes hinder the growth of fungal mycelium (**Fig. 3.2 g**)

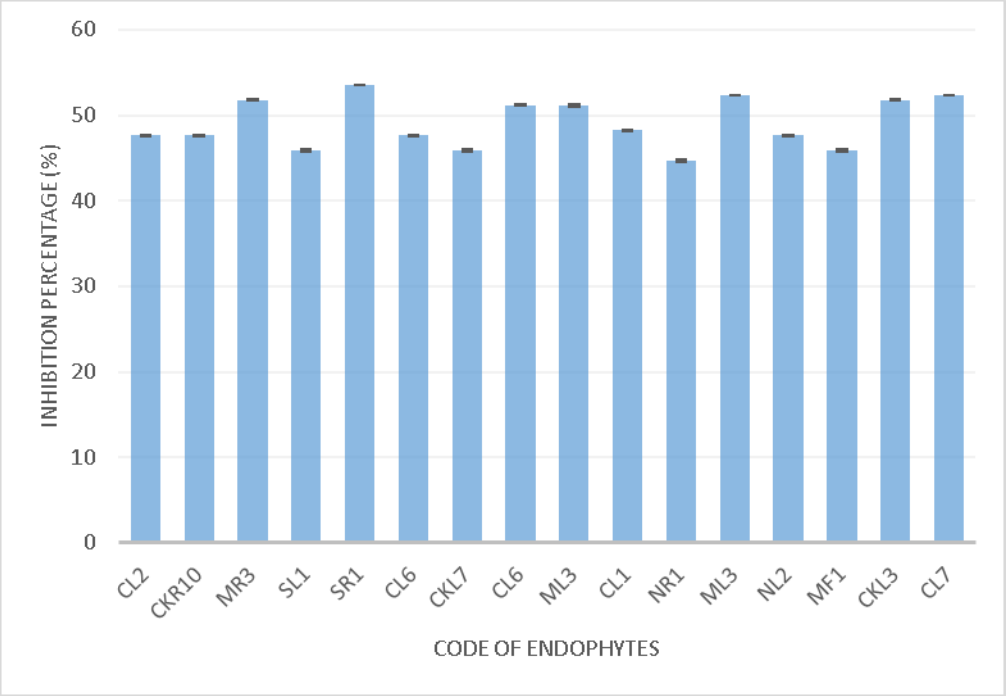


Fig. 3.7. Inhibition percentage of *Colletotrichum* sp. by antagonistic endophytes in dual culture agar plate assay. Values are means of ten independent readings.

3.2.11 Bio-control Activity of Endophytic Bacteria against *Fusarium oxysporum*

Out of 70 endophytes, 25 isolates displayed inhibition percentages within the 10-63% range for *Fusarium oxysporum*, soil-borne fungi that cause chilli wilt disease (**Fig.3.2 h**). ML1, isolated from moringa leaf, could inhibit more than 60% of *F. oxysporum* growth on agar medium (**Fig. 3.8**).

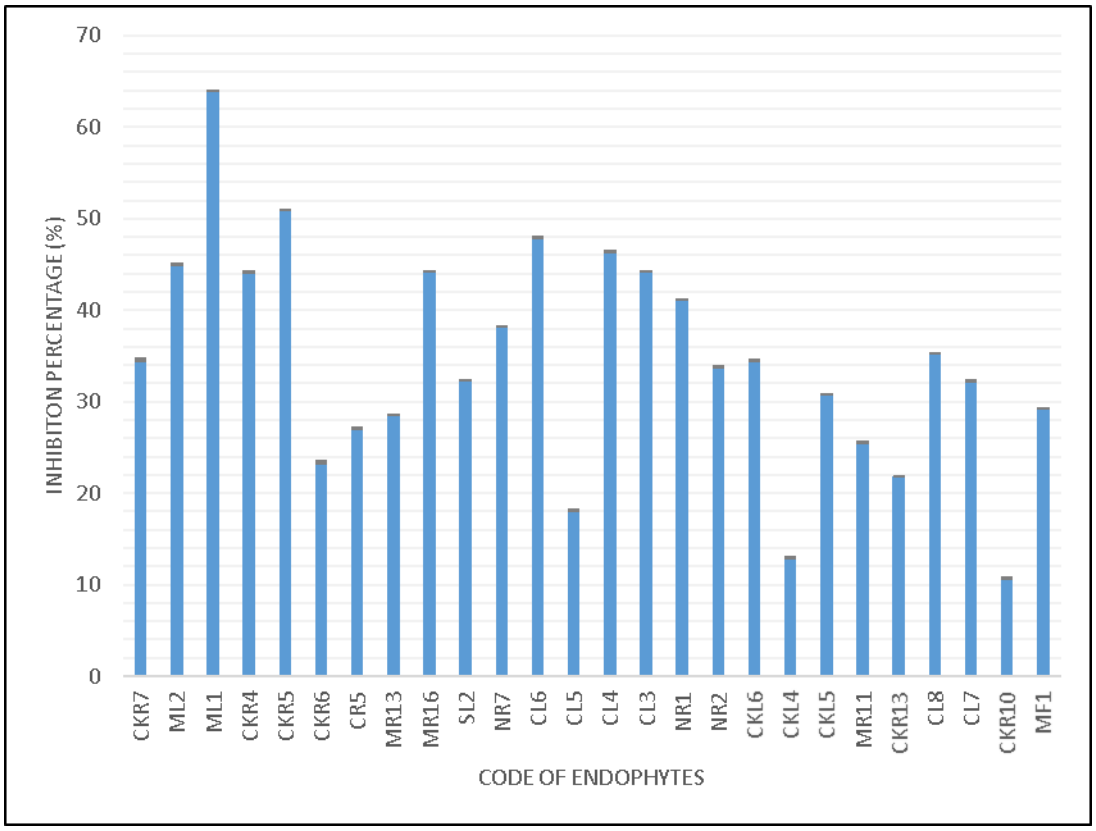


Fig. 3.8. Inhibition percentage of *Fusarium* sp. by the antagonistic activity of endophytes in a dual culture plate assay. Values are means of three independent readings.

3.2.12 Biofilm Production by Endophytic Bacteria

Root colonization related to biofilm is a fundamental trait of plant growth-promoting bacteria as it facilitates the absorption of nutrients from soil to root. All the potential endophytes with multiple beneficial characteristics were subjected to the biofilm assay using 96-well microplates. The results showed that all endophytes could produce a high amount of biofilm, which is indicated by the absorbance within the range of 1.38 -3.36 as compared to the control that recorded an absorbance value of 0.52 (**Fig. 3.9**). Among all the endophytic bacteria that could produce biofilm, CKL7 exhibited the highest amount of biofilm that was determined based on the highest absorbance reading (3.36 A). There were no significant differences among other endophytic bacteria that could produce a high biofilm concentration, such as MR5, CKR8, MR14, SL3, MR3, and CKL7. On the other hand, bacteria that had irrelevant amounts of

biofilm were MR13, ML1, and ML3, as the values were similar to the control value.

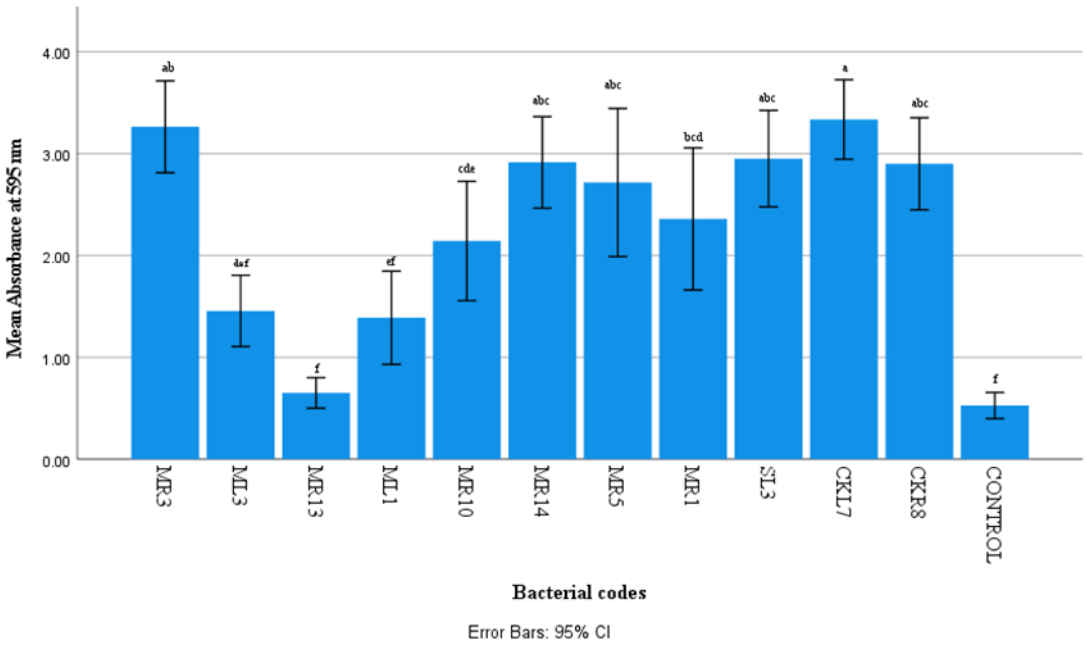


Fig. 3.9. Exopolysaccharides (Biofilm) production by endophytic bacteria. The values are an average of eight independent readings. According to the Tukey HSD test, means with different letters are significantly different at $p<0.05$. (IBM SPSS statistics, version 28).

Biofilm produces extracellular polymeric substances, including polysaccharides, proteins, nucleic acids, and lipids. Extracellular polysaccharides are essential in maintaining water potential, aggregating soil particles, ensuring attachment to plant roots, and resisting the adverse effects of environmental stresses (Gouda, Kerry, et al., 2018). In contrast, bacteria that cannot produce biofilm, such as *Streptomyces* (MR13), could produce vegetative hyphae to form a complex mycelium that can grow and colonize the host (Vurukonda, Giovanardi, & Stefani, 2018).

3.3 Conclusion

It is concludedd from this experiment that bacterial endophytes from moringa roots exhibited multiple and the majority of the beneficial traits based on the

qualitative and quantitative assays. This could be due to the chemotaxis signals produced by root exudates of moringa roots to attract helpful bacteria to their root microbiome. Only seven bacteria were selected for future experiments among all 70 endophytic bacteria isolated from moringa, sesbania, neem, and chilli roots and leaves. The selection of the promising endophytes focussed on isolates that exhibited multiple plant growth-promoting traits and represented at least one strong beneficial trait. The selected endophytes were MR1, MR3, MR10, and MR13 from moringa roots, ML1 from moringa leaf, CKR8 from chilli root and CKL1 from chilli leaf. These endophytic bacteria were screened and selected as they exhibited *in vitro* plant growth-promoting traits. MR1 and MR10 could produce ACC deaminase; ML1 could produce indole acetic acid; CKR8 and MR13 could solubilize phosphorus; MR3 showed bio-control activity, and CKL7 could produce siderophores.

The study's first objective is to select the most promising endophytic bacteria to develop bio-stimulant. Although seven potential isolates were selected from the plant growth-promoting biochemical assays, a preliminary validation of the beneficial traits must be done using a model crop. The biochemical tests alone could not be used as plant-growth-promoting indicators as the bacteria could act differently during the interaction with the plant. Biochemical screening for beneficial traits has to be corroborated with seed germination and the vegetative seedling growth analyses to confirm their potential as plant growth-promoting endophytes. Furthermore, the biosafety of these bacterial isolates must be considered before selecting the potent bacterial isolate for bio-stimulant development. Thus, the subsequent studies conducted to determine the potential endophytic bacteria, were described in **Chapter 4**.

CHAPTER 4
CHARACTERIZATION OF PLANT GROWTH-PROMOTING ENDOPHYTES AND THE EFFICIENCY IN PROMOTING THE GROWTH OF CHILLI SEEDLINGS

4.0 Introduction

Plant growth-promoting endophytic bacteria can promote plant growth and development at different growth stages, such as seed germination, vegetative growth, and yield, and enhance the quality of the produce. They act as plant bio-stimulant to promote the development of plants either directly by facilitating the plant's acquisition of nitrogen, phosphorus, and potassium or by modulating phytohormone production (Cochard et al., 2022).

In the previous chapter (Chapter 3), the potential microorganisms were screened for multiple plant growth-promoting traits. However, the *in vitro* characteristics must be verified by *in vivo* experiments. *In vitro* plant growth-promoting activities are insufficient to influence plant growth even under controlled conditions (Costa-Gutierrez, Adler, Espinosa-Urgel, & de Cristóbal, 2022). Although plant growth-promoting isolates are often selected based on *in vitro* tests, those do not necessarily correspond to the mechanisms, which influence growth promotion *in vivo* (Bergottini et al., 2015). Mere primary screening of isolates for PGPR traits does not guarantee efficacious plant growth-promotion under field conditions, as it is also possible that isolates that exhibit less *in vitro* growth-promoting activities might possess different plant growth-promotion strategies (Basu et al., 2021). Generally, microorganisms could demonstrate the maximum potential in producing plant growth hormones or enzymes that facilitate the biochemical assays as the growth conditions and medium will be optimized to trigger the beneficial activities of microorganisms. Conversely, in the actual plant condition, there might not be enough precursor produced by the plant to activate microbial biosynthesis of protein and enzymes that mediate the beneficial activities for plant growth. Thus, it is crucial to characterize the plant

growth-promoting traits by testing the potential bacteria in the actual plant at various growth stages.

Chapter four focuses on the preliminary validation of the seven good endophytic bacteria selected from the previous study and the characterization of potential plant growth-promoting endophytic bacteria using molecular identification, root colonization efficiency, and plant growth-promoting metabolites production. Seed germination analysis was done to confirm that the bacteria were safe to use for plants. The first selection of potential bacteria was made based on seed germination time and energy. Selected bacteria were tested as individual and mixed cultures to study the effect of seedlings' vegetative growth. Concurrently, molecular identification was made to eliminate bacteria regarded as pathogens and opportunistic pathogens in plants, humans, and animals. Finally, bacteria with no biosafety issues and showed positive effects on seedlings' growth were tested on root colonization efficiency. The production of metabolites related to plant growth were also evaluated. Exopolysaccharide (biofilm) production could indicate root colonization but does not guarantee to enter the root cells, as the bacteria must compete with the dominant root microbiome and be compatible with root exudates. In past studies, much less attention was given to the early stages of interaction between bacteria and plant roots, which may be essential for successful root colonization (Costa-Gutierrez et al., 2022). Finally, the effect of the selected bacteria was tested in a shade-house study to determine the efficiency of single and mixed cultures of the selected endophytic bacteria in promoting the vegetative growth and yield of chilli planted in a soilless medium.

4.1 Materials and Methods

4.1.1 Preparation of Endophytic Bacterial Culture

Selected pure cultures of bacteria with desired characteristics were inoculated in Tryptic Soy Broth (TSB) (Merck, Germany) and incubated for 5 days at 90 rpm on an incubator shaker (Wisecube, Faculty of Science & Engineering, University of Nottingham, Malaysia) at room temperature. The culture was centrifuged at 4,000 rpm for 15 minutes (Refrigerated centrifuge, Eppendorf, CEPB laboratory,

University of Nottingham, Malaysia), and the cells were washed with distilled water. It was then diluted x10 for seed germination and seedling growth analysis.

4.1.2 Assessment of the Compatibility between the Selected Endophytic Bacteria

One bacterial endophyte was streaked as a line in the middle of the agar plate, and the other isolates were streaked perpendicularly across this line on a separate Tryptic Soy Agar (TSA) (Merck, Germany). The bacteria were incubated at room temperature for one week to observe for inhibition of bacterial growth. The compatible bacteria were cultivated in TSB for three days and re-streaked on TSA to check the survival of the bacterial cells in a mixed culture.

4.1.3 Priming of Chilli Seeds with Endophytic Bacteria

SJ2-461, F1 hybrid hot chilli seeds were purchased from SAKATA Corp. Chilli seeds (SAKATA 461) were soaked in 70% ethanol for 1 minute, followed by soaking in 1% sodium hypochlorite for 10 minutes and finally rinsed with sterile distilled water five times. After the sterilization, seeds were primed in 10 ml of bacterial cells for 1 hour (Roslan et al., 2020) in sealed conical flasks. The seeds were spread out on filter paper to blot out the treatment solution at room temperature for 30 min. Then, the seeds were placed on a petri dish containing sterile Whatman filter paper (No.42, 90 mm) saturated with 2 mL of sterile distilled water. The Petri dishes were sealed with parafilm and covered with aluminium foil. The plates were kept at 25 ± 2°C and observed for 14 days. Twenty seeds with three replicates were used for each potential endophytic bacteria.

4.1.4 Effect of Endophytic Bacterial Treatment on Chilli Seed Germination

Germination was recorded every day, and the germination percentage, mean germination time (MGT), seed germination energy and seed vigor index was estimated based on the formula below (Muthu-Pandian Chanthini et al., 2019).

$$Germination\ percentage\ (\%) = \frac{Number\ of\ seeds\ germinated}{Total\ number\ of\ seeds} x\ 100$$

$$Mean\ germination\ time\ (MGT) = \frac{\sum(nT)}{\sum n}$$

n = Number of newly germinated seeds at time T (25°C)

T = Hours from the beginning of the germination test

∑n = Final germination

$$Seed\ germination\ energy\ (\%), GE = \frac{Number\ of\ germinated\ seeds\ for\ 3\ days}{Total\ number\ of\ seeds}\ x\ 100$$

$$Vigor\ index = Seed\ germination\ (\%) x\ (Mean\ root\ length\ + Mean\ shoot\ length)$$

The treatments for seed germination are described in **Table 4.1**

Table 4.1 Seed germination treatments using the selected endophytic bacteria based on superior *in vitro* beneficial traits

Treatments	Endophytic bacterial code	Beneficial traits
T1	MR13	P-solubilizing endophytic bacteria
T2	CKR8	P-solubilizing endophytic bacteria
T3	ML1	Indole acetic acid-producing endophytic bacteria
T4	CKL7	Siderophore-producing endophytic bacteria
T5	MR1	ACC deaminase-producing endophytic bacteria
T6	MR10	ACC deaminase-producing endophytic bacteria
T7	MR3	Endophytic bacteria with bio-control activity
T8	Distilled water	Control

4.1.5 Effect of Selected Endophytic Bacterial Treatment from Seed Germination Test on the Vegetative Growth of Chilli Seedling

Four endophytic bacteria, namely, CKR8, MR3, MR10, and MR13, were selected for seedling growth study based on their potential to enhance seed germination (**section 4.2.2**). For the vegetative growth analysis, chilli seeds were soaked in water overnight before sowing into polybags containing wet peat moss. The polybags containing peat moss were covered with black plastic sheets and kept at room temperature in a dark room until the chilli seeds germinated. The black sheets were removed once the radicle was visible, and the polybags were observed in the shade until true leaves emerged. Peat moss was sprayed with water every two days once or when dry. When 2-4 leaves emerged from the seedlings, 2 ml of the bacterial treatment (1 x 10^8 cfu/mL) was applied to the peat moss. Seedling growth analyses were done three weeks after germination. The experiment was conducted using a completely randomized design. Each treatment was replicated thrice with five plants in each replicate. The treatments are described in **Table 4.2**.

Table 4.2 The details of the individual and mixed culture treatments of four endophytic bacterial inoculants selected from seed germination test on chilli seedlings' vegetative growth

Code of treatment	Bacterial inoculant used in the treatment
T1	MR13 (P-solubilizing endophytic bacteria)
T2	CKR8 (P-solubilizing endophytic bacteria)
T3	MR10 (ACC deaminase-producing endophytic bacteria)
T4	MR3 (Endophytic bacteria with bio-control activity)
T5	MR13 + MR10 + MR3
T6	MR13 + MR3 + CKR8
T7	MR13 + MR10 + CKR8
T8	CKR8 +MR13
T9	MR13 + MR3
T10	MR13 + MR3 + MR10 +CKR8

T11	MR 10 + MR 13
T12	Distilled water (Control)

4.1.5.1 Effect of Endophytic Bacteria on the Relative Chlorophyll Content of Chilli Seedlings

Relative chlorophyll content was measured on each treatment's third, fully expanded leaf from the shoot apex. Relative Chlorophyll Content was measured using the SPAD-502 chlorophyll meter (SPAD 502, Minolta-Camera Co., Osaka, Japan). The data points were recorded at five positions along the leaf blade, and then the data points were averaged as a single value.

4.1.5.2 Effect of Endophytic Bacteria on the Length and Surface Area of Chilli Root

The roots were separated from the shoot and placed into clean plastic bags. Peat moss attached to chilli seedlings was removed gently by shaking and washing the roots in clean water several times. WinRhizo root scanner (EPSON Flatbed Scanner EPSON Expression 11000XL) was used to capture the image of chilli roots and measure the total root length and total root surface area. Chilli roots were placed in clean water to view under the root imaging system.

4.1.5.3 Effect of endophytic Bacteria on the Height of Chilli Seedlings

Seedling height was measured above the soil level until the shoot tip using a measuring tape. The average height of five seedlings was recorded.

4.1.5.4 Effect of Endophytic Bacteria on the Leave Area Meter of Chilli Seedlings

All the leaves were collected in paper bags. Each leaf was spread well before placing it on the Leaf area meter (Licor, CEPB laboratory, University of Nottingham, Malaysia).

4.1.5.5 Effect of Endophytic Bacteria on the Root-to-Shoot Ratio of Chilli Seedlings

The leaves, roots, and stems of the plant were partitioned separately and placed in an oven for three days at 60°C. Eight plants representing each treatment were selected for this analysis. Root- to-shoot ratio was calculated using the formula below.

$$\frac{Root}{Shoot} = \frac{Root\ dry\ weight}{Shoot\ \&\ stem\ dry\ weight}$$

4.1.6 Molecular Identification of the Selected Potential Endophytes Based on Seedlings' Vegetative Growth and Bioinformatics Analysis

Selected endophytic bacteria were identified based on 16S rRNA gene sequencing. Endophytic bacteria were grown on Luria Bertani agar (MERCK, USA) for a pure colony. One pure colony was picked to inoculate 5 mL of Luria Bertani broth (MERCK, USA) and incubated overnight at 30°C. Genomic DNA was extracted using Presto Tm Mini gDNA Bacteria Kit (www.geneaid.com). The following primers were used for the amplification of 16 S ribosomal DNA. 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (3'-CTA CGG CTA CCT TGT TAC GA-5') (Szymańska et al., 2016). Polymerase chain reaction (PCR) was carried out using MyTaq^{TM} Red Mix (Bioline, UK) in 50 µl reaction mixture containing 25 µl of MyTaq^{TM} Red Mix (Bioline, UK), 22 µl of molecular grade water, 1µl of each primer (10 mM) and 1 µl of genomic DNA. The following thermal conditions were used: 95°C for 2 min, followed by 94°C for 1 min, 55°C for 1 min, 72°C for 2 min in 30 cycles, and a final extension step at 72°C for 5 min. PCR amplification products were purified using NucleoSpin Gel and PCR Cleanup kit (Macherey- Nagel, Germany) according to the manufacturer's protocol and visualized on a 1% gel red using Syngene (USA). The fragments were outsourced for sequencing service using primers 27F and 1492R (Apical Scientific Sdn. Bhd, Serdang, Malaysia). The acquired sequence was analyzed using reference sequences deposited in the GenBank nucleotide database. A minimum of 99% similarity was required for appropriate

identification. All of the DNA sequences of endophytic bacteria were submitted to GenBank. Using BLAST Search, the 16R rRNA gene sequence was compared with available gene sequences of endophytic bacteria in the National Centre for Biotechnology Information (NCBI) GenBank. A neighbor-joining phylogenetic tree was constructed using MEGA 12.0 with bootstrap analysis based on 1000 replicates.

4.1.7 Metabolites Production by the Identified Endophytic Bacteria

Each pure bacterial culture was inoculated in 50 mL of Tryptic Soy broth (Merck, Germany) for 48 h at room temperature, as described in **section 4.1.1**. The washed cells were kept overnight in a -80°C freezer (Faculty of Science and Engineering, University of Nottingham, Malaysia) before placing them in a freeze dryer (Faculty of Science and Engineering, University of Nottingham, Malaysia) for 48 h. About 0.3 mg of the lyophilized cell of bacteria was weighed separately into a 2.0 mL microcentrifuge tube. An internal standard, 3-phenyl butyric acid (10 µl) of 50 µl was added using a 100 µl glass syringe and followed by 1 mL of the extraction solvent, 80% methanol. The microcentrifuge tubes were placed in a vibration mill, and the parameters were set at 1800 rpm for 5 min on each side. Later, the microcentrifuge tubes were incubated at 70°C for 15 minutes at 1400 rpm in a Thermomixer Comfort, Eppendorf (Faculty of Science and Engineering, University of Nottingham, Malaysia), followed by centrifugation at 14 000 rpm for 20 min. The supernatant was transferred using a glass Pasteur pipette into a glass GC-sample vial and evaporated to total dryness in a nitrogen stream. Then, 50 ul of 20 mg/ml of methoxyamine hydrochloride (LiChropur™) solution in pyridine (Sigma Aldrich, USA) was added into a GC-sample vial using a glass syringe, capped, and heated for 90 min at 37°C in a Thermomixer. About 80 µl of BSTFA with 1% TMCS (LiChropur™) was added using a glass syringe and heated again for 90 min at 37°C using a Thermomixer. Finally, the sample was transferred into a new GC-sample vial.

The analysis used GC-MS, Perkin Elmer GC Clarus 680 SQ8 (Block N, Faculty of Science and Engineering, University of Nottingham). Gas Chromatogram

equipped with capillary column Elite-5MS (30 m x 0.25 mm internal diameter x 0.25µm). An electron ionization system with ionizing energy of 70 eV was used. Helium gas (99.99%) was used as the carrier gas at a constant flow rate of 1 mL/min, and an injection volume of 1 ml was employed at a split ratio of 50:1, injector temperature was at 250°C and ion source temperature was at 200°C. The oven temperature was raised from 10°C/min to 300°C after 3 min of constant heating at 80°C. Mass spectra were acquired using a full scan and a monitoring mode with a mass scan range of 50 to 550 m/z with a scan speed of 0.2 s after a solvent delay of 7 min. The spectra for each chromatogram peak were compared with those in the NIST database library.

4.1.8 Root Colonization Analysis by the Selected and Identified Endophytic Bacteria

Based on the molecular identification, endophytic bacteria regarded as safe for humans and the environment were selected for further study. Bacteria identified as opportunistic pathogens for humans and animals through an online database search were excluded from further investigation.

4.1.8.1 Total Bacterial Population in Chilli Roots Inoculated with the Selected Endophytic Bacteria

The taproots were separated from the primary root system. Roots were washed several times in sterile water to remove the adhered planting material. One gram of tips of taproot was weighed into distilled water and sonicated for 10 min at 19 kHz in an ultrasonicator. The root solution was incubated overnight at 28°C. The roots were homogenized in a pre-sterilized mortar and pestle before serial dilution until 10^6 and plated on Potato Dextrose Agar (PDA) (Merck, Germany).

4.1.8.2 Root Colonization by Single and Mixed Cultures of the Selected Endophytic Bacteria

Two non-pathogenic endophytic bacteria identified as *Bacillus* sp. (MR3) and *Streptomyces* sp. (MR13) were used for the root colonization (RC) study using

Field Emission Scanning Electron Microscopy (FESEM) (FEI, Quanta 400F/Oxford FESEM, Faculty and Science and Engineering, University of Nottingham, Malaysia). The bacterial inoculum was prepared as described in **section 4.1.1**. The treatments consisted of single and mixed cultures of *Bacillus* sp. (MR3) and *Streptomyces* (MR13). The inoculum was applied during two growth stages for *Streptomyces*: vegetative and sporulating. *Streptomyces* has a complex developmental process in solid and liquid cultures. Therefore, the growing method and culture medium for *Streptomyces* were evaluated in this study. The spores were collected using 0.1% tween 80 from mature colonies of *Streptomyces* in solid agar plates, while pellets of hyphae were obtained from the liquid cultures. Fifty microliters of inoculum were applied to the seedlings twice a month. Then, the roots were removed for analysis. The treatments were as follows:

RC (a): *Streptomyces* spores
RC (b): *Streptomyces* mycelium
RC (c): *Bacillus* cells
RC (d): *Streptomyces* spores and *Bacillus* cells
RC (e): *Streptomyces* mycelium and *Bacillus* cells
RC (f): No-inoculation (control)

For the observation under a scanning electron microscope, the roots were removed from the soil and washed several times to discard the planting materials attached to the roots. Then, root tips were cut into a bottle filled with sterile distilled water as a final washing step, followed by sonication for 2 min at 8 Hz at room temperature. Then, the roots were dried on tissue paper. Several roots were immersed in 3% glutaraldehyde buffered with 0.1 M phosphate buffer for 24 hrs. Later, the roots were rinsed thrice with 0.1 M of phosphate buffer, pH 7.2, for about 10 min per wash. After the fixation, the root samples were placed in 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.2, for about 2 hours at room temperature in a loosely capped bottle. Then, the washing step for 10 minutes was repeated three times using 0.1 M phosphate buffer, pH 7.2. For the dehydration process, a graded ethanol solution of 30%, 50%, 70%, 80%, 90%, and 95% (v/v) was prepared, and the roots were placed in each solution for 15

minutes. Later, the roots were transferred into 1: 2 (HMDS: 95% ethanol solution) and left for 20 minutes. This step was followed by immersing the root samples into 2: 1 (HMDS: 95% ethanol) and 100% HMDS for 20 minutes each. Finally, the sample was transferred to fresh HMDS and left in a fume hood overnight to evaporate HMDS. The next day, the samples were cut into tiny pieces with a 0.3- 0.5 cm length and placed on double-sided conductive carbon tape. The samples were sputtered with platinum for 30 seconds before viewing under a Field emission scanning electron microscope (FEI, Quanta 400F/Oxford FESEM, Faculty and Science and Engineering, University of Nottingham, Malaysia).

4.1.9 Effect of Single and Mixed Cultures of the Identified Endophytic Bacteria on Chilli Growth and Yield

A preliminary shade-house experiment was conducted to evaluate the effect of single and mixed cultures of identified endophytic bacteria on chilli's vegetative growth and yield. The seeds were soaked in water overnight before sowing into wet peat moss. The polybags were covered with black plastic sheets and kept at room temperature in a dark room until they germinated. The black sheets were removed once the radicle was visible, and the polybags were observed in the shade until true leaves emerged. Peat moss was sprayed with water every two days once or when dry. After the 14th day of sowing, fertilizer AB with an EC reading of 0.9 was poured into a tray, and the seedling tray was placed on it for maximum absorption of nutrients.

Transplanting of seedlings was done 28 days after sowing. The planting medium, cocoa peat and peat moss were prepared in an 8" x 12" cm polybag. A completely randomized block design was adopted for this experiment. The independent variable is AB liquid fertilizer doses, and the dependent variable is the endophytic bacterial treatment. The bacterial culture (100ml/plant) corresponding to $1x\ 10^{7}$ cfu/ml was applied during transplanting and twice a week by drenching the planting media. There were five treatments, with three replications of each treatment. Each replication consists of three plants.

4.1.10 Effect of Single and Mixed Cultures of Identified Endophytic Bacteria on the Vegetative Growth of Chilli Plants

Plants were removed from the planting material 40 days after transplanting. The shoot length was measured. The aboveground shoot and root were separated and transferred to the lab in separate plastic bags. The aboveground parts were placed in large brown envelopes before drying in the oven (CEPB laboratory, University of Nottingham) at 70°C for three days. The weight of dried aboveground biomass was measured using a weighing balance (CEPB laboratory, University of Nottingham).

For root samples, the planting material attached to the roots was removed by washing the roots in tap water. After cleaning the roots, they were scanned via WinRhizo root scanner (EPSON Flatbed Scanner EPSON Expression 11000XL, CEPB laboratory, University of Nottingham). The root imaging system measured the total root length and total root surface area.

4.1.11 Effect of Single and Mixed Cultures of Identified Endophytic Bacteria on the Yield of Chilli Plants

The effect of single and mixed bacterial culture on the yield of chilli plants was estimated by the fruit weight 40 days after transplanting.

4.1.12 Statistical Analysis

Seed germination was analyzed using binomial logistic regression in IBM SPSS Statistics 25. Binomial logistic regression estimates the probability of seed germination **Table A4** (**Appendix**).

Statistical analysis was performed using the univariate analysis with Tukey's HSD test ($p < 0.05$) by the SPSS software (IBM Statistics, version 28, USA) to determine the significance of the data. According to Tukey's HSD, bars sharing the same letters are not significantly different ($p \leq 0.05$). The vertical bar in the graphs indicates the standard error.

4.2 Results and Discussion

4.2.1 Assessment of the Compatibility between the Selected Endophytic Bacteria

All seven endophytic bacteria exhibited co-existence ability in this experiment. An example of the compatibility test between three bacterial isolates is shown in **Fig. 4.1**. Positive compatibility of the isolates was recorded as no inhibition zones were observed near the isolates streaked on agar medium. Among all the 70 endophytic bacteria isolated from the roots and leaves, only seven were selected for *in vivo* experiments. The selection process was based on the potential of endophytic bacteria to demonstrate more vigorous beneficial activity, as determined by the size of clear zones and quantified plant growth-promoting beneficial traits (**Chapter 3**). Compatibility is a necessary test in formulating bio-inoculants. It was also stated that research should be rationally designed by mixing bacteria with no negative interaction, which could enhance synergistic effects among members of the consortium (Costa-Gutierrez et al., 2022).

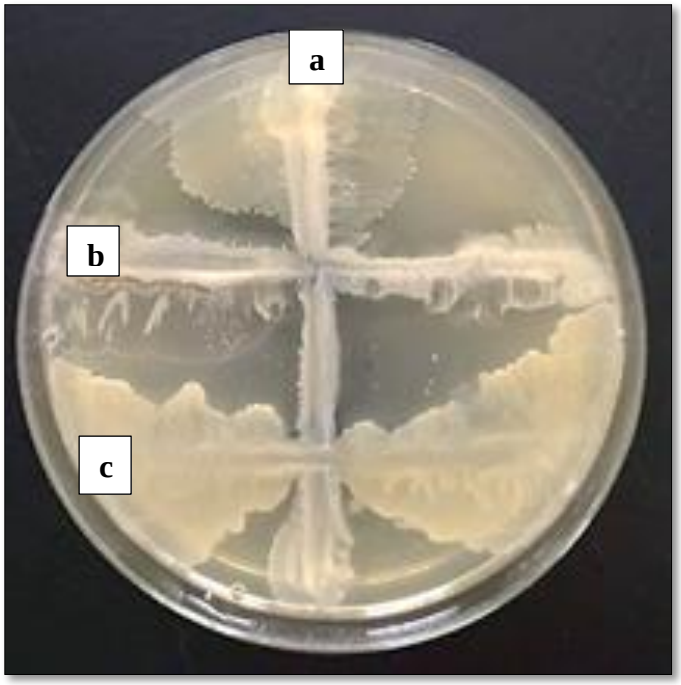


Fig. 4.1. Compatibility test between three isolates of endophytic bacteria. (a) MR10, (b) CKR8 and (c) MR3 shows no inhibition of growth / no antagonistic activity

4.2.2 Effect of Endophytic Bacterial Treatment on Chilli Seed Germination

In this study, endophytic bacteria (1 x 10^8 cfu/ml) were treated on chilli seeds to check their efficiency and toxicity on chilli growth. The germination percentage was 100 % for all chilli seeds treated with endophytic bacteria. A binary logistic regression was performed to ascertain the effects of endophytic bacteria on the germination of chilli seeds. The logistic regression model was not statistically significant, $\chi 2$ (1) = 2.809, p=0.95. The model explained between 0.7 % (Cox & Snell R2) to 10 % (Nagelkerke R2) of the variance in seed germination and correctly classified 73.4 % of cases. Sensitivity was 100%, specificity was 0%, positive predictive value was 98.3 %, and negative predictive value was 0% (**Table 4.3**).

In this study, MR13 and distilled water exhibited the lowest mean germination time, followed by CKR8, MR3, and MR1 (**Fig. 4.2**). Mean germination time (MGT) demonstrates the rate and the time taken for germination, while germination energy corresponds to the speed and uniformity of seedling emergence. The lower the mean germination time, the earlier the seeds could germinate. Low MGT of MR13 and control seeds treated with water indicates that the seeds germinated quickly and uniformly. In contrast, a high MGT demonstrates that the seeds took longer to grow and were less uniform in their germination timing. MR13 and control did not show a significant difference in mean germination time, while other bacterial treatments have demonstrated low MGT despite all treatments showing 100% germination. Once the radicle has emerged, the effects of bacteria on seed metabolism are usually limited. This is because the initial germination process involves activating enzymes and repairing cellular damage, largely independent of bacterial activity. Additionally, the seedling begins to rely on its metabolic processes for growth and development, which bacterial interactions may not influence. Thus, MR13 has contributed to the rapid radicle elongation, while other bacteria do not affect the emergence of the first radicle. When seeds are primed, they absorb water, which leads to a sudden increase in weight. This increase in water content helps to repair cell membranes and proteins, as well as mitochondrial and DNA damage. Additionally, it suppresses germination inhibitors and allows small

molecules to diffuse throughout the seed to be used for growth. As a result, the radicle, which is the embryonic root, begins to grow due to a rapid elongation of its cells, followed by slower cell division-driven growth (Rozier, Gerin, Czarnes, & Legendre, 2019).

MR13 also exhibited the highest germination energy, followed by distilled water, MR1, MR3, CKR8, and MR10 (**Fig. 4.3**). Germination energy is an essential measure of seed quality as it provides information on the percentage of viable seeds that have the potential to germinate and produce healthy seedlings. Based on the germination energy result, MR13 exhibited the potential to improve seed quality, leading to a successful crop establishment.

The vigor index (VI) is a measure of indication of the total performance of the seed. Taking into consideration the root and shoot length of the seedlings, MR10 showed the highest vigor index (12 457 VI), followed by MR3 (9450 VI), MR13 (8730 VI), and CKR8 (7120 VI). The Vigour index is a helpful tool for evaluating seed quality and predicting the growth potential of seedlings, which can be essential for crop management and yield. As the radicle grows, the seed actively takes in water, causing its weight to increase again. The stored energy in the seed, such as starch, proteins, and lipids, will only be consumed after germination to provide the necessary energy for seedling development. MR10 and MR3 could contribute to the seedling quality compared to other treatments.

Seed germination analysis reveals both the toxicity and efficiency of endophytes on chilli. It was proven that the endophytes were not harmful but helped the early germination of seeds and could enhance the overall seed quality. Based on these observations, MR13, MR3, MR10, and CKR8 were selected for the seedling growth analysis.

In this experiment, using PGP endophytes improved chilli seed germination and seedling growth. The results of this study provide further support for the idea that PGPR can be a valuable alternative to traditional, energy-demanding physical treatments for seeds. *A. lipoferum*, a PGPR isolate, was reported to bear agronomic potential as a bio-priming agent (Rozier et al., 2019). Likewise, *B.*

megaterium strains improved seed germination and initial seedling growth and were recommended as an environmentally friendly strategy to prime seeds before field planting (Miljaković et al., 2022).

Table 4.3 Logistic regression predicting the likelihood of seed germination based on treatments

Variables in the Equation

							95% C.I.for EXP(B)	
		B	S.E.	Wald	Sig.	Exp(B)	Lower	Upper
Step 1[a]	Endophytic bacterial code	.083	.050	2.793	.095	1.087	.986	1.198
	Constant	-1.385	.254	29.830	<.001	.250		

a. Variable(s) entered on step 1: Endophytic bacterial code.

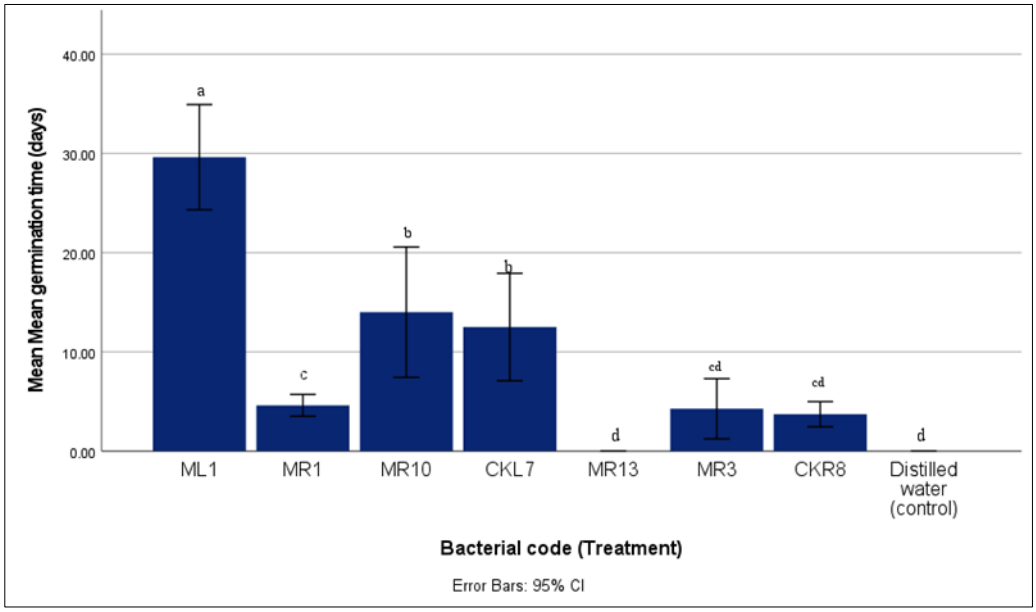


Fig. 4.2 Graph shows the mean germination time (MGT) of chilli seeds treated with potential endophytic bacteria. Each value is the mean of four replicates (n=4), and bars sharing the same letters are not significantly different ($p \leq 0.05$) according to Tukey's HSD. The vertical bar indicates the standard error

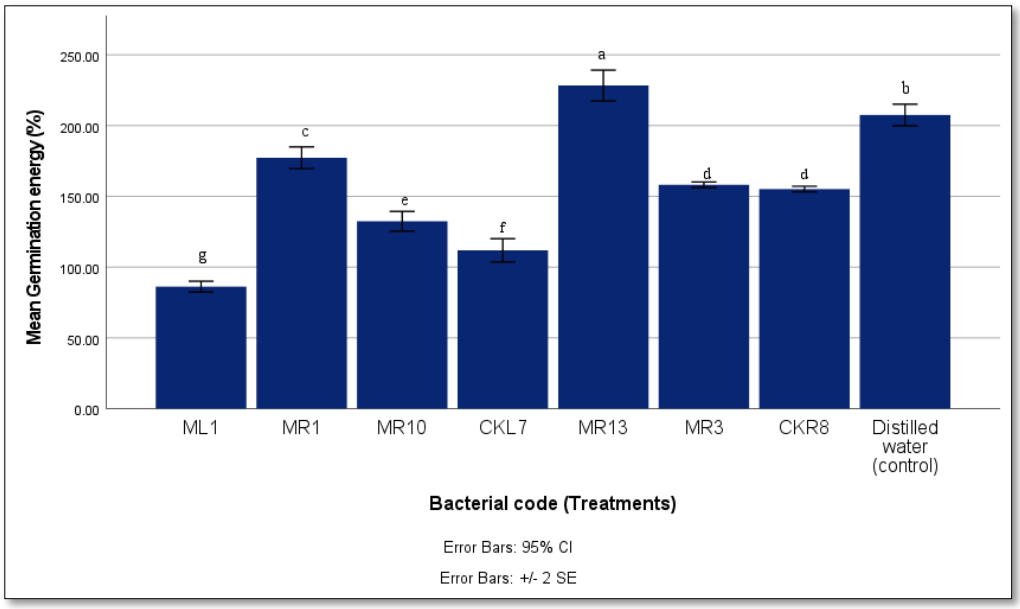


Fig. 4.3 Graph depicts the germination energy (%) of chilli seeds treated with potential endophytic bacteria. Each value is the mean of four replicates (n=4), and bars sharing the same letters are not significantly different ($p \leq 0.05$) according to Tukey's HSD. The vertical bar indicates the standard error

4.2.3 Effect of the Selected Endophytic Bacterial Treatment on the Vegetative Growth of Chilli Seedlings

Based on the seed germination results, only four endophytic bacteria, MR3, MR13, CKR8, and MR10, were selected for further study. Vegetative growth analysis, such as seedlings' height, root length, root surface area, and relative chlorophyll content, was done to select the most efficient bacteria to enhance the vegetative growth of chilli (**Table 4.4**). Based on the seed germination analysis, T1 (MR13), T2 (CKR8), T3 (MR10), and T4 (MR3) were selected for single inoculant treatment and mixed culture treatment to determine the efficiency of chilli seedlings' development.

All the endophytic bacterial treatments have increased the height of chilli seedlings significantly compared to the control. The highest height of chilli seedlings was recorded by T8 (CKR8+MR13), followed by T5 (MR13+MR10+MR3) and T6 (MR13+MR3+CKR8). Seedling height is a direct parameter that demonstrates the efficiency of bacterial inoculants in promoting plant growth.

The total root length and surface area of chilli seedlings demonstrated a significant increase in chilli seedlings inoculated with T3 (MR10). The rest of the treatments did not show a significant difference. T7 (MR13+MR10+CKR8) has caused root length and surface area reduction, which is lower than the control (**Table 4.4**). This finding shows that there could be antagonistic activity among these three bacteria where the metabolites produced by bacterial culture could have retarded the root growth of the seedlings. Although the compatibility test did not show this antagonism effect in the earlier trial, the active growth stage in a liquid medium could have produced unfavorable metabolites for the growth of the seedlings. Furthermore, the period from seed germination to seedling change is a critical plant growth stage. A small amount of toxicity could be very harmful to the development of the seedlings during this vulnerable growth stage. Previously, it was reported that co-inoculation of *Bacillus subtilis* and *Enterobacter cloacae* was not conducive to the growth of *B. subtilis*. Among all the beneficial traits tested, only siderophore-related genes in *Bacillus subtilis* were upregulated (Yan Li et al., 2021). This shows that the compatibility of bacterial relies on the chemical interaction between them.

The root images depict the effect of the endophytes inoculation on root growth (**Fig. 4.4**). It is evident from the root images that inoculation with mixed bacterial cultures shows the development of thick root collar stem and thick roots with high density (**Fig. 4.4 g-l**) as compared to single bacterial culture and control (**Fig. 4.4 a-f**). In general, all bacterial cultures have enhanced root development by increasing the root length, hair, density, and thickness compared with non-inoculated seedlings (control). A similar finding was found in the past that co-inoculation of different genera of bacteria promoted the growth of maize and peanut crops (Anzuay et al., 2017). In another study, co-inoculation of *B. cepacia* and *R. palustris* increased peanuts' growth, quality, and yield (Y. Wang et al., 2021).

SPAD meter readings revealed that T11 (MR10+MR13) treatment resulted in high related chlorophyll readings. The chlorophyll content in a plant determines the photosynthetic capacity, leaf nitrogen level, and general plant health.

Relative chlorophyll content can be measured using a non-destructive method with a SPAD meter. The relative SPAD meter values are proportional to the chlorophyll content of the leaves.

Root development and the modification of root architecture are the fundamental criteria for beneficial endophytic bacteria, which have a high affinity towards the root exudates and colonize the host plant's root efficiently. *B. subtilis* and *B. japonicum* were reported to improve root growth and positively change soybean's root architecture to aid the uptake of essential nutrients (Araujo, Bonifacio, Bavaresco, Mendes, & Araujo, 2021). An extended root surface could increase plant roots' nutrient acquisition and water uptake (Ribeiro et al., 2018). Two-way chemical interactions were observed between endophytes and root hair cells (Chang, Kingsley, & White, 2021). Endophytes consume root exudates rich in organic acids, carbohydrates, amino acids, and vitamins, while plants use microorganisms as their source of nutrients. Endophytes also produce ethylene that enhances root hair growth and elongation, which could strengthen nutrient acquisition by plant roots. Once enters the plant root cells, endophytes become non-walled protoplast due to the degradation of the bacterial cell wall by superoxide produced by plants (White, Kingsley, Verma, & Kowalski, 2018), and the nutrients from the endophytic microorganisms will be extracted by plant cells while the endophytes proliferate and enhance root elongation and root hair growth inside the root cells. As the nutrients deplete, the endophytes will be ejected from the root and scavenge nutrients in the soil. The endophytes can form a new cell wall in the rhizosphere.

Table 4.4 Vegetative growth analysis of chilli seedlings with beneficial endophytic bacteria

Endophytic bacterial code	Seedling's height (cm)	Root length of seedlings (cm)	Root surface area of seedling (cm^2)	Relative Chlorophyll content (SPAD-value)
MR13 (T1)	15.7±1.46^{ab}	71.8±6.5^{abc}	11.48±0.97^{ab}	31.7
CKR8(T2)	15.9±1.34^{a}	55.3±14.8^{bc}	9.11±1.77 ^{ab}	33.8
MR10 (T3)	15.3±1.09^{ab}	109.33±2.92^{a}	13.53±0.13^{a}	31.1
MR3 (T4)	14.4±1.5^{ab}	86.1±14.2 ^{abc}	12.4±0.77 ^{ab}	30.4
MR13+MR10+MR3 (T5)	16.8±1.03^{a}	69.1±18.7 ^{abc}	10.47±1.01 ^{ab}	29.9
MR13+MR3+CKR8 (T6)	16.1±2.56^{a}	80.7±17.7 ^{abc}	11.33±2.33 ^{ab}	36.8
MR13+MR10+CKR8(T7)	15.5±1.39^{ab}	47.9±11.02^{c}	8.62±2.26^{b}	31.4
CKR8+MR13 (T8)	17.0±1.47^{a}	76.3±6.7 ^{abc}	10.17±0.77 ^{ab}	31.5
MR13+MR3 (T9)	16.1±1.66^{a}	76.4±5.43 ^{abc}	11.18±1.53 ^{ab}	36.2
MR13+MR3+MR10+CKR8 (T10)	15.13±2.7^{ab}	71.8±25.2 ^{abc}	11.18±1.63 ^{ab}	35.7
MR10+MR13 (T11)	13.5±1.78^{ab}	100.4±8.54^{ab}	13.09±0.48 ^{ab}	40.3
Control (T12)	12.6±0.58^{b}	63.47±12.03 ^{abc}	10.16±0.18 ^{ab}	29.9

*The data was calculated as the average of five replications. Means with different superscript letters differ significantly at $p<0.05$ (Tukey HSD).

The total root length and root surface area of chilli seedlings demonstrated a significant increase in chilli seedlings inoculated with T3 (MR10). The rest of the treatments were not different significantly. T7 (MR13+MR10+CKR8) has caused root length and surface area reduction which is lower than the control

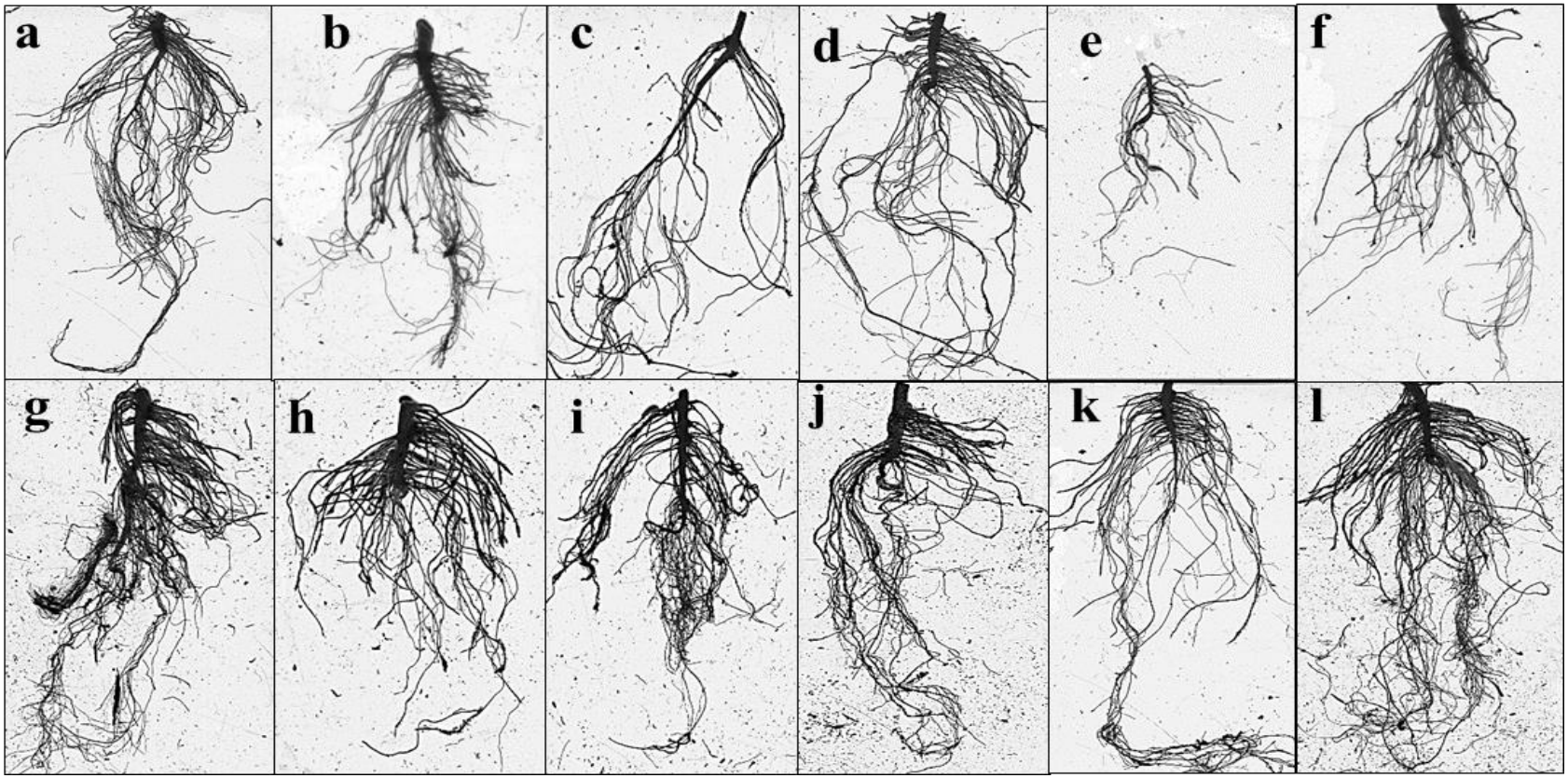


Fig. 4.4 Root images of chilli seedlings inoculated with endophytic bacteria captured using WinRhizo Root Scanner. (a) MR13, (b) CKR8, (c) MR10, (d) MR3 and (e) Control (f) MR13+MR10+MR3 (g) MR13+MR3+CKR8 (h) MR13+MR10+CKR8 (i) CKR8+ MR13 (j) MR13+ MR3 (k) MR13+ MR3+MR10+CKR8 (l) MR10+MR13

Compatibility is an essential criterion for preparing a mixed culture of microorganisms. Bacteria that showed various beneficial traits might be harmful to other species or a genus of bacteria that co-exist in a solution. The secondary metabolites produced by some bacteria might delay, inhibit or destroy other bacterial cells. Therefore, the compatibility of selected bacteria was observed in an agar plate assay to ensure that the bacteria do not show antagonistic activity against each other. This study justifies that the mixed culture of bacteria may have both inhibition and stimulation effects. The beneficial characteristics of genes of interest could be upregulated or downregulated in the mixed culture.

4.2.4 Molecular Identification of the Selected Endophytic Bacteria and Phylogenetic Analysis

BLAST search revealed the identification of the endophytic bacteria as described in **Table 4.5.** Based on 16S rRNA sequence analysis, the endophytic bacterial isolates were classified as the genus *Proteus*, *Enterobacter*, *Bacillus*, and *Streptomyces*.

Table 4.5 Molecular identification of endophytic bacteria based on National Centre for Biotechnology Information (NCBI) GenBank database BLAST search

Endophytic bacterial code	Molecular Identification according to GenBank database	Percentage of identification similarity	E-value
ML1	*Proteus mirabilis* (CP053685.1)	96.85 %	0.00
MR13	*Streptomyces panaciradicis* (NR_134200.1)	100.0 %	0.00
MR10	*Enterobacter cloaceae* (OK083761.1)	98.00 %	0.00
CKR8	*Enterobacter cloaceae* (MT679256.1)	98.64 %	0.00
MR1	*Enterobacter cloaceae* (CP040827.1)	100.0 %	0.00
CKL7	*Bacillus sp.* (LC202628.1)	80.49 %	0.00
MR3	*Bacillus subtilis* (CP054177.1)	100.0 %	0.00

Due to the ambiguous classification of human pathogenic and plant growth-promoting bacteria, only MR13 and MR3 could be regarded as potential bio-inoculants for the development of chilli bio-stimulant. The evolutionary history for MR3 and MR13 was determined using the Neighbour-Joining method (**Fig. 4.5**). The phylogenetic analysis also confirmed that MR3 is closely related to *Bacillus* sp. (**Fig. 4.6**). In contrast, MR 13 is closely associated with *Streptomyces* sp. (**Fig. 4.7**). An essential characteristic of plant growth-promoting bacteria is not harmful to the environment or humans (Basu et al., 2021). The DNA sequences were deposited in the NCBI database under the accession numbers OM714810 (**Fig. A7**) (**Appendix**) and OM001090 (**Fig. A8**) (**Appendix**).

The published sequences in the National Center for Biotechnology Information (NCBI) can be found at: https://www.ncbi.nlm.nih.gov/nuccore/OM714810 https://www.ncbi.nlm.nih.gov/nuccore/OM001090

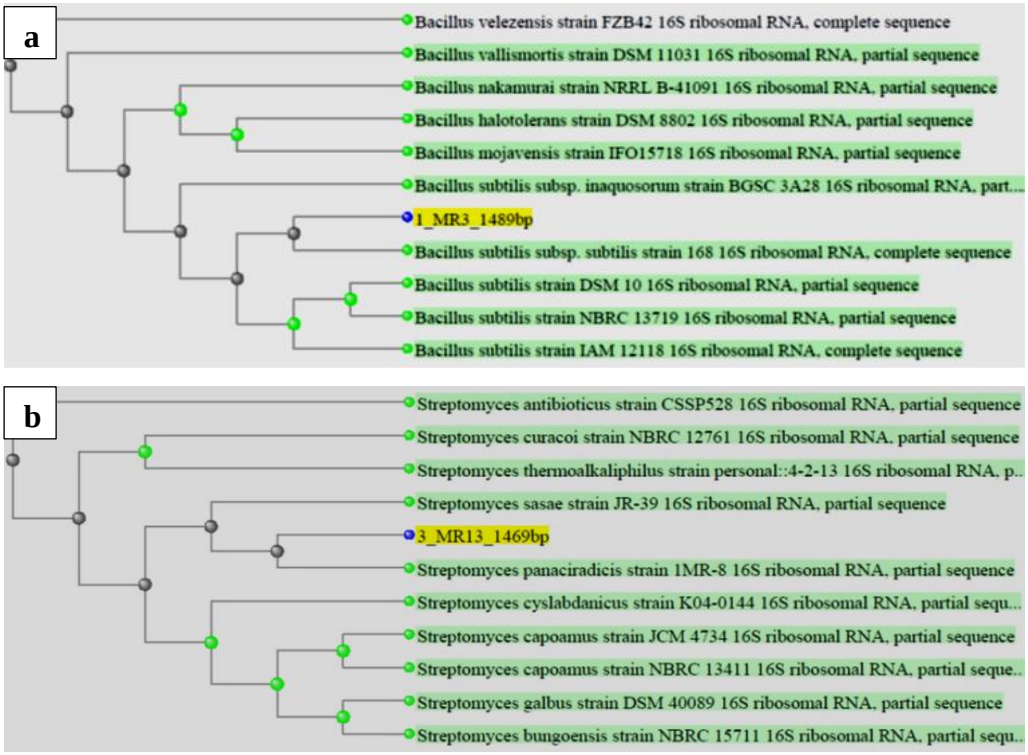


Fig. 4.5 Phylogenetic tree of the identified endophytic bacteria using the Neighbour-Joining method. The bootstrap consensus tree inferred from 1000 replicates represents the evolutionary history of (a) MR3 and (B) MR13 taxa. This analysis involved twelve nucleotide sequences. Evolutionary studies were conducted in the MEGA11 software.



Fig. 4.6 Morphology of *Bacillus subtilis* in potato dextrose agar

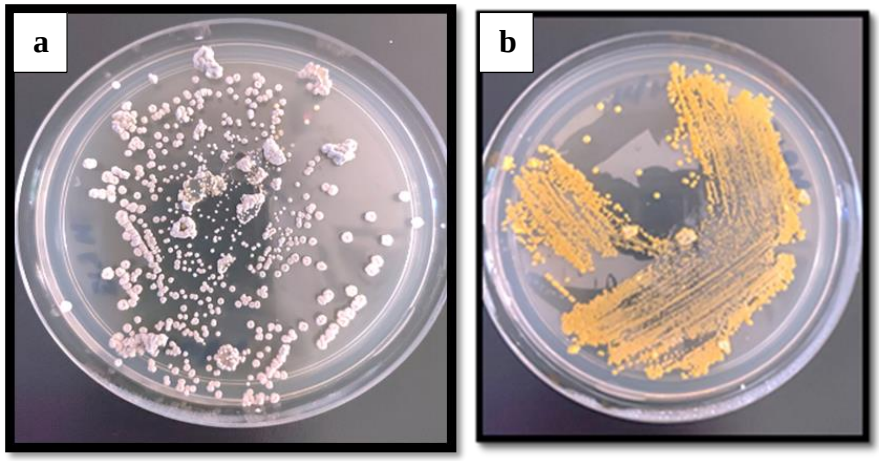


Fig. 4.7 Morphology of *Streptomyces panaciradicis* in potato dextrose agar (a) Early sporulating stage of *Streptomyces panaciradicis* (b) Matured spores after 3-4 weeks incubation

Identification of bacteria is vital to avoid human pathogens in the study. Numerous studies have reported that efficient PGPR with a positive effect on plants and soil was attributed to several opportunistic pathogens for humans and animals or clinical isolates (Chauhan, Bagyaraj, Selvakumar, & Sundaram, 2015). Therefore, it is essential to eliminate pathogens from the study during laboratory experiments. ML1, which was identified as *Proteus mirabilis*, has also been reported as a pathogen (Ghramh, Khan, & Alshehri, 2019; E. I. Hussein et al., 2020) and plant growth-promoting bacteria (Amaresan, Jayakumar, Kumar, & Thajuddin, 2021; Zia et al., 2021). MR3 and CKL7 were

identified as *Bacillus* sp. where this species is well known for crop growth enhancement (Araujo et al., 2021; de Lima, Bonifacio, de Alcantara Neto, Araujo, & Araujo, 2022; Amrit Kumar, Rabha, & Jha, 2021; Sehsah et al., 2021). MR10, CKR8, and MR1 were identified as *Enterobacter* sp. that has been reported to boost plant growth (Ghosh et al., 2022; Ludueña et al., 2019; Panigrahi, Mohanty, & Rath, 2020). *Streptomyces* have been reported to survive in extreme and unfavorable conditions due to the production of filaments and spores. Furthermore, they influence soil fertility by breaking down complex nutrients into simple and available forms via multiple enzyme activities (Vurukonda et al., 2018). *Streptomyces panaciradicis* was previously reported as a β-glucosidase-producing bacterium isolated from Ginseng (the root of Panax ginseng) used as a traditional medicine in Asian countries (Lee, Cho, Chung, & Whang, 2014). This is the first report on *S. panaciradicis* as an efficient phosphate-solubilizing endophyte or phyto-stimulating agent for chilli. *S. panaciradicis* has been reported very little, making it the least studied bacteria in these contexts.

The biosafety of the microbial inoculant is an essential criterion for selecting the potential microorganism for glasshouse or field application. Most microorganisms are ubiquitous and can be found as pathogens or beneficial strains in diverse places. The function of the microorganism might change according to the host's condition. It is common to find human pathogens as beneficial bacteria for plant growth in laboratory experiments. However, the biosafety issue will arise regarding commercialization or large-scale production. There is also a need for proper guidelines on testing the virulence of the bacterial species categorized as a pathogen and beneficial bacteria. The distinction between pathogens and phylogenetically related PGPR can be unveiled via comparative genomics and pathogenesis study using nematodes (Igiehon & Babalola, 2018).

4.2.5 Metabolites Production by the Identified Endophytic Bacteria

GC-MS results revealed that many unknown compounds were produced by *Bacillus* sp. (MR3) and *Streptomyces* sp. (MR13). **Fig. A9** (**Appendix**) and **Fig.**

A10 (**Appendix**) depicts the GC-MS chromatograms of MR3 and MR13, respectively. The chromatograms show the retention time and abundance of metabolites produced at the specific retention time. The top three metabolites produced by *Bacillus* sp. (**Fig. A11**) (**Appendix**) and *Streptomyces* sp. (**Fig. A12**) (**Appendix**) are listed in **Table A5** (**Appendix**). Based on literature reviews, many compounds were identified in the PGP- endophytes belonging to bio-control and medicinal compounds such as anti-cancer, anti-diabetes, anti-viral, antibiotics, stimulant drugs, etc. (**Table A6 and Table A7**) (**Appendix**). The reason for the similar characteristics of the two plant growth-promoting endophytes may be attributed to their source of isolation, which is the root of the moringa plant. Moringa is known for its high nutritional and medicinal properties, and the endophytes that exist within the same plant may possess similar beneficial traits. Therefore, the similarities observed in the two endophytes may be linked to their association with the moringa plant. Endophytes have been found to produce extracellular compounds that have potential applications in agriculture, including as bio-pesticides, bio-control agents, antioxidants, herbicides, plant growth regulators, plant micronutrients, and plant adaptation enhancers. The unknown compounds of MR3 were listed in **Table A8** (**Appendix**).

Endophytes produce functional metabolites such as alkaloids, terpenoids, steroids, quinone, isocoumarin derivatives, flavonoids, phenols, phenolic acids, and peptides (D. S. Arora & Kaur, 2019). This research has demonstrated that *Bacillus* and *Streptomyces* can produce a wide range of active compounds with diverse biological activities. These compounds can potentially be beneficial in synthesizing essential products for various industries, including agriculture, medicine, pharmaceuticals, agrochemicals, food, and cosmetics. The finding of this study corroborated by another study that investigated the metabolites produced by the genus *Bacillus,* where it was reported to be among the majority of bacteria isolated from medicinal plants and a potent producer of antibiotics which possess the inhibitory effect against different pathogens (Ntemafack et al., 2022). The root extract of *Chenopodium album* was also found to contain an antifungal compound, 9-Octadenoic acid that was found in *Streptomyces* sp. cell extract (Osman Mohamed Ali et al., 2017). Likewise, 1-(5-

bicyclo[2.2.1]heptyl)ethylamine is an alkaloid extracted from *Petiveria alliaceae* L. leaves and tested for antibacterial and antiviral properties was also found in *Bacillus* sp.(Durgawale, Khanwelkar, & Durgawale, 2018). Tripenoids were reported as defensive compounds against pathogenic microbes (Sawai & Saito, 2011). Antimicrobial compounds produced by endophytes could help control pathogenic microbes and prevent the spread of diseases in crops, livestock, and humans. Natural pigments and antioxidants produced by endophytes could be used as natural food additives and cosmetics to treat various conditions. Although many species of actinomyces, particularly those of the genus *Streptomyces*, inhibit many fungal pathogens and are considered efficient bio-control agents, their metabolic products can influence plant growth (Passari et al., 2015).

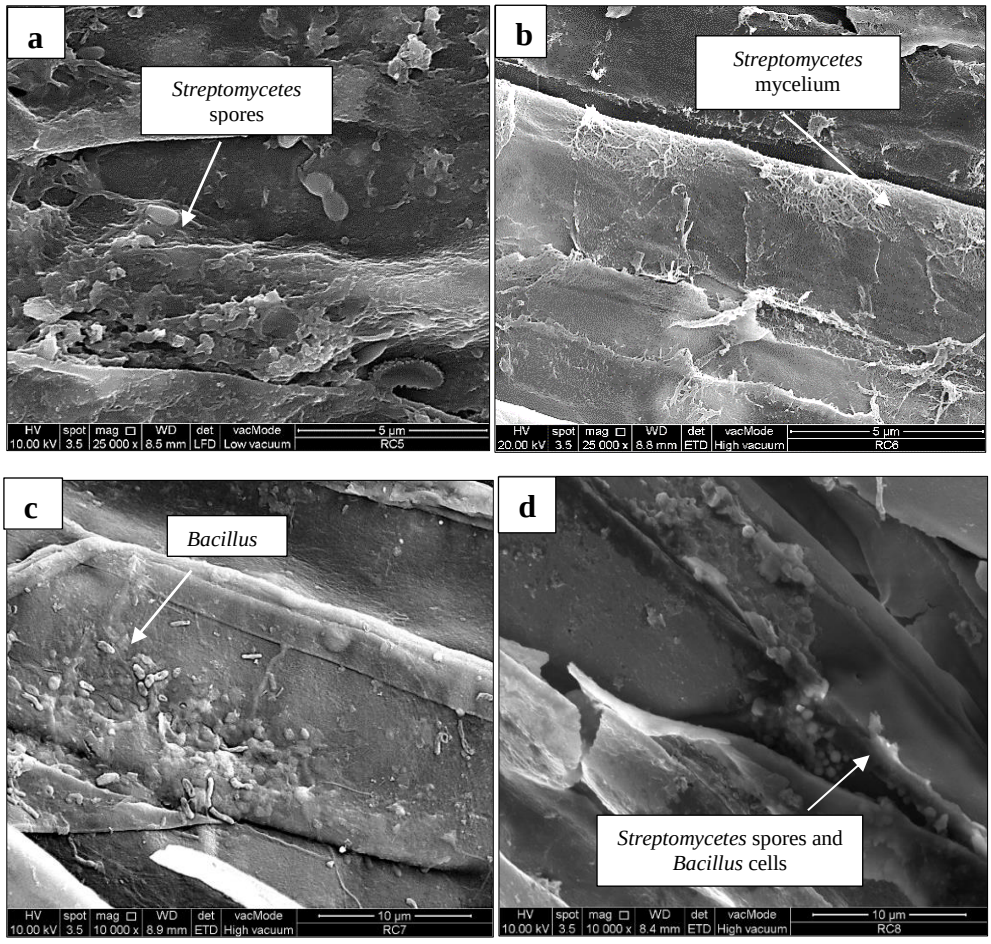
Some of the biological compounds produced by *Bacillus* and *Streptomyces* were found to be associated with plant growth, health, and quality. For example, *Bacillus* sp. could produce rhodopsin and lycoxanthin, while *Streptomyces* sp. could produce echinenone, zeaxanthin, carotene, lycoxanthin, astaxanthin, rhodoxanthin, rhodophin, and rubixanthin acetate. The presence of zeaxanthin and echinoneno reduces the extent of photoinhibition of a pigment-protein complex that converts light energy to chemical energy (PSII system) by protecting the repair of PSII (Kusama et al., 2014). Rhodoxanthin carotenoids found in *P. crispus* played the role of anti-oxidants and immune system regulators in plants (Fu et al., 2023). Carotenoids are pigments in the light-harvesting complexes of plants and microbes that provide photo-protection and light-absorbing functions and can be used as antioxidants (Saejung & Apaiwong, 2015). Pigments produced by microorganisms protect the cells against oxidative damage and impart coloration in plants (Kirti, Amita, Priti, Mukesh Kumar, & Jyoti, 2014). Apart from this, astaxanthin produced by bacteria is ketocarotenoids that accumulate the pigment intracellularly for its photo-protection role against excessive light (de Carvalho et al., 2022). Carotenoids function as light-harvesting pigments, extend the range of light absorption, and participate in photoprotection in photosynthetic tissues while acting as colorants and precursors for isoprenoids in non-photosynthetic tissues (Swapnil et al., 2021).

Endophytic bacteria also produced compounds as a precursor for the biosynthesis of various functional proteins and enzymes in plants. Cysteine is a precursor for essential bio-molecules such as vitamins and co-factors, antioxidants, and many plant defense compounds (Álvarez, Ángeles Bermúdez, Romero, Gotor, & García, 2012). Nickel, a vital plant micronutrient from *Streptomyces*, could facilitate urea hydrolysis in plant tissues (Fabiano, Tezotto, Favarin, Polacco, & Mazzafera, 2015). Likewise, molybdenum is utilized by selected enzymes to carry out redox reactions in the plant's biochemical processes (Kaiser, Gridley, Brady, Phillips, & Tyerman, 2005). Zirconium was reported to stimulate or inhibit enzymes in plant cells (Shahid, Ferrand, Schreck, & Dumat, 2013). Additionally, glycine was reported to be the most widely used amino acid that stimulates physiological processes in plants to increase yield and quality (Zargar Shooshtari, Souri, Hasandokht, & Jari, 2020). Allantoin that *Streptomyces* produced also has a role in increasing stress tolerance in plants (Kaur, Chowrasia, Gaur, & Mondal, 2016) while boron was identified as a part of the bacterial quorum sensor autoinducer and cell wall of higher plants (Goldbach & Wimmer, 2007).

4.2.6 Chilli Root Colonization by Single and Mixed Cultures of the Selected Endophytic Bacteria

For the root inoculation study, *Streptomyces* sp. (MR13) and *Bacillus* sp. were selected based on their efficiency in promoting chilli seedlings' vegetative growth and bio-safety properties. A total plate count was done for all the root samples, and the results indicated that the bacterial population was 10^8 and 10^{10} in all the inoculated chilli roots (results are not shown). Non-inoculated chilli roots also exhibited bacterial count at a lower dilution of 10^6. This could be due to the native microbiome in chilli roots. Total plate count results indicated that there could be some indigenous microorganisms at the exterior part of the root that dislodged from the root surface during the sonication process. Thus, total plate count analysis alone could not determine bacterial colonization. The slight increase in bacterial population in the inoculated roots indicates a successful colonization process.

FESEM analysis revealed that non-inoculated roots do not show distinct bacterial colonization in the interior part of the root tips. However, some rod-shaped bacterial growth was detected in control roots (**Fig. 4.8 f**). This again confirms the presence of indigenous microorganisms that reside in the interior part of chilli roots. FESEM images clearly show endophytic bacteria's colonization in the root tips' internal part. FESEM results show that single culture was well established in chilli roots (**Fig. 4.8 a-c**), but mixed culture colonization was dominated by *Streptomyces* sp. (**Fig. 4.8 d-e**).



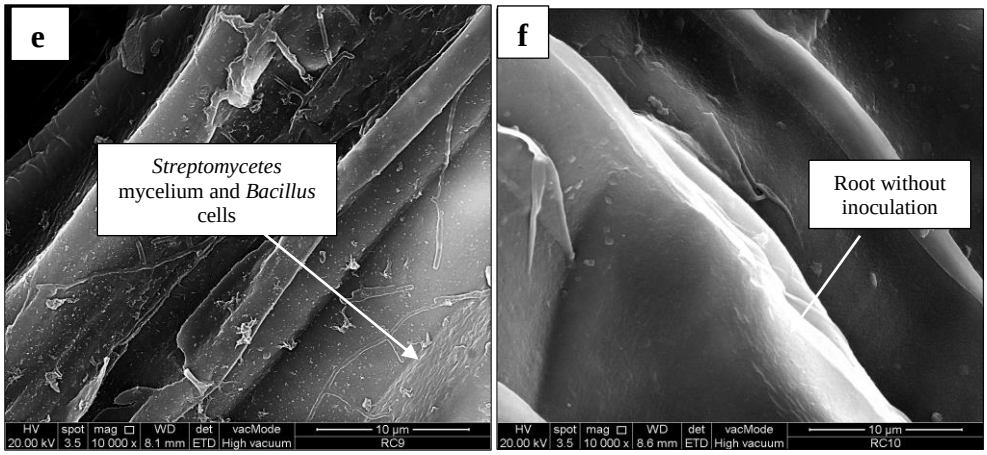


Fig. 4.8 Field Emission Scanning Electron Microscopy (FESEM) of chilli roots colonized with endophytic bacteria. (a) *Streptomyces* spores' colonization in chilli roots; (b) *Streptomyces* mycelium colonization in chilli roots; (c) *Bacillus* cells colonization in chilli roots; (d) Mixed culture (*Streptomyces* spores and *Bacillus* cells) colonization in chilli roots; (e) Mixed culture (*Streptomyces* mycelium and *Bacillus* cells) colonization in chilli roots; (f) Non-inoculated chilli roots (control)

In the current study, the establishment of *Streptomyces* sp. (MR13) supersedes *Bacillus* sp. (MR3) when the chilli seedlings were treated with a mixed culture. The difference between the colonization competence and the growth performance of crops inoculated with single and mixed cultures indicates that the interaction and mechanism of single and mixed cultures with crops are very complex. In contrast to the vegetative growth of the root, FESEM images of root tips revealed that single bacterial culture has better colonization efficiency than mixed culture. Furthermore, the root colonization study shows that mixed cultures of MR3 and MR13 did not show apparent colonization as demonstrated by single cultures. This could be due to the different mechanisms, growth stages, and establishment patterns of bacteria in the root, as they must compete for efficient colonization. The 'hitchhiking' model was reported as the possible mechanism of co-inoculation of root-colonizing microbes, the immotile fungus, and motile bacterium (Muok, Claessen, & Briegel, 2021). *Streptomyces* has a complex lifecycle that involves the formation of aerial hyphae that differentiate into spores, and it was unclear how the spores were transported to plant roots (Muok et al., 2021). It was demonstrated that *Streptomyces* spores could use the motility machinery of motile microbes by directly attaching to their flagella (Muok et al., 2021). This explains the reason behind the low distribution and

establishment of *Streptomyces* spores inside chilli roots (**Fig 4.8** (a) and (d). Additionally, previous research reported that arbuscular mycorrhizal fungus demonstrated different life cycle stages in different parts of wheat roots from week 2 to week 5 (Fester, Maier, & Strack, 1999). In a solid medium, *Streptomyces* develop into compartmentalized mycelium and produce spores, whereas in liquid cultures, no aerial mycelium or spores appear, but hyphae form pellets and clumps (Manteca & Yagüe, 2018). Therefore, the inoculant was prepared in spore and mycelium form to check its colonization capability. It was proven that mycelium grown in liquid culture has better colonization efficiency than spores, as well-established mycelium was observed in chilli root inoculated with single and mixed cultures. Co-inoculation does not guarantee the successful colonization of plant roots, although it could benefit the plant via various mechanisms such as acidosis and nutrient mobilization (Razzaghi Komaresofla, Alikhani, Etesami, & Khoshkholgh-Sima, 2019).

Root colonization is also an essential criterion for the commercialization of bio-inoculants. Most plant growth-promotion trials in field conditions failed to exhibit good plant growth performance due to poor root colonization by the bio-inoculants (Costa-Gutierrez et al., 2022). The foremost important step in plant growth promotion is microbes' colonization of plant roots (Basu et al., 2021). Bacterial endophytes from the rhizosphere environment colonize root hairs due to the secretion of root exudates and rhizo-deposits. According to a research report, the xylem vascular system is the main transport route for systemic colonization of internal plant compartments. In contrast, other endophytes colonize the intercellular spaces of plant cells (Hardoim et al., 2015). Both endophytic bacteria in this study could colonize the roots when applied as a single culture.

4.2.7 Effect of Single and Mixed Cultures of Identified Endophytic Bacteria on the Vegetative Growth of Chilli Plants

The vegetative growth, such as plant height, fruit length, root length, root surface area, root diameter, root volume, aboveground biomass, and root biomass, were evaluated to determine the efficiency of the endophytic bacteria as a bio-

stimulant for chilli growth. Plants applied with *Bacillus* bio-stimulant exhibited the most significant height (78.5 cm) compared to the control plant (60 cm) treated with AB fertilizer. Other treatments were not significantly different (**Fig. 4.9**). The height of plants treated with endophytic bacteria showed an increase in the plant height that fell within a range of 10-30.8 % compared to the control. The average chilli length was most significant in plants treated with *Streptomyces* spores (14.9 cm) and mixed culture of *Streptomyces* spores and *Bacillus* bio-stimulant (14.5 cm). In comparison, the average chilli length in control plants was 11.9 cm (**Fig. 4.10**). The increase in fruit length in the 9.24-25.2 % range was associated with endophytic bacteria. Root length and surface area were similar and insignificant between all treatments. Bio-stimulant did not affect the root growth of chilli significantly. However, bio-stimulant treatments showed a better increase of root length (**Fig. 4.11 and 4. 12**). In contrast, the root diameter of plants treated with *Streptomyces* spores was smaller than other treatments (**Fig. 4.13**). However, the root volume of the plant treated with *Bacillus* bio-stimulant was more significant, while *Streptomyces* spores' application showed the lowest value (**Fig. 4.14**). The root volume of plants treated with *Bacillus* increased by 6.5% compared to the control. Generally, the aboveground biomass was more significant for single bacterial cultures than mixed cultures (**Fig. 4.15**). The highest plant biomass was observed in plants treated with *Streptomyces* spores (MR13 spores) which recorded a 3.75 increase compared to the control. On the other hand, root biomass increased significantly in plants treated with *Streptomyces* spores and a mixed culture of *Streptomyces* mycelium and *Bacillus* culture (**Fig. 4.16**). An increase of 2.5% was observed in the root biomass of plants treated with *Streptomyces* spores (4.49 g) as compared to the control (treated with AB fertilizer only) (4.38 g).

Overall, the positive effects of endophytic bacteria as bio-stimulant were observed in promoting plant height, chilli yield, chilli fruit length, root biomass, and aboveground biomass. Root growth in length, volume, surface area, and diameter did not show significant differences between treatments. This indicates that endophytic bacteria from *Bacillus* and *Streptomyces* could exhibit bio-stimulating effects on chilli seed germination, seedling growth, yield, and aboveground vegetative growth.

4.2.8 Effect of Single and Mixed Cultures of Identified Endophytic Bacteria on the Yield of Chilli Plants

Chilli plants were applied with single and mixed cultures of endophytic bacteria and AB fertilizers for about 40 days. Chilli fruits that turned red were harvested for about two weeks. The yield of chilli fruits was weighed, and the results are shown in **Fig. 4.17**. Plants treated with *Streptomyces* spores (MR 13 spores) and *Bacillus* sp. (MR3) gave the highest total yield of chilli fruits (983.3 g in 2 weeks), followed by mixed cultures of *Streptomyces* spores (MR13 spores) (743.9. g in 2 weeks) as compared to control plants (626.2 g in 2 weeks) that were treated with AB fertilizer alone.

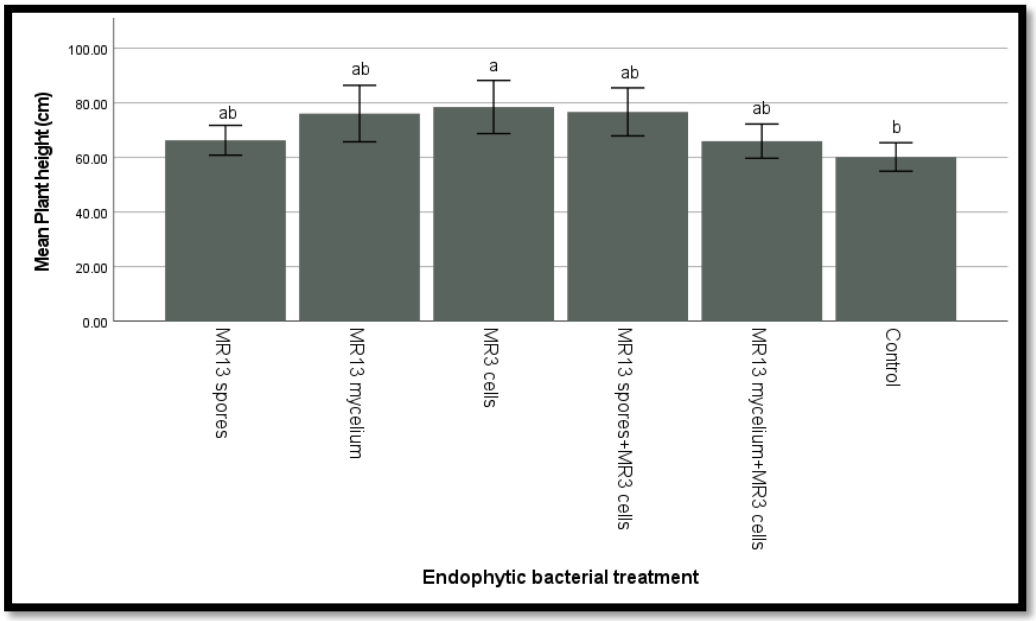


Fig. 4.9 Efficiency of *Bacillus* and *Streptomyces* endophytic bacteria to increase the height of the chilli plants. According to Tukey's HSD, bars sharing the same letters are not significantly different ($p \leq 0.05$). The vertical bar in the graphs indicates the standard error.

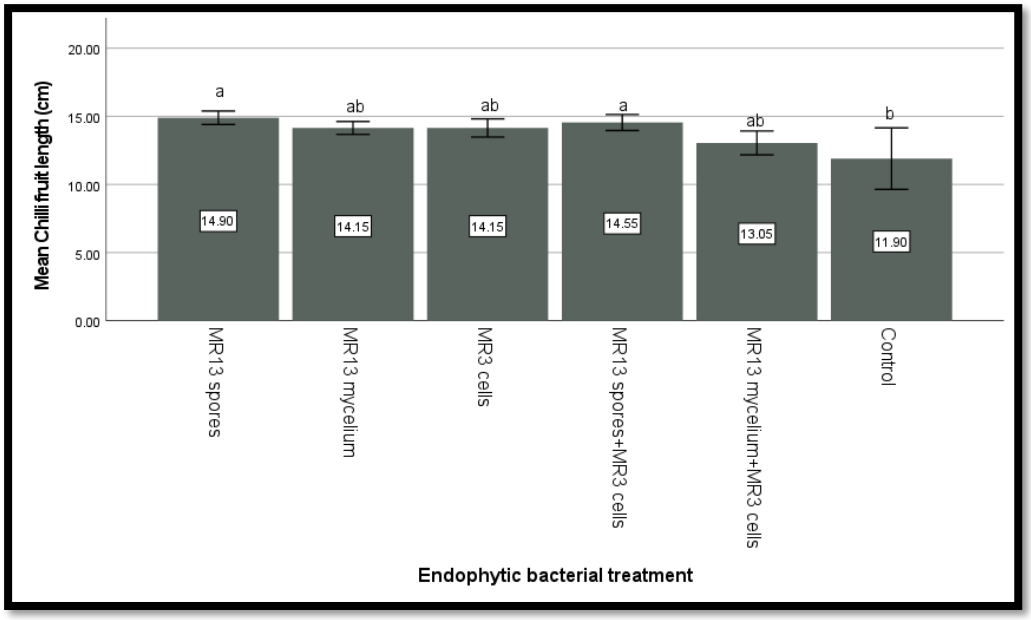


Fig. 4.10 Efficiency of *Bacillus* and *Streptomyces* bio-stimulant to increase the length of chilli fruits. According to Tukey's HSD, bars sharing the same letters are not significantly different ($p \leq 0.05$). The vertical bar in the graphs indicates the standard error.

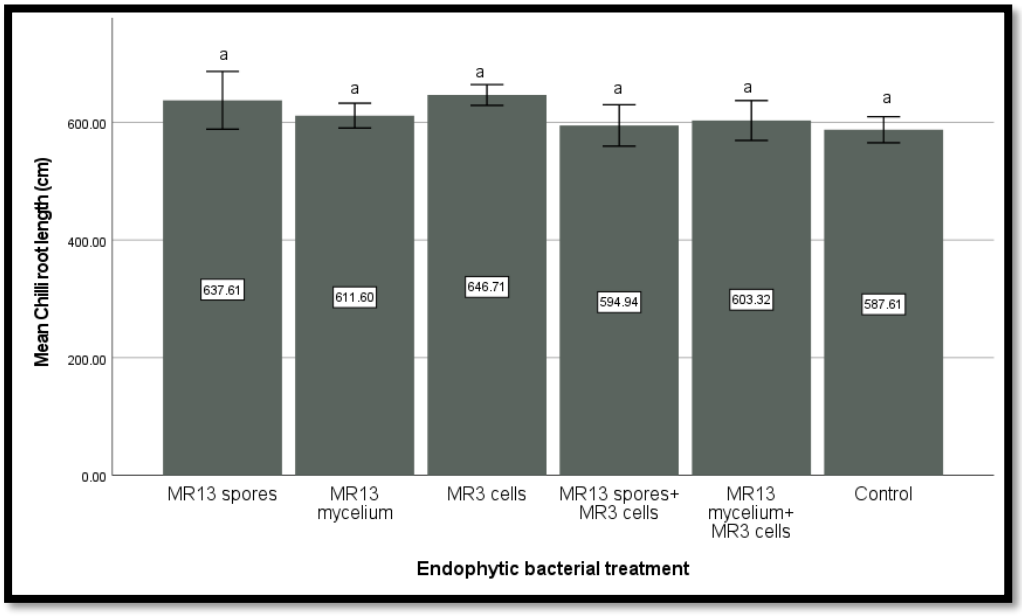


Fig. 4.11 Efficiency of *Bacillus* and *Streptomyces* bio-stimulant to increase the length of chilli roots. According to Tukey's HSD, bars sharing the same letters are not significantly different ($p \leq 0.05$). The vertical bar in the graphs indicates the standard error.

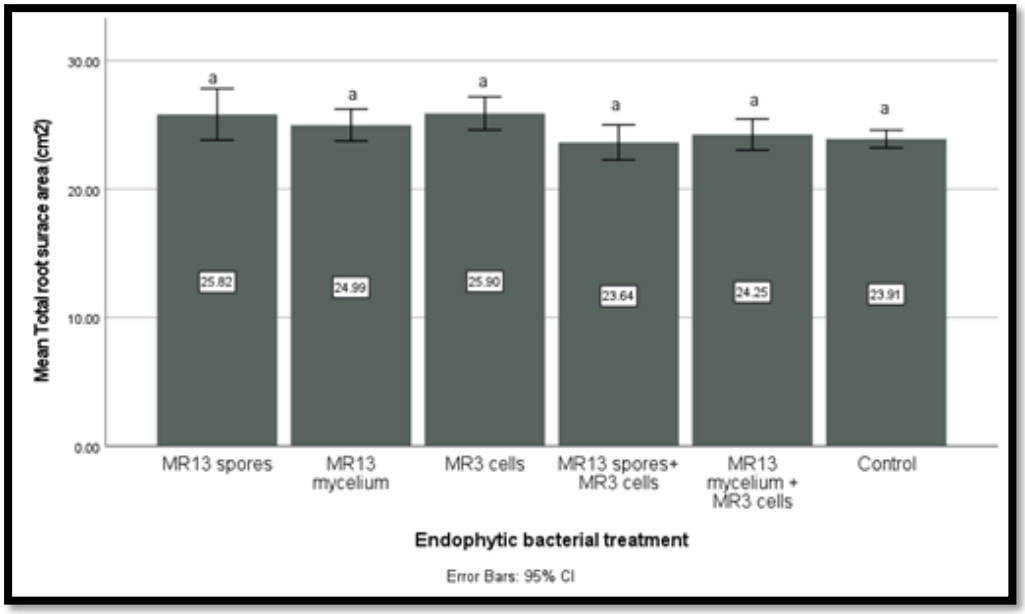


Fig. 4.12 Efficiency of *Bacillus* and *Streptomyces* bio-stimulant to increase the total area of chilli root surface. According to Tukey's HSD, bars sharing the same letters are not significantly different ($p \leq 0.05$). The vertical bar in the graphs indicates the standard error

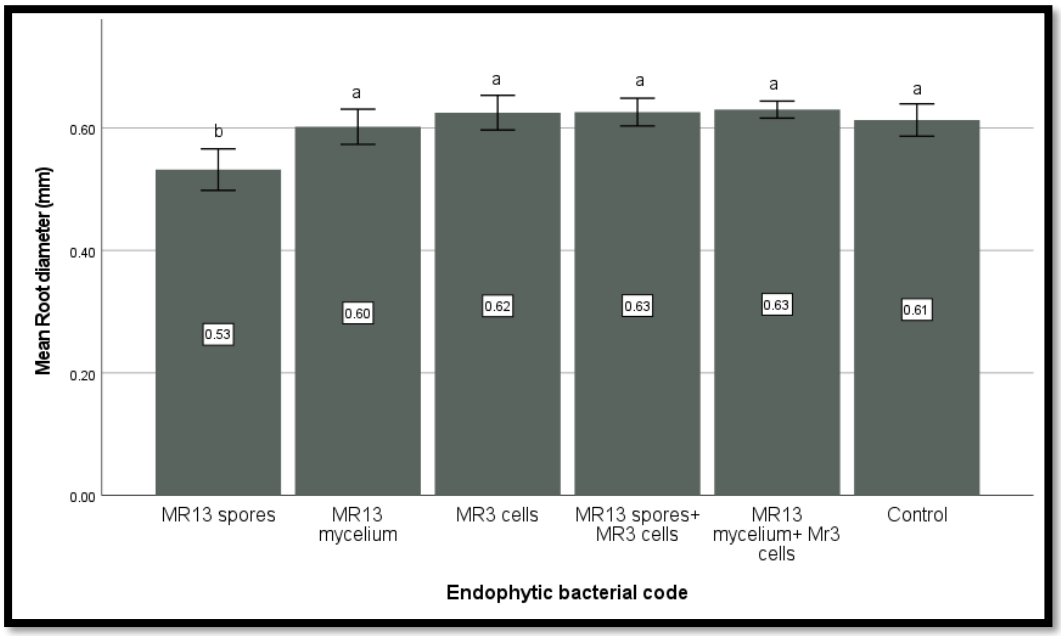


Fig. 4.13 Efficiency of *Bacillus* and *Streptomyces* bio-stimulant to increase the chilli root diameter. According to Tukey's HSD, bars sharing the same letters are not significantly different ($p \leq 0.05$). The vertical bar in the graphs indicates the standard error.

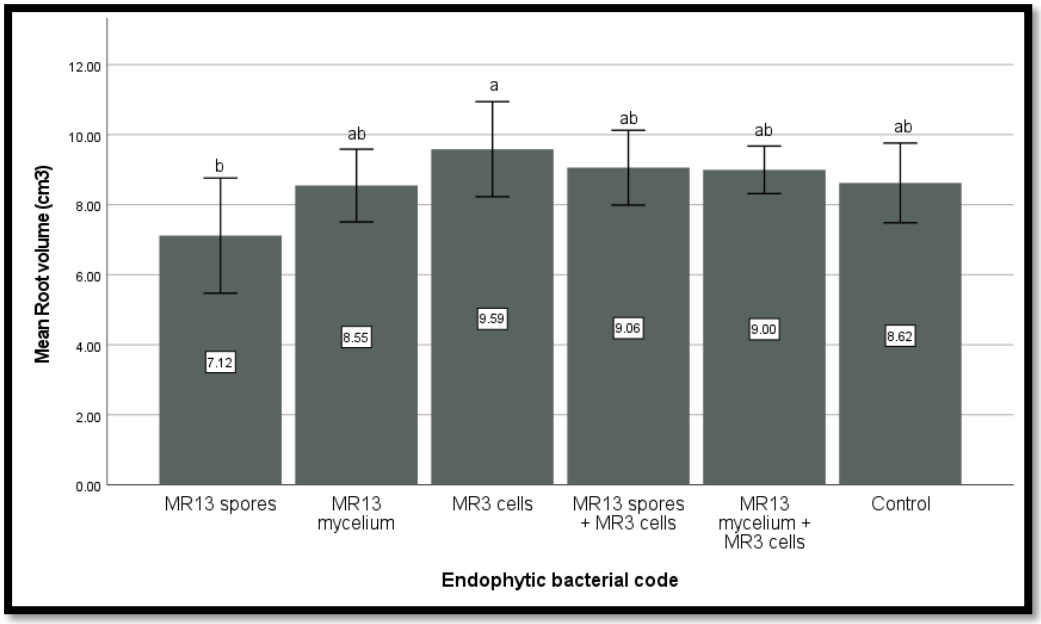


Fig. 4.14 Efficiency of *Bacillus* and *Streptomyces* bio-stimulant to increase the chilli root volume. According to Tukey's HSD, bars sharing the same letters are not significantly different ($p \leq 0.05$). The vertical bar in the graphs indicates the standard error.

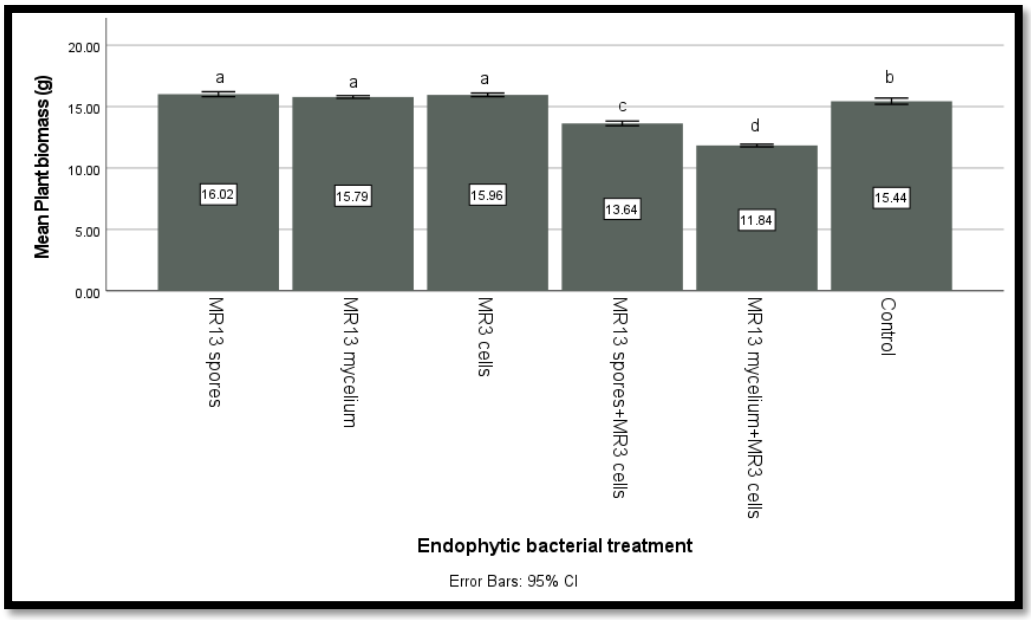


Fig. 4.15 Efficiency of *Bacillus* and *Streptomyces* bio-stimulant to increase the aboveground biomass of chilli plants. According to Tukey's HSD, bars sharing the same letters are not significantly different ($p \leq 0.05$). The vertical bar in the graphs indicates the standard error.

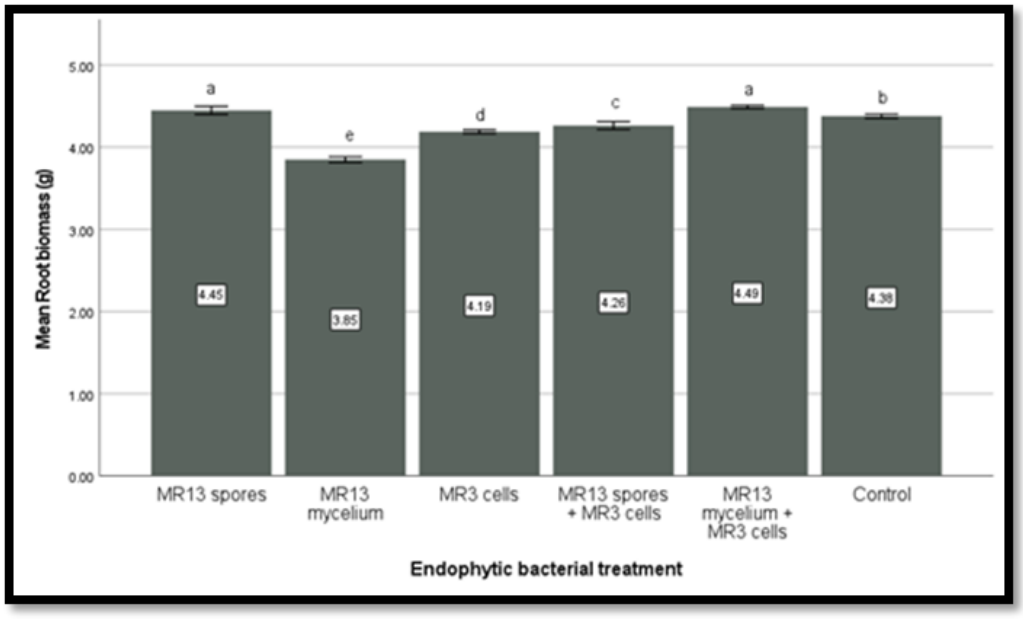


Fig. 4.16 Efficiency of *Bacillus* and *Streptomyces* bio-stimulant to increase the root biomass of chilli plants. According to Tukey's HSD, bars sharing the same letters are not significantly different ($p \leq 0.05$). The vertical bar in the graphs indicates the standard error.

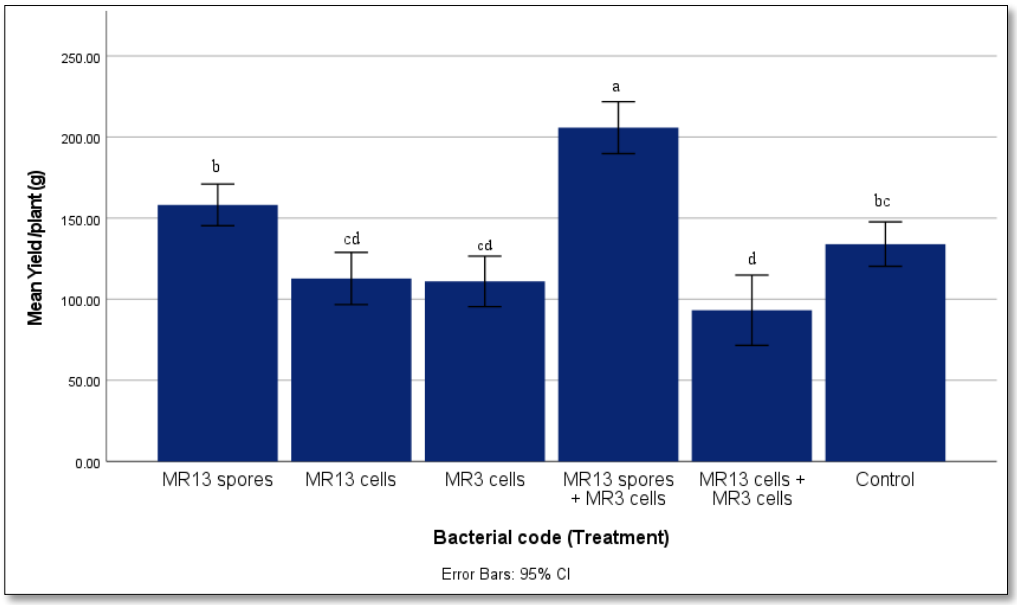


Fig. 4.17 Yield of five chilli plants 40 days after transplanting. Each value is the mean of five replicates (n=5), and bars sharing the same letters are not significantly different ($p \leq 0.05$) according to Tukey's HSD. The vertical bar indicates the standard error.

4.2.9 Conclusion

About seven endophytic bacterial isolates with multiple plant growth-promoting traits were proven to enhance seed germination. MR13 and CKR8 recorded the lowest mean germination time, while MR10 and MR3 recorded the highest vigor index. This result was used as the pre-selection criterion for chilli seedling growth analysis. The four pre-selected endophytes belong to *Streptomyces*, *Bacillus*, and *Enterobacter* groups. Although the endophytic bacteria belonging to the *Enterobacter* group was found to be an efficient bio-stimulating agent for chilli seedlings, they were not suggested to be used as bio-inoculant in field experiments due to biosafety issues. Hence, *Bacillus subtilis* (MR3) and *Streptomyces panaciradicis* (MR13) were considered potential endophytes for bio-stimulant formulation exclusively for chilli seedlings. Mixed culture demonstrated better root development during the seedling developmental stage than a single-culture inoculant. This study has proven that non-native endophytic bacteria from moringa roots could enhance chilli seedlings' vegetative growth. This indicates the potential of non-native endophytic bacteria to be applied to various crops for growth and productivity enhancement. The compatibility of non-native beneficial endophytes and the host is determined by the root exudates produced by the host plant upon inoculation with the non-native endophytic bacteria. There is a possibility that endophytic bacteria has special characteristics which makes them compatible with non-native crops. However, further reseach has to be done to prove that non-native endophytic bacteria have a unique feature to adapt and colonize in various crops to exert their beneficial effects on crops. If proven so, the bioprospection of beneficial bacteria could be aimed to screen endophytic microorganisms for better plant colonization efficiency. Nevertheless, it might not be the same with all endophytic bacteria where some endophytic bacteria could be exclusively recruited by the host via specific natural selection and unsuitable to live in non-native hosts. Thus, a proper real-time compatibility screening is vital to use non-native endophytes in different crops. GC-MS analysis unveiled that these endophytic bacteria could produce many essential and useful metabolites that can be used for plant growth. Additionally, *Streptomyces* and *Bacillus* exhibited bio-stimulating effects in increasing chilli yield, chilli length, root biomass, and aboveground biomass

when applied as mixed culture except for plant height, which was triggered by single culture inoculant consisting of *Bacillus* sp. In general, mixed culture was proven to promote chilli's vegetative growth and yield. A mixed culture of various bacterial strains would have a different impact on plant growth than a single culture, as the interaction between various bacterial cultures allows the production of different metabolites that have unique effects on crop growth. Quorum sensing communication between bacteria could lead to the secretion of biological compounds that can impede plant growth. On the other hand, secondary metabolites produced by bacteria in the mixed culture could be beneficial or harmful to the other bacteria in the consortium. Therefore, the synergistic effect of the mixed culture has to be studied in detail to ensure that the mixed culture is not harmful or hamper the plant growth. This is an important criterion in developing bio-stimulants consisting of mixed bacterial cultures. The study's first objective was achieved by selecting *Streptomyces* and *Bacillus* as promising candidates for bio-stimulant development. Furthermore, the endophytic bacterial applied via the drenching method of soilless medium is proven effective in promoting the vegetative growth and yield of chilli compared to the control treated with inorganic liquid fertilizer alone.

CHAPTER 5
CO-APPLICATION OF THE SELECTED ENDOPHYTIC BACTERIA AND MORINGA LEAF EXTRACT ON THE VEGETATIVE GROWTH AND YIELD OF CHILLI

5.0 Introduction

Moringa leaf extract is considered a natural bio-stimulant due to its high amount of cytokinin (zeatin), gibberellic acid, ascorbates, phenolics, and minerals (Refai et al., 2023). It has gained research priority in recent years as it could show promising results on seed germination, nutrient use efficiency, plant growth and yield under abiotic stress, and good quality of post-harvest (Abd El-Mageed et al., 2017; Alkuwayti et al., 2020; Batool et al., 2020; Desoky et al., 2019; El-Serafy & El-Sheshtawy, 2020; Elrys et al., 2018; Howladar, 2014; Nasir et al., 2016; Rady et al., 2019; Shaukat et al., 2017; ShM et al., 2017; Waqas et al., 2017; Zulfiqar, Casadesús, et al., 2019).

However, very few studies have exploited the effect of the co-application of bacterial bio-stimulant and botanical bio-stimulant on crops' growth and yield. Previous studies have explored the synergistic impact of algal extracts and their combination with the plant growth-promoting bacterium on the germination and establishment of lettuce seedlings (Julia, Oscar, Analía, Zocolo Guilherme, & Virginia, 2020). The study has proven that applying bacteria and algae individually or in combination exerted a positive effect. Likewise, the efficiency of nitrogen-fixing bacteria, moringa leaf extract, and their combinations have been evaluated on fennel plant growth, yield, and oil components (El-Serafy & El-Sheshtawy, 2020). It was revealed in another study that single or combined additives significantly increased the vegetative and physiological growth of plants (Eltahawy, Awad, Ibrahim, Merwad, & Desoky, 2022). However, the previous studies applied moringa leaf extract as a foliar treatment. This study would be a pioneer attempt that reports the effect of the co-application of moringa leaf extract, endophytic bacteria, and their combinations in the soilless medium using a drenching method.

This chapter compared the potential of aqueous extraction of fresh and dry moringa leaves with 50% ethanol and 50% methanol extraction. Polar solvent extraction is a standard method used to extract bioactive compounds from plants, but it can be expensive due to the cost of the solvents and the equipment required for the extraction process. It was hypothesized that aqueous extraction, which is generally cheaper and more environmentally friendly, is comparable with polar solvents in promoting the vegetative growth of the seedlings.

A preliminary shade-house experiment was conducted to assess the efficiency of the aqueous extract of moringa leaf as a potential bio-stimulant for promoting the vegetative growth and yield of chilli as a single treatment and as a combination with the endophytic bacteria selected from the previous chapters. Additionally, the application method adopted in this study was drenching a soilless medium. The main objective of this experiment is to assess the synergistic effect of moringa leaf extract and endophytic bacteria in a soilless medium and to choose the best treatment for nano-formulation of bio-stimulant.

5.1 Materials and Methods

5.1.1 Preparation of Moringa Leaf Extract (MLE)

Moringa leaf for the entire study were collected from a 20-year-old moringa tree in Senawang, Negeri Sembilan, Malaysia. Dark, mature leaves were collected in sterile autoclavable bags, transferred to the laboratory for immediate use, or kept at 10°C in a chiller for 24 h. Fresh leaves were used directly after weighing the appropriate amount into the beaker or kept at 10°C and used the next day. Other fresh leaves were placed in large envelopes and dried in a hot oven at 40°C for 3 days. Dry leaves were ground using an electric blender into powder and kept at 10°C. The nutrient content of dry moringa leaf was as follows: 15.68% ash, 3.93% nitrogen, 0.30% phosphorus, 3.23% potassium, 3.14% calcium, and 0.24% magnesium.

The extraction process was done using both dry (DL) and fresh leaf (FL), and the methods described by (Vongsak et al., 2013) were modified. This study used three extraction methods: maceration, infusion, and decoction. The solvents were distilled water, 50% ethanol v/v, and 50% methanol v/v. For maceration, 10 g of dry leaf sample and 35 g of fresh leaf were mixed with 450 mL of solvent and left in a shaker for 72 h at 100 rpm. Fresh leaves were homogenized using a pestle and mortar before extraction. Pestle and mortar were used, as only 10 g of moringa leaves were used for the extraction. An electric blender was used to extract a large amount of moringa leaves. The extract was filtered using a muslin cloth, and the marc was re-extracted by the same process and solvent three times. The solvents, such as ethanol and methanol, were evaporated using a rotary evaporator. After the evaporation process, the remaining balance was collected and stored at -20°C until further use. For the infusion method, distilled water (50°), cold methanol (50%), and cold ethanol (50%) were used. About 10 g of dry leaf sample and 35 g of fresh leaf sample were mixed with 450 mL of solvent and left for 72 h at room temperature. The extract was filtered, and the marc was re-extracted by the same process and solvent three times. The solvents, such as ethanol and methanol, were evaporated using a rotary evaporator. After the evaporation process, the remaining balance was collected and stored at -20°C until further use. For the decoction method, 10 g of dry leaf sample and 35 g of fresh leaf sample were boiled with 450 mL of distilled water for 30 minutes and left overnight. The extract was filtered using a muslin cloth and stored at -20°C until further use. Moringa leaf extraction methods and solvents used for extraction that represents treatments applied to evaluate the effect on seedlings' growth was described in **Table 5.1**

Table 5.1 Moringa leaf extraction using decoction, maceration and infusion methods

Treatment code	Leaf type	Solvent & Condition	Method
T1	DL	50% methanol	Maceration
T2	DL	50% ethanol	Maceration
T3	DL	Distilled water	Maceration
T4	FL	50% methanol	Maceration
T5	FL	50% ethanol	Maceration
T6	FL	Distilled water	Maceration
T7	DL	Cold 50% methanol	Infusion
T8	DL	Cold 50% ethanol	Infusion
T9	DL	Distilled water 50°C	Infusion
T10	FL	Cold 50% methanol	Infusion
T11	FL	Cold 50% ethanol	Infusion
T12	FL	Distilled water 50°C	Infusion
T13	DL	Distilled water 100°C	Decoction
T14	FL	Distilled water 100°C	Decoction

*DL: Dry leaf; FL: Fresh leaf

5.1.2 Phytochemical Analysis of Moringa Leaf Extract

Water-based treatments were centrifuged at 12,000-x g for 20 min. Then, the supernatant was collected and stored at -20°C. Treatments with solvent centrifuged at 12,000-x g for 20 min and the supernatant was collected and kept in a water bath set at 40° C for three days. The solvent evaporated using a rotary evaporator at 40°C for 24 h. The balance solution after evaporation was stored at -20° C until further analysis.

5.1.2.1 Phenolic Content in Moringa Leaf Extract

A method described by (Hossain et al., 2013) was modified to determine the total phenolic content in the moringa leaf extract. Test tubes containing 100 µL of plant extract, 500 µL of water, and 100 µL of Folin-Ciocalteu reagent were prepared in triplicates. The mixture was allowed to stand for 6 minutes before adding 1mL of sodium carbonate solution (7.5 % w/v) and 500 µL of water. Then the tubes were kept in a dark room at RT for 30 min. Absorbance was recorded against a reagent blank at 760 nm using a UV-Vis spectrometer after 30 min of incubation in a dark room. Total phenolic content was calculated using the standard calibration curve of gallic acid in methanol and expressed as mg gallic acid equivalent/g.

5.1.2.2 Total Flavonoids in Moringa Leaf Extract

A method described by (Al-Matani et al., 2015) was modified to determine the total flavonoids in the moringa leaf extract. Plant extract (100 µL) was mixed with 500 µL of distilled water and then with 100 µL of 5% sodium nitrate in test tubes and allowed to stand for 6 minutes. Then, 150 µL of 10% aluminum chloride solution was added and allowed to stand for 5 min, and finally, 200 µL of 1M sodium hydroxide was added. The absorbance of the samples was measured at 510 nm using a UV-Vis spectrometer. Blank contained all reagents except Al_2CI_3, which was replaced with distilled water. The results were calculated using the standard calibration curve of quercetin in methanol and expressed as quercetin equivalents (mg/g).

5.1.2.3 Free Radical Scavenging Activity of 2, 2-diphenyl-1-picryl-1-phenyl hydrazyl-hydrate (DPPH) of Moringa Leaf Extract

A method described by (M. K. Swamy et al., 2017) was modified to determine DPPH free radical scavenging activity in moringa leaves extract. A fresh 0.02 % DPPH solution was prepared in methanol. A 50 µL of moringa leaf extract and 3 mL of DPPH solution were added to the test tube and mixed vigorously. It was allowed to stand in the dark at 37°C for 30 min. The decrease in the absorbance was measured in each solution at 517 nm using a UV-Vis spectrometer. A standard graph was prepared using gallic acid of different concentrations. Blank was prepared without gallic acid. The concentration of sample required to scavenge 50% of DPPH was determined from the curve of percentage inhibition. Free radical scavenging activity was calculated as per the formula below.

$$Free\ radical\ scavenging\ activity = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control}\ x\ 100$$

5.1.3 Effect of Moringa Leaf Extracts on the Vegetative Growth of Chilli Seedlings

Chilli seeds (Sakata 461) were pre-germinated in sterile water and transferred into polybags filled with peat moss. It was allowed to germinate for about one week or until 2-4 leaves emerged from the seedlings. Then 2 ml of the moringa leaf extract (T1-T14) and distilled water (T15-control) were applied to the peat moss every two weeks once. Each treatment was prepared with five replications. Thirty days after seed germination, the seedlings were subjected to plant physiology analyses such as height, root length, root surface area, relative chlorophyll content, and root shoot ratio.

5.1.3.1 Relative Chlorophyll Content of Chilli Seedlings

Relative chlorophyll content was carried out on each treatment's third, fully expanded leaves from the shoot apex. Relative chlorophyll content was

measured using the SPAD-502 chlorophyll meter (SPAD 502, Minolta-Camera Co., Osaka, Japan). The data points were recorded at five positions along the leaf blade, and then the data points were averaged as a single value.

5.1.3.2 Root Length and Root Surface Area of Chilli Seedlings

WinRhizo root scanner (EPSON Flatbed Scanner EPSON Expression 11000XL) was used to capture the image of chilli roots and measure the total root length and total root surface area. Chilli roots were washed thoroughly in tap water to remove the soil debris and placed in clean water to view under the root imaging system.

5.1.3.3 Plant Height and Root: Shoot Ratio of Chilli Seedlings

Seedlings' height was measured from the collar of the stem to the highest leaf. The roots and shoots were separated, washed, and dried in an oven at 70°C for three days before measuring the dry weight of root biomass and shoot biomass.

5.1.4 Assessment of the Compatibility between Moringa Leaf Extract and Endophytic Bacteria

Two endophytic bacteria described in **Chapter 4**, namely MR3 and MR13, were tested for their compatibility with the aqueous extraction of moringa leaf. Moringa leaf (50g) was mixed with 1 L water for extraction via maceration and decoction methods and left overnight before filtering out the extracted solution (**Fig. 5.1**). Plant extracts without dilution was used to test the compatibility with endophytic bacteria. One milliliter of moringa leaf extract was mixed with 1 mL of the endophytic bacteria culture in TSB. The mixture was left overnight at room temperature, followed by plating on TSA. The plates were incubated for five days, and the colony-forming units were calculated as soon as they were formed on the agar medium.

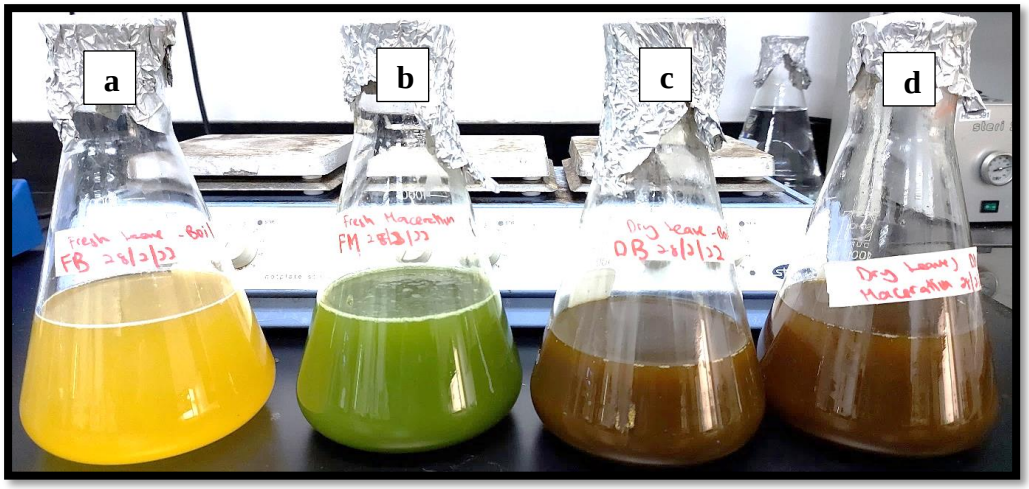


Fig. 5.1 Moringa leaf extraction using aqueous decoction and maceration methods. (a) Fresh leaf extraction using boiling method (FB); (b) Fresh leaf extraction using maceration methods (FM); (c) Dry moringa leaf extracted using boiling method (DB); (d) Dry moringa leaf extracted using maceration methods (DM)

5.1.5 Synergistic Effect of Moringa Leaf Extract and Endophytic Bacteria on the Vegetative Growth of Chilli

Chilli seeds were soaked in water overnight before sowing into wet peat moss. The polybags containing peat moss were covered with black plastic sheets and kept at room temperature in a dark room until the seeds germinated. The black sheets were removed once the radicle was visible, and the polybags were observed in the shade until true leaves emerged. Peat moss was sprayed with water every two days once or when dry. After the 14th day of sowing, fertilizer AB with an EC reading of 0.9 mS/cm was poured into a tray, and the polybags were placed on it for maximum absorption of nutrients. Transplanting of seedlings was done 28 days after sowing. The planting medium, cocoa peat and peat moss were prepared in an 8" x 12" cm polybag. A completely randomized block design was adopted for this experiment. The independent variable is AB liquid fertilizer doses, and the dependent variable is the moringa leaf extract and endophytic bacterial treatment. Diluted moringa leaf extract (1:1000), and bacterial culture (100ml/plant) corresponding to 1x 10^7 cfu/ml, were applied for two weeks once by drenching the planting media. There were 25 treatments, with three replications of each treatment. Each replication consists of five plants. The treatments are described in **Table 5.2.**

Table 5.2 The details of the treatments containing various combinations of aqueous extract of moringa leaf and endophytic bacteria

Treatment code	Moringa leaves extraction method	Endophytic bacteria
T1	Fresh moringa leaf powder/Decoction or Boiling method using distilled water (FB)	-
T2	Fresh moringa leaf powder/Maceration method using distilled water (FM)	-
T3	Dry moringa leaf powder/ Decoction or Boiling method using distilled water (DB)	-
T4	Dry moringa leaf powder/ Maceration method using distilled water (DM)	-
T5	Fresh moringa leaf powder/Maceration method using distilled water (FM)	*Bacillus* sp. (MR3)
T6	Fresh moringa leaf powder/Maceration method using distilled water (FM)	*Streptomyces* sp. mycelium (MR13-M)
T7	Fresh moringa leaf powder/Maceration method using distilled water (FM)	*Streptomyces* sp. spores (MR13-S)

T8	Fresh moringa leaf powder/Maceration method using distilled water (FM)	*Bacillus* sp. (MR3) and *Streptomyces* sp. mycelium (MR13-M)
T9	Fresh moringa leaf powder/Maceration method using distilled water (FM)	*Bacillus* sp. (MR3) and *Streptomyces* sp. spores (MR13-S)
T10	Dry moringa leaf powder/ Maceration method using distilled water (DM)	*Bacillus* sp. (MR3)
T11	Dry moringa leaf powder/ Maceration method using distilled water (DM)	*Streptomyces* sp. mycelium (MR13-M)
T12	Dry moringa leaf powder/ Maceration method using distilled water (DM)	*Streptomyces* sp. spores (MR13-S)
T13	Dry moringa leaf powder/ Maceration method using distilled water (DM)	*Bacillus* sp. (MR3) and *Streptomyces* sp. mycelium (MR13-M)
T14	Dry moringa leaf powder/ Maceration method using distilled water (DM)	*Bacillus* sp. (MR3) and *Streptomyces* sp. spores (MR13-S)

T15	Fresh moringa leaf powder/Decoction or Boiling method using distilled water (FB)	*Bacillus* sp. (MR3)
T16	Fresh moringa leaf powder/Decoction or Boiling method using distilled water (FB)	*Streptomyces* sp. mycelium (MR13-M)
T17	Fresh moringa leaf powder/Decoction or Boiling method using distilled water (FB)	*Streptomyces* sp. spores (MR13-S)
T18	Fresh moringa leaf powder/Decoction or Boiling method using distilled water (FB)	*Bacillus* sp. (MR3) and *Streptomyces* sp. spores (MR13-S)
T19	Fresh moringa leaf powder/Decoction or Boiling method using distilled water (FB)	*Bacillus* sp. (MR3) and *Streptomyces* sp. mycelium (MR13-M)
T20	Dry moringa leaf powder/ Decoction or Boiling method using distilled water (DB)	*Bacillus sp.* (MR3)
T21	Dry moringa leaf powder/ Decoction or Boiling method using distilled water (DB)	*Streptomyces* sp. spores (MR13-S)
T22	Dry moringa leaf powder/ Decoction or Boiling method using distilled water	*Streptomyces* sp. mycelium (MR13-M)

	(DB)	
T23	Dry moringa leaves powder/ Decoction or Boiling method using distilled water (DB)	*Bacillus* sp. (MR3) and *Streptomyces* sp. spores (MR13-S)
T24	Dry moringa leaf powder/ Decoction or Boiling method using distilled water (DB)	*Bacillus* sp. (MR3) and *Streptomyces* sp. mycelium (MR13-M)
T25	Water (Control)	-

Chillies were harvested, and plants were removed from the planting material 50 days after transplanting. The shoot length was measured. The aboveground shoot and root were separated and transferred to the lab in separate plastic bags. The aboveground parts were placed in brown envelopes before drying in the oven (CEPB laboratory, University of Nottingham) at 60°C for three days. The weight of dried aboveground biomass was measured using a weighing balance (CEPB laboratory, University of Nottingham).

For root samples, the planting material attached to the roots was removed by washing the roots in tap water. After cleaning the roots, they were scanned via WinRhizo root scanner (EPSON Flatbed Scanner EPSON Expression 11000XL, CEPB laboratory, University of Nottingham). The root imaging system measured the total root length and surface area.

5.1.6 Synergistic Effect of Moringa Leaf Extract and Endophytic Bacteria on the Yield of Chilli

The effect of single treatments of aqueous extraction of fresh and dry moringa using decoction and maceration methods and their combination with endophytic bacteria selected from the previous experiments were evaluated based on the

yield of chilli plants. The fruit weight estimated the yield after 40 days after transplanting.

5.1.7 Statistical Analysis

Statistical analysis was performed using the Univariate analysis with Tukey's HSD test ($p < 0.05$) by the SPSS software (IBM Statistics, version 28, USA) to determine the significance of the data. Bars sharing the same letters are not significantly different ($p \leq 0.05$). The vertical bar in the graphs indicates the standard error.

5.2 Results and Discussion

5.2.1 Phytochemical Contents in Moringa Leaf extract

In this study, the antioxidant properties such as phenols, flavonoids, and DPPH radical scavenging activities of moringa leaf extract of various extraction methods revealed that apart from polar solvents that are commonly used for the extraction of antioxidant compounds, water extraction has also resulted in similar antioxidant activities like polar solvents (**Table 5.3**).

The DPPH radical scavenging activity range was 26.41 – 73.96 µg/mL. The least free radical scavenging activity was recorded by maceration of fresh moringa leaf with 50% of methanol. All the other extraction methods exhibited more than 50% free radical scavenging activity, corresponding to more than 8.39 µL/mL of DPPH concentration. Notably, aqueous extraction of fresh moringa leaf using the boiling method (T14) showed 44.50 µg/mL of DPPH scavenging activity; meanwhile, dry moringa leaf extracted using maceration and boiling method showed 47.39 µg/mL and 43.22 µg/mL of DPPH scavenging activities respectively. DPPH (2, 2-diphenyl-1-picrylhydrazyl) is a stable free radical commonly used to assess the antioxidant activity of compounds. DPPH scavenging activity measures a substance's ability to neutralize this free radical by donating an electron to it, thereby reducing it to a stable, non-radical form. IC50 value was used to indicate the antioxidant capacity where the concentration

of the sample that causes a 50% reduction of the initial DPPH concentration was calculated based on a linear regression graph (**Fig. A9**) (**Appendix**) of the mean percentage of antioxidant activity against the concentration of gallic acid (µg/ml).

The total phenolic content in all the extraction methods ranged between 0.16 – 0.72 gallic acid equivalent (GAE) mg/L, while total flavonoid content ranged from 0.25–75.44 quercetin equivalent mg/mL. The calculation was based on the standard gallic acid graph (**Fig. A9**) (**Appendix**) and quercetin standard graph (**Fig. A10**) (**Appendix**). Polar solvent extraction using methanol and ethanol has shown better antioxidant activities than water extraction. For example, infusion of the fresh leaf using cold 50% methanol showed the highest antioxidant activity; maceration of dry leaf using 50% methanol exhibited 0.72 gallic acid equivalent (GAE) mg/L highest phenolic content, and fresh leaf infusion using cold 50% ethanol showed 75.44-quercetin equivalent mg/mL. Methanol extraction using the maceration method (T1) showed the highest phenolic content, and water extraction using the infusion method (T9) showed the lowest phenolic content. Extraction methods using ethanol (T11) and water (T3) exhibited the highest flavonoid content, followed by cold methanol infusion (T10) and water boiling of the fresh leaf (T14). Treatment T4 (maceration using methanol) and T12 (Water infusion at 50°C) had the lowest flavonoid content. Fresh leaf extraction has recorded high flavonoid content except for sample T3 and high DPPH free radical scavenging activity except for samples T7 and T8. Dry leaf extraction showed high phenolic content. This finding agrees with (Vongsak et al., 2013), whereby dry leaf maceration with 70% ethanol showed the highest phenolic, flavonoid, and DPPH-scavenging activity. However, in this study, dry leaf infusion with 50% cold methanol exhibited the highest antioxidant activities. The optimal extraction method should be simple, rapid, economical, and applicable to a larger-scale industry, while high-quality extraction methods using solvents are usually recommended for pharmaceutical and nutraceutical industries (Vongsak et al., 2013).

Among aqueous extraction methods, maceration of dry leaves, decoction of fresh leaves, and decoction of dry leaves showed 47.39 µg/mL, 43.22 µg/mL, and

44.50 µg/mL of DPPH scavenging activities, respectively. For phenolic content, the decoction method of dry leaf, maceration method of fresh leaf, and decoction method of fresh leaf produced 0.52, 0.32, and 0.31 of gallic acid equivalent (GAE) mg/L, respectively. Flavonoid content results exhibited that maceration of dry leaf, a decoction of fresh leaf, and maceration of fresh leaf produced 74.89, 70.78, and 69.82 of quercetin equivalent mg/mL, respectively. Overall, a decoction of fresh leaf and maceration of dry leaf showed comparable phytochemical content, especially flavonoid content, compared to solvent extraction methods.

Medicinal plants are rich in antioxidants, which could alleviate environmental stresses and lead to plant growth. Moringa leaf extract was used in this experiment to evaluate its efficiency on chilli plant growth. Past literature reviews have reported the potential of moringa leaf to stimulate seed germination, seedling growth, stress alleviation, and plant yield (Ahmed, Elezz, & Khalid, 2021; Zulfiqar, Casadesús, Brockman, & Munné-Bosch, 2020). The extract of moringa leaf contains a broader range of phytochemicals, and the effect of moringa leaf extract varies with the solvent used for extraction (Zulfiqar, Casadesús, et al., 2019).

Table 5.3 Phytochemical analysis of moringa leaf extract

Treatment	DPPH scavenging activity %	Phenolic content (mg/ml)	Flavonoid content (mg/ml)
T1	63.70± 0.23	0.72±0.01	18.61±11.98
T2	61.25± 0.35	0.50±0.00	69.60±1.60
T3	47.39 ± 0.94	0.16±0.00	74.89±0.40
T4	66.13 ± 0.32	0.34±0.00	0.35±0.02
T5	70.29 ± 0.18	0.27±0.00	13.1±11.13
T6	26.41 ± 1.02	0.32±0.00	69.82±1.51
T7	70.58 ± 0.04	0.67±0.00	74.42±0.60
T8	71.73 ± 0.05	0.60±0.00	0.29±0.03
T9	54.91 ± 0.85	0.12±0.00	12.65±12.02
T10	73.96 ± 0.01	0.30±0.00	70.14±2.11
T11	72.38 ± 0.03	0.28±0.00	75.44±1.83
T12	69.32 ± 0.00	0.24±0.00	0.25±0.01

T13	43.22 ± 0.78	0.52±0.00	12.83±12.37
T14	44.50 ± 0.00	0.31±0.00	70.78±2.26

* Standard deviation of five independent measured were calculated for each analysis

5.2.2 Effect of Moringa Leaf Extracts on the Vegetative Growth of Chilli Seedlings

The plant growth parameters such as relative chlorophyll content, seedling height, total root length, total root surface area, and the root-to-shoot ratio were measured for moringa leaf extracted using various extraction methods. The relative chlorophyll content was not significant among treatments except for T2 (50% ethanol maceration of dry leaf); T7 (cold methanol infusion of dry leaf), and T9 (water infusion of dry leaf at 50°C) (**Fig. 5.2**). Relative chlorophyll content is a non-destructive and indirect method that indicates the health and nutritional status of the plant. Applications of moringa leaf extract were reported to enhance chlorophyll content and photosynthesis rate in plants under normal and stress conditions (Zulfiqar, Casadesús, et al., 2019).

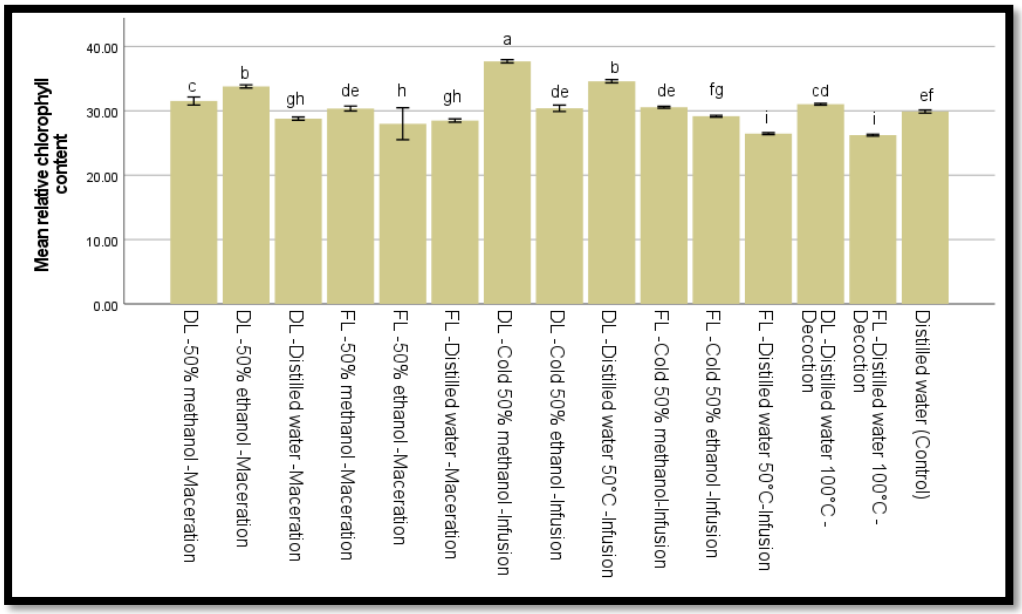


Fig. 5.2 Relative chlorophyll content of chilli seedlings treated with moringa leaf extract of various extraction methods. The values are an average of five independent measurements. According to Tukey's HSD, bars sharing the same letters are not significantly different ($p \leq 0.05$). The vertical bar in the graphs indicates the standard error.

Seedlings' height was significantly higher in all treatments than the control, particularly in sample T1 (methanol maceration of dry leaf) and T6 (water maceration of fresh leaf). The seedling's height ranged between 12.1 cm- 16.5

cm (**Fig. 5.3**). Total root length and total root surface area are essential indicators that could show the direct effect of bio-stimulant on chilli seedlings. Total root surface measurements were not significantly different among all the treatments (**Fig. 5.4**). Root length and proliferation of root hairs enhance nutrient absorption and nutrient use efficiency. The highest root length was observed in samples T2 (dry leaf of ethanol extraction), T10 (fresh leaf of methanol extraction), T8 (dry leaf of ethanol extraction), and T9 (dry leaf of water extraction) (**Fig. 5.5**).

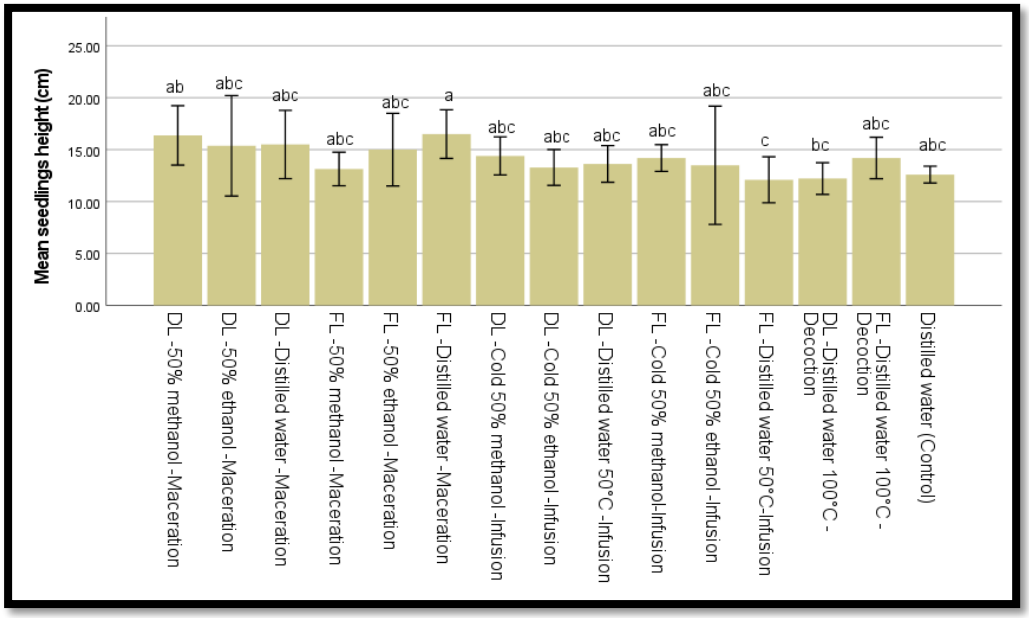


Fig. 5.3 The height of chilli seedlings treated with moringa leaf extracts of boiling, infusion, and maceration methods. The values are an average of five independent measurements. According to Tukey's HSD, bars sharing the same letters are not significantly different ($p \leq 0.05$). The vertical bar in the graphs indicates the standard error.The Shapiro-Wilk test significance value of 0.047 shows that the data is significantly deviate from a normal distribution.

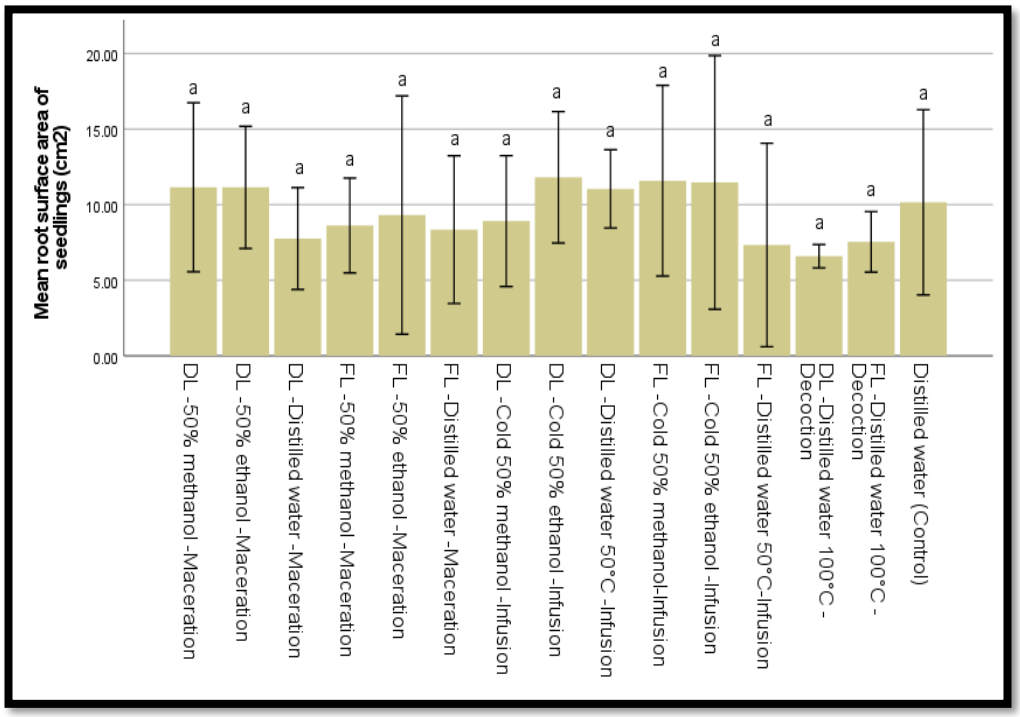


Fig. 5.4 Total root surface area of chilli seedlings treated with moringa leaf extract of various extraction methods. The values are an average of five independent measurements. Bars sharing the same letters are not significantly different ($p \leq 0.05$) according to Tukey's HSD. The vertical bar in the graphs indicates the standard error. The Shapiro-Wilk test significance value of 0.026 shows that the data is significantly deviate from a normal distribution.

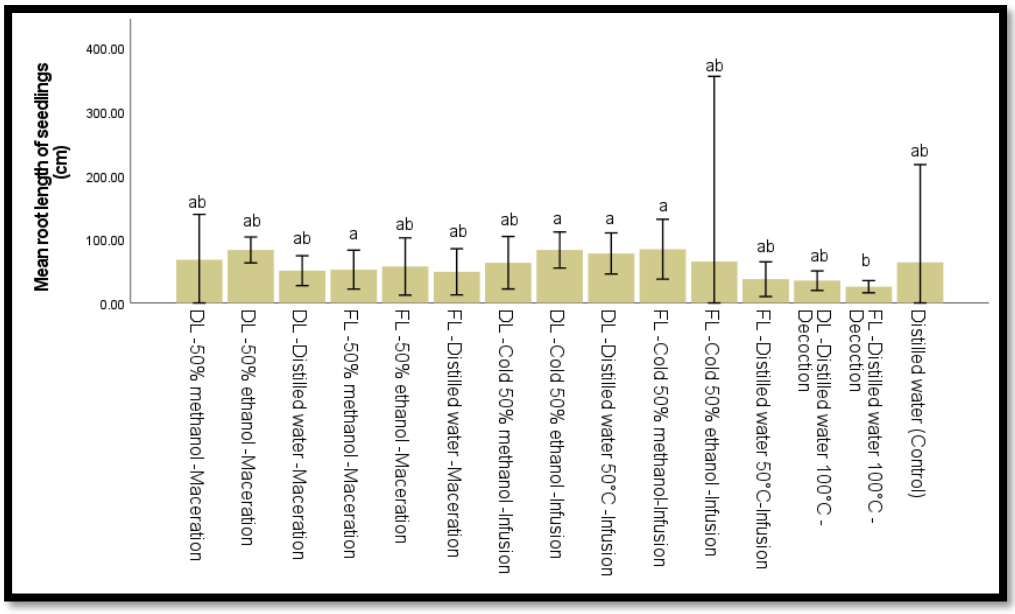


Fig. 5.5 Root length of chilli seedlings treated with moringa leaf extract of various extraction methods. The values are an average of five independent measurements.

According to Tukey's HSD, bars sharing the same letters are not significantly different ($p \leq 0.05$). The vertical bar in the graphs indicates the standard error. The vertical bar in the graphs indicates the standard error. The Shapiro-Wilk test significance value of 0.228 shows that the data is normal.

Root: shoot ratio is a vital criterion for assessing plant health. Chilli seedlings treated with dry moringa leaf extracted with 50% cold ethanol via infusion method showed a significant root-to-shoot ratio compared to all other treatments (**Fig. 5.6**).

Chilli seedlings treated with dry moringa leaf extracted with 50% ethanol using the maceration method showed the highest root biomass, which is significant as compared to other treatments (**Fig. 5.7**). Besides that, fresh leaf maceration and infusion with 50% methanol; dry leaf infusion with 50% ethanol and dry leaf decoction with water also demonstrated higher root biomass as compared to the control.

The highest shoot biomass was observed in chilli seedlings treated with dry moringa leaf extracted with 50% ethanol using the maceration method (**Fig. 5.8**). Apart from that, fresh leaf infusion with 50% cold methanol, dry leaf infusion with 50° C water and 50% cold methanol dry leaf maceration with 50% ethanol and 50% methanol also exhibited higher shoot biomass. A similar finding supporting the study is the application of moringa leaf extract at 50% concentration that increased maximum plant height, fresh and dry weights, and root fresh and dry weight of sunflower plants (Iqbal et al., 2020). This suggests that the phytochemicals in the moringa leaf extract significantly impact plant growth and development. Apart from that, the growth-enhancing potential of water-extract moringa leaf significantly influenced the growth of sunflower seedlings and plants, including the vegetative growth (Iqbal et al., 2020).

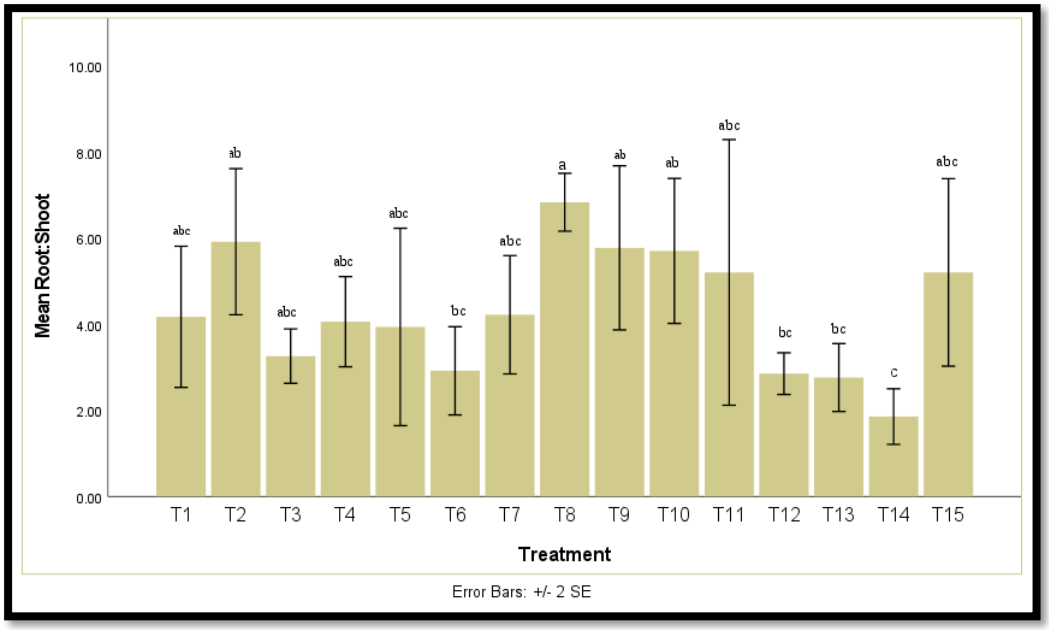


Fig. 5.6 Root-to-shoot ratio of chilli seedlings treated with moringa leaf extract of various extraction methods. The values are an average of five independent measurements. Bars sharing the same letters are not significantly different ($p \leq 0.05$) according to Tukey's HSD. The vertical bar in the graphs indicates the standard error. The Shapiro-Wilk test significance value of 0.230 shows that the data is normal.

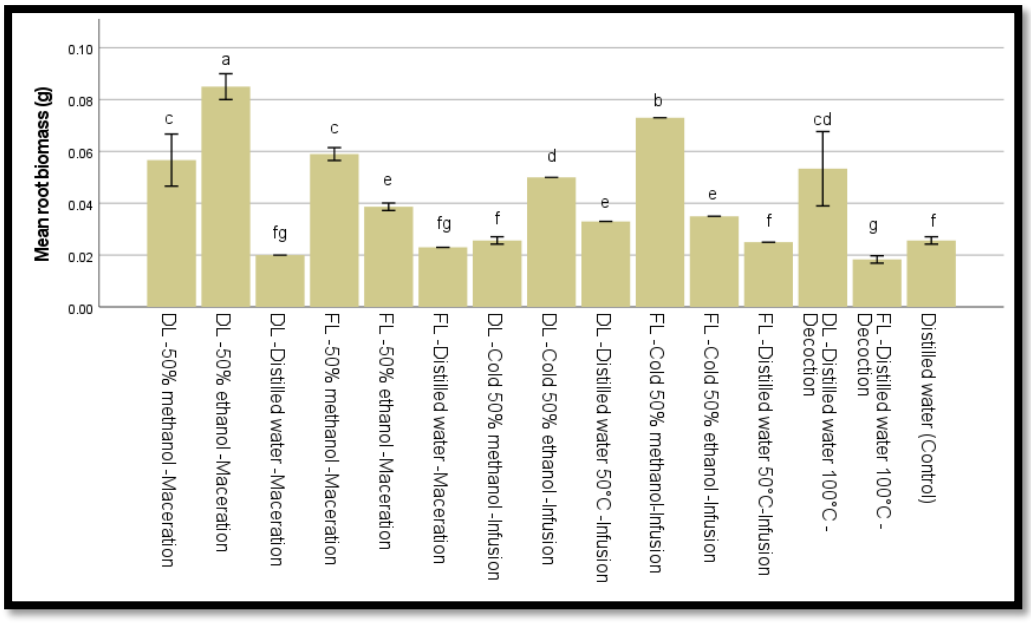


Fig. 5.7 Root biomass of chilli seedlings treated with moringa leaf extract of various extraction methods. The values are an average of five independent measurements. According to Tukey's HSD, bars sharing the same letters are not significantly different ($p \leq 0.05$). The vertical bar in the graphs indicates the standard error.

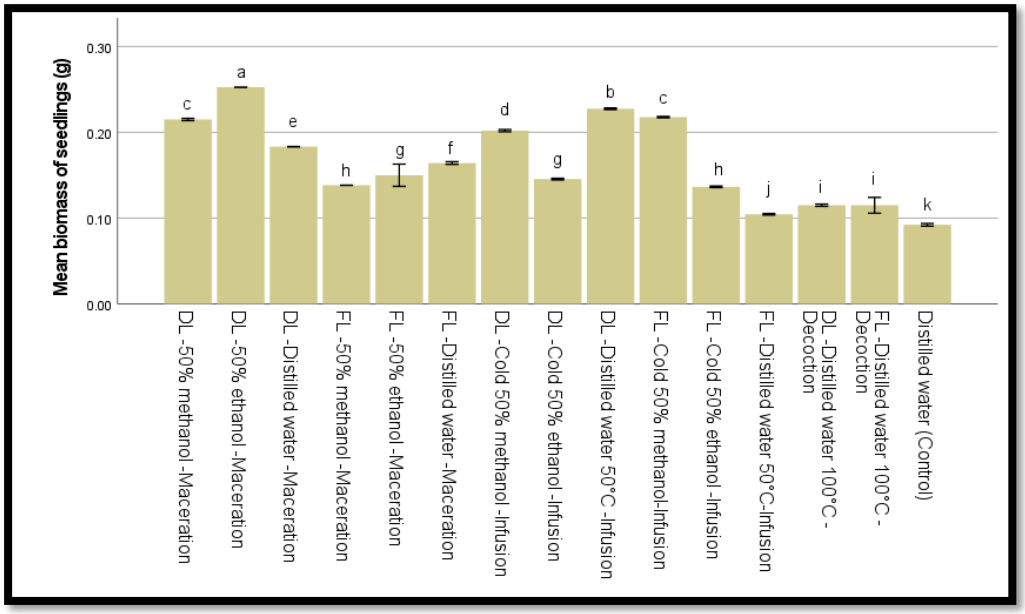


Fig. 5.8 Shoot biomass of chilli seedlings treated with moringa leaf extract of various extraction methods. The values are an average of five independent measurements. According to Tukey's HSD, bars sharing the same letters are not significantly different ($p \leq 0.05$). The vertical bar in the graphs indicates the standard error.

Polar solvent extraction is a standard method to extract bioactive compounds from natural products. Still, it can be expensive due to the cost of the solvents and the equipment required for the extraction process. On the other hand, aqueous extraction uses water as the solvent, which is generally cheaper and more environmentally friendly. While polar solvent extraction was more effective at promoting seedling growth and exhibited high antioxidant activities, the efficiency of aqueous extract was also comparable with polar solvent extract in specific analyses. For example, aqueous extraction increased the relative chlorophyll content, the height of seedlings, root length, and shoot biomass of chilli seedlings. Since the choice of extraction method will determine the cost of bio-stimulant production, aqueous extraction methods were selected for further study. Although the polar extraction method could give the best result, it will not be suitable for long-term bio-stimulant preparation due to its feasibility in agriculture. Therefore, aqueous extraction methods were compared with the polar extraction method to highlight the ease of use, safety, or environmental impact that is important in bio-stimulant preparation.

5.2.3 Assessment of the Compatibility between Moringa Leaf Extract and Endophytic Bacteria

The subsequent experiments used dry and fresh moringa leaf aqueous extracts prepared with decoction and maceration. The *Streptomyces* spores, *Bacillus*, and *Streptomyces* mycelial cell populations of the pure cultures were 1x 10^9 spores/ml, 1 x 10^{10} cfu/ml, and 1x 10^9 cfu/ml, respectively (**Table 5.4**). Compatibility of endophytic bacteria and moringa leaf extract using total plate count unveiled that decoction and maceration of fresh moringa leaf did not affect much the population of *Bacillus* and *Streptomyces*(**Table 5.4**). In contrast, decoction and maceration of dry moringa leaf extract were incompatible when mixed with crude moringa leaf extract (**Fig. 5.9**). This was obvious in the *Bacillus* population where it was reduced to 10^6 cfu/ml (Decoction) and *Streptomyces* population was reduced to 10^7 cfu/ml and 10^6 cfu/ml in both maceration and decoction preparations respectively. *Streptomyces* spore count was reduced to 10^6 cfu/ml in decoction preparation of dry leaf and no growth was observed in macerated dry moringa leaf extract. Although the total population was almost similar to the pure cultures, drastic changes were observed in the size of the colonies. The difference in the size of bacterial colonies indicates the mild antibacterial effect caused by moringa leaf extract on the survival and propagation of endophytic bacteria. The reduction in the number and size of the colonies shows the incompatibility of dry moringa leaf crude extract with endophytic bacteria. This could be due to the hormesis phenomenon, a diphasic dose response in which a low dose stimulates growth while a high dose inhibits it. The application of plant water extracts has an inhibitory effect at higher concentrations while stimulating at lower concentrations (Ibrahim, Khaliq, Hussain, & Haq, 2023). In general, the inhibitory and stimulating effect of plant extract can be associated with the high flavonoid content. Past study has reported high flavonoid content in dry moringa leaf extract (Vongsak et al., 2013). Furthermore, moringa leaf extract has antibacterial activity due to the presence of saponins, flavonoids, tannins, alkaloids, phenolics, and triterpenoids, which have the mechanism of destroying bacteria (Zulfiqar, Casadesús, et al., 2019). However, diluted leaf extract could reduce the antibacterial effects on the endophytic bacteria. The moringa leaf extracts were diluted x 100, and the

compatibility was tested with endophytic bacteria. It was found that *Streptomyces* sp. and *Bacillus* sp. were compatible with diluted dry moringa leaf extract, as no inhibition of bacterial colonies was observed in potato dextrose agar (**Fig. 5.10**). The effect of dilution of a plant extract, *A. subviscida* extract was observed on *A.alternata* colonies, in which the inhibitory effect was changed to growth promotion when treatments were diluted (Lira-de León et al., 2014). Therefore, a detailed study on the effect of diluted plant extract is vital to optimize the dosage and application methods of bio-stimulants derived from plants.

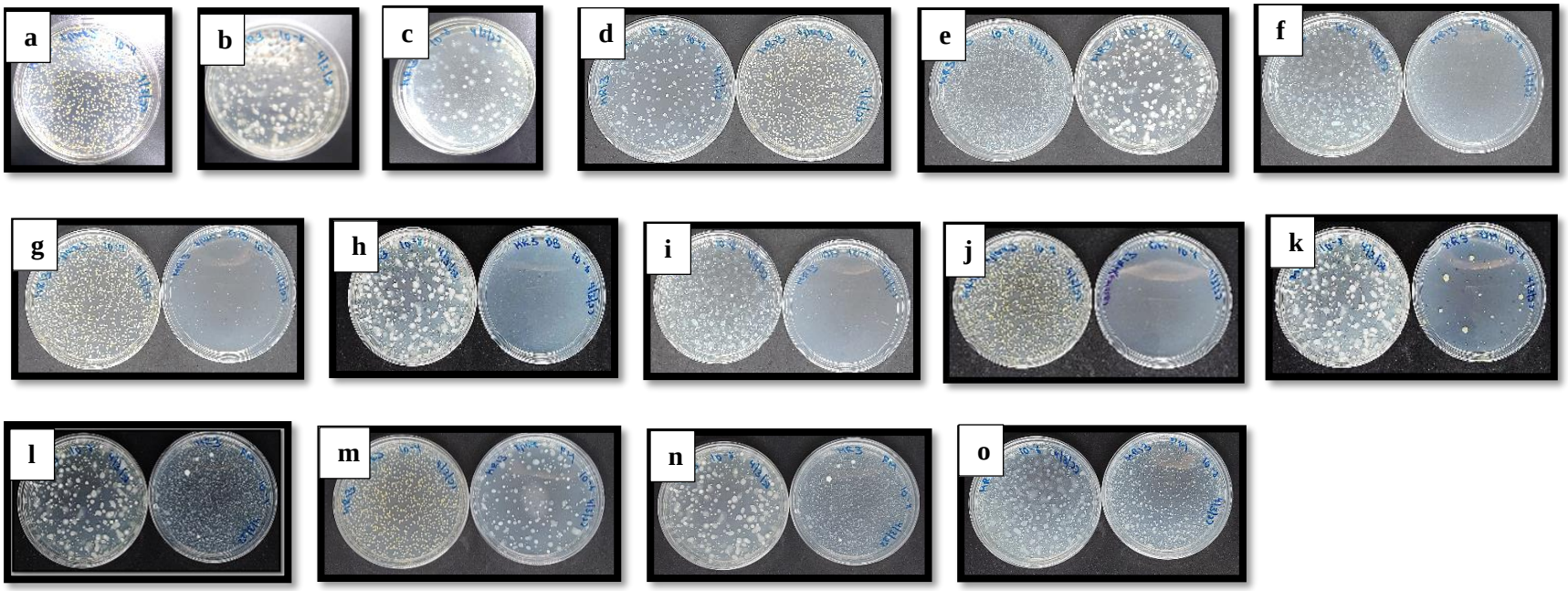


Fig. 5.9 Morphology of endophytic bacteria tested with moringa leaf extracted using boiling and maceration methods. (a) *Streptomyces* spores; (b) *Bacillus*; (c) *Streptomyces* vegetative cells; (d) *Streptomyces* + Fresh moringa leaf extracted using boiling method; (e) *Bacillus* + Fresh moringa leaf extracted using boiling method (f) *Streptomyces* vegetative cells + Fresh moringa leaf extracted using boiling method; (g) *Streptomyces* + Dry moringa leaf extracted using boiling method; (h) *Bacillus* + Dry moringa leaf extracted using boiling method (i) *Streptomyces* vegetative cells + Dry moringa leaf extracted using boiling method; (j) *Streptomyces* + Dry moringa leaf extracted using maceration method; (k) *Bacillus* + Dry moringa leaf extracted using maceration method (l) *Streptomyces* vegetative cells + Dry moringa leaf extracted using maceration method; (m) *Streptomyces* + Fresh moringa leaf extracted using maceration method; (n) *Bacillus* + Fresh moringa leaf extracted using maceration method (o) *Streptomyces* vegetative cells + Fresh moringa leaf extracted using maceration method

Table 5.4 Compatibility of endophytic bacteria with moringa leaf extracted using boiling and maceration methods

Bacteria	Plant extract	Method of extraction	Code	cfu/ml
***Bacillus* sp.**	-	-	MR3	4.47 x 10^{10}
***Streptomyces* vegetative stage**	-	-	MR13	1.29 x ^{9}
***Streptomyces* sporulating stage**	-	-	MR13 (spores)	1 x 10^{9}
***Streptomyces* vegetative stage**	Dry moringa leaf	Decoction	MR13 –DB	1 x 10^{6} Tiny colonies
***Bacillus* sp.**	Dry moringa leaf	Decoction	MR3-DB	1 x 10^{6} Tiny colonies
***Streptomyces* sporulating stage**	Dry moringa leaf	Decoction	MR13 (spores)-DB	1 x 10^{6}
***Streptomyces* vegetative stage**	Fresh moringa leaf	Decoction	MR13-FB	1 x 10^{10} Tiny colonies
Streptomyces sporulating stage	Fresh moringa leaf	Decoction	MR13 (spores)-FB	1 x 10^{9}
***Bacillus* sp.**	Fresh moringa leaf	Decoction	MR3-FB	1 x 10^{9} Tiny colonies
***Bacillus* sp.**	Dry moringa leaf	Maceration	MR3-DM	5.9 x 10^{9}
***Streptomyces* sporulating stage**	Dry moringa leaf	Maceration	MR13 (spores)-DM	No growth
***Streptomyces* vegetative stage**	Dry moringa leaf	Maceration	MR13-DM	4.4 x 10^{7} Tiny colonies
***Bacillus* sp.**	Fresh moringa leaf	Maceration	MR3- FM	1 x 10^{9}

***Streptomyces* vegetative stage**	Fresh moringa leaf	Maceration	MR13-FM	1 x 10^{9}
***Streptomyces* sporulating stage**	Fresh moringa leaf	Maceration	MR13 (spores)-FM	1 x 10^{6}

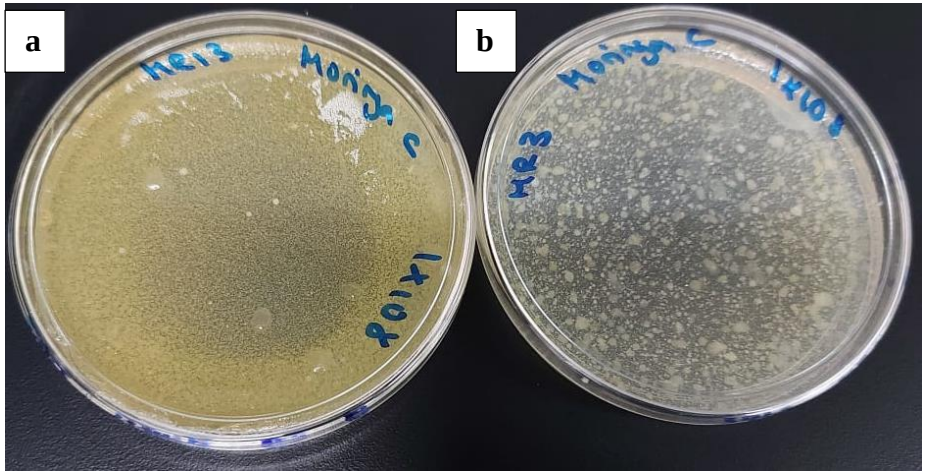


Fig. 5.10. Pure cultures of *Streptomyces* and *Bacillus* show no growth inhibition when mixed with diluted (1:100) moringa leaf extract (a) *Streptomyces* sp. on potato dextrose agar and (b) *Bacillus* sp. on potato dextrose agar

5.2.4 Synegistic Effect of Moringa Leaf Extract and Endophytic Bacteria on the Vegetative Growth of Chilli

Chilli treated with fresh moringa leaf juice extracted by maceration showed significantly higher height than other treatments. Fresh moringa leaf extract obtained via maceration and *Bacillus* sp. (FM + MR3) showed the highest chilli root length (**Fig. 5.11**). The application of fresh moringa leaf extracted by maceration (FM) alone showed lower root length, but it increased significantly when applied together with endophytic bacteria.

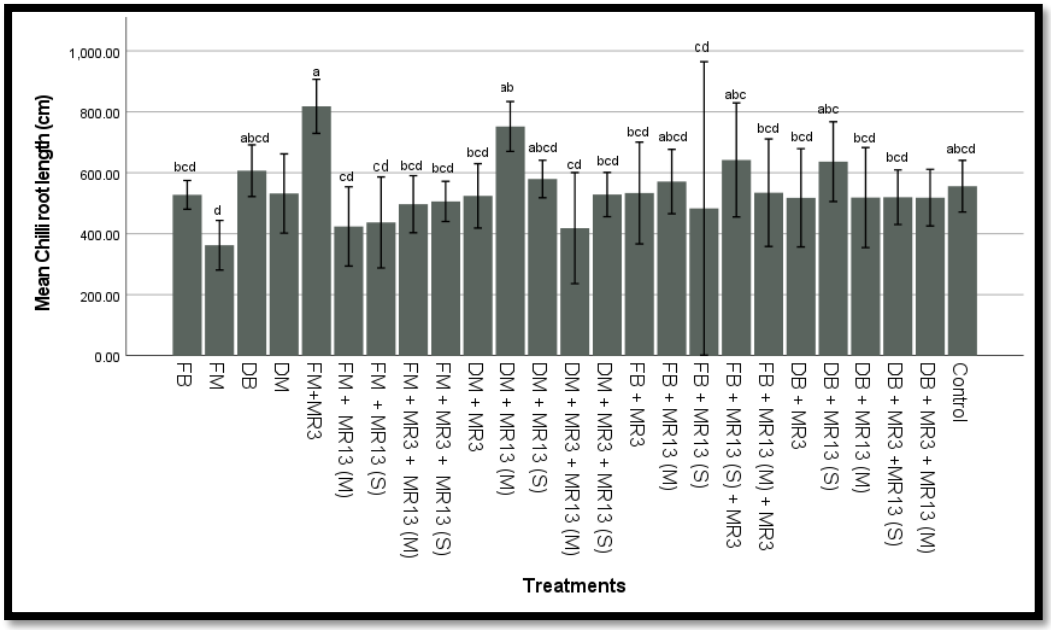


Fig. 5.11 Root length of chilli plants treated with moringa leaf extract and endophytic bacteria. The measurement was calculated automatically by Winrhizo root scanner, which included the mean length of 15 individual plants' entire root system. According to Tukey's HSD, bars sharing the same letters are not significantly different ($p \leq 0.05$). The vertical bar in the graphs indicates the standard error. The Shapiro-Wilk test significance value of 0.16 shows that the data is normal.

Likewise, the lowest value of root surface area of chilli seedlings was attained when the plants were treated with fresh moringa leaf extracted via maceration (**Fig. 5.12**). Fresh and dry moringa leaf extracted via maceration showed lower root length and surface area than the same treatment co-applied with endophytic bacteria.

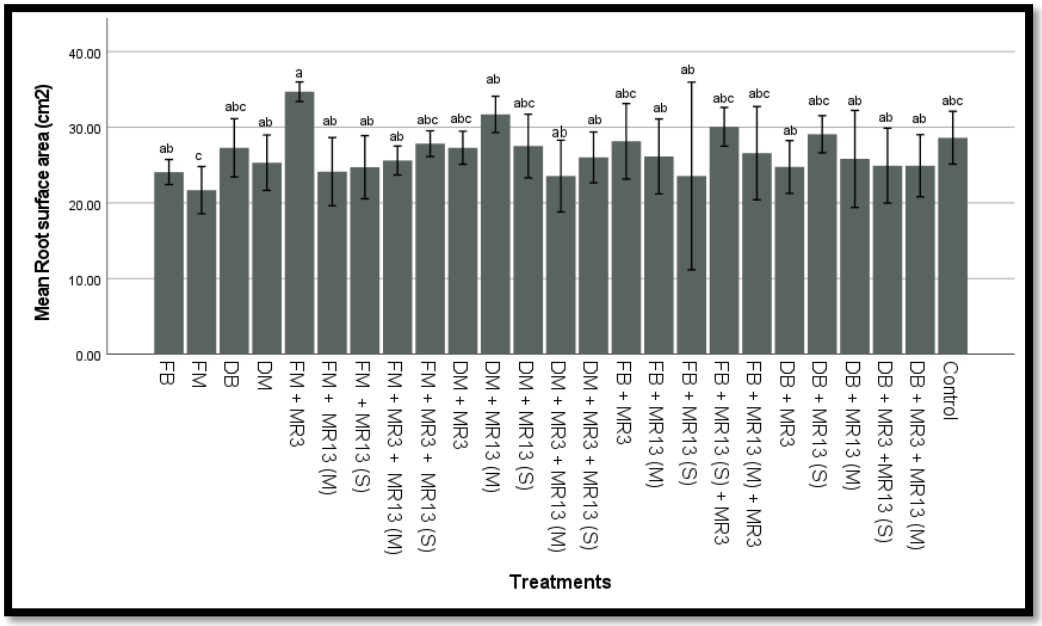


Fig. 5.12 Root surface area of chilli plants treated with moringa leaf extract and endophytic bacteria. The measurement was calculated automatically by Winrhizo root scanner, which included the mean length of 15 individual plants' entire root system. According to Tukey's HSD, bars sharing the same letters are not significantly different ($p \leq 0.05$). The vertical bar in the graphs indicates the standard error. The Shapiro-Wilk test significance value of 0.265 shows that the data is normal.

On the other hand, the plants treated with dry moringa leaf extract obtained via the decoction method and endophytic bacteria showed the most significant aboveground biomass of chilli plants (**Fig. 5.13**). Similarly, the root biomass also increased significantly when applied with dry moringa leaf decoction extract and various combinations of endophytic bacteria (**Fig. 5.14**).

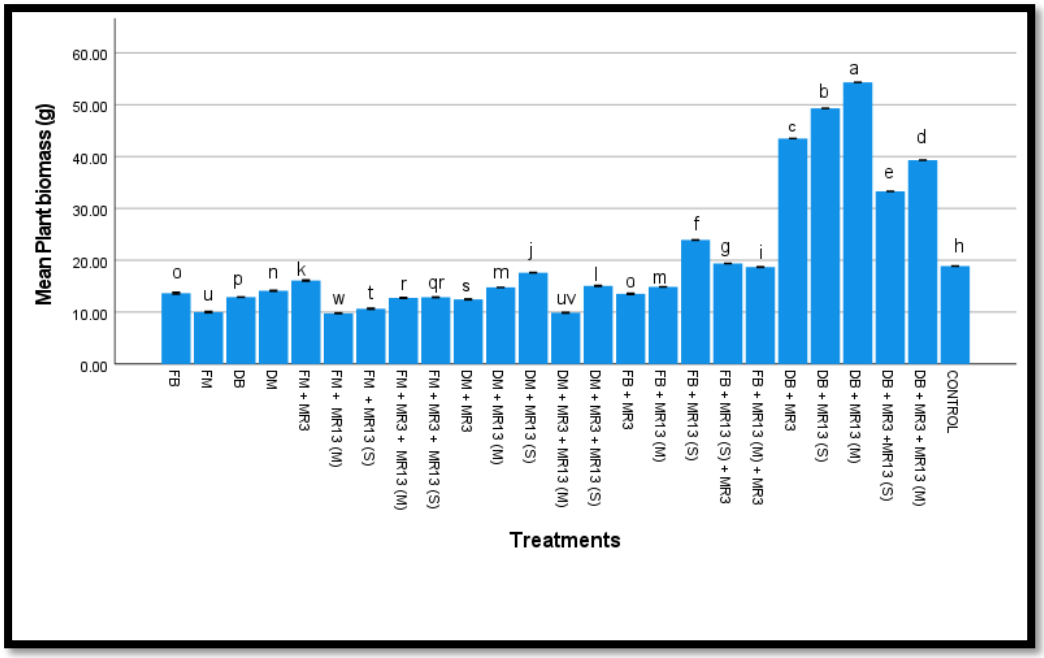


Fig. 5.13 Aboveground biomass of chilli plants treated with moringa leaf extract and endophytic bacteria. Each value is the mean of fifteen replicates (n=15), and bars sharing the same letters are not significantly different ($p \leq 0.05$) according to Tukey's HSD. The vertical bar in the graphs indicates the standard error.

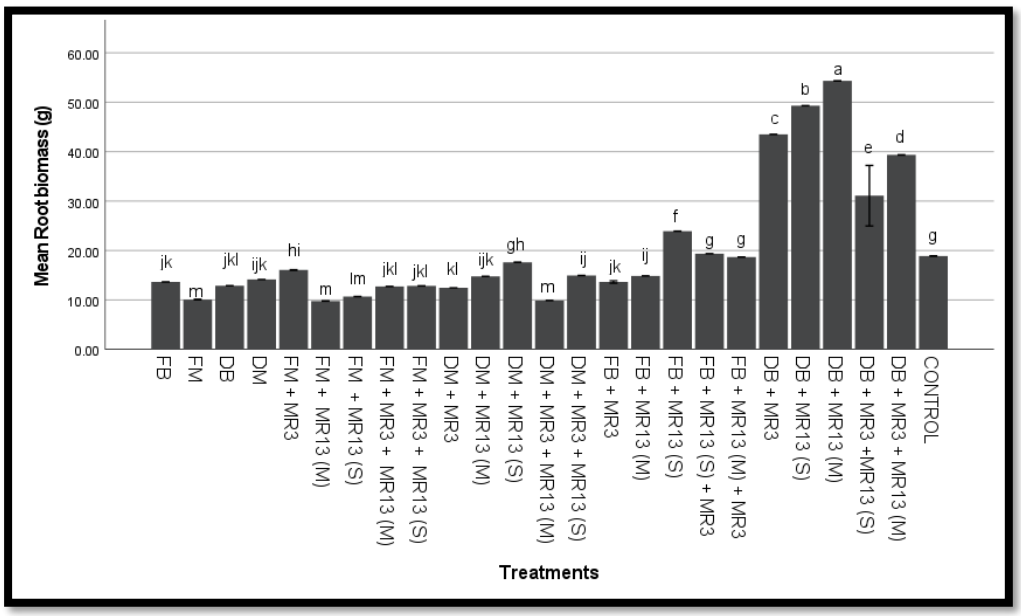


Fig. 5.14 Root biomass of chilli plants treated with moringa leaf extract and endophytic bacteria. Each value is the mean of fifteen replicates (n=15), and bars sharing the same letters are not significantly different ($p \leq 0.05$) according to Tukey's HSD. The vertical bar in the graphs indicates the standard error.

5.2.5 Synegistic Effect of Moringa Leaf Extract and Endophytic Bacteria on the Yield of Chilli

The yield of chilli per plant showed that plants treated with a dry leaf of moringa extracted via maceration alone (DM) and co-applied with *Bacillus* sp. (DM + MR3) could give the highest produce, 691.1g and 650.1g, respectively. This was followed by dry moringa leaf extract via decoction method (DB) and fresh moringa leaf extracted via decoction method (FB), 603.7g and 599.3g, respectively (**Fig. 5.15**).

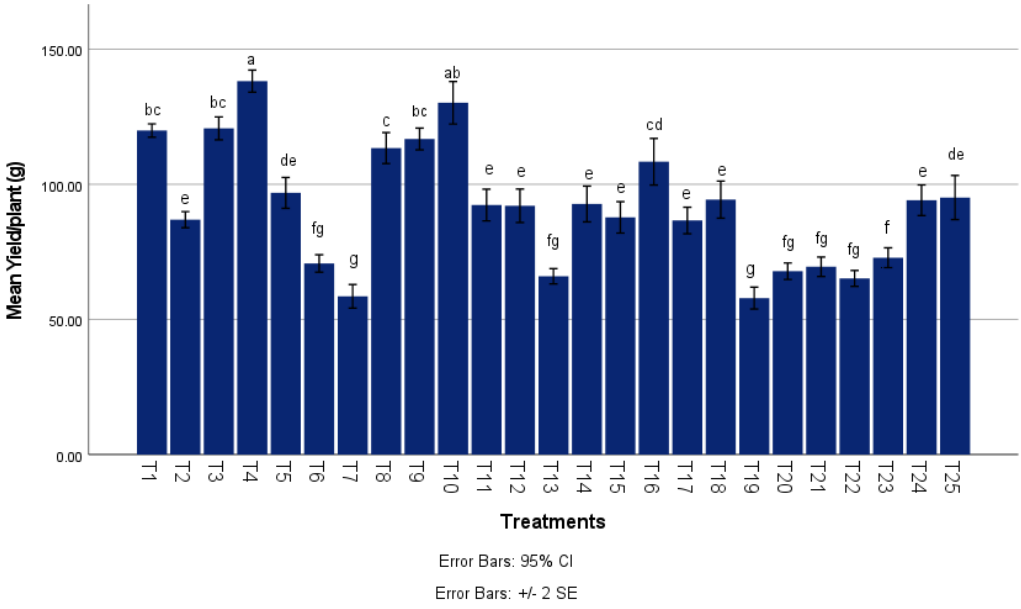


Fig. 5.15 Yield per chilli plant treated with combined application of moringa leaf extract and endophytic bacteria. Each value is the mean of fifteen replicates (n=15), and bars sharing the same letters are not significantly different ($p \leq 0.05$) according to Tukey's HSD. The vertical bar in the graphs indicates the standard error.

A growing trend in agriculture involves the utilization of bio-stimulants that incorporate a mixture of various sources. For example, foliar application of bio-stimulant containing moringa leaf extract, seaweed extract, and fulvic acid could positively improve the vegetative growth, fruit set percentage, fruit yield, and fruit physical and chemical characteristics as well as leaf nutritional value (Mosa et al., 2023). The combined application of arbuscular mycorrhiza fungi and seaweed extract also positively affected the vegetative and phytochemical traits in lettuce more than their sole application (Rasouli et al., 2022). On the other

hand, adding plant-derived soy lecithin and flavonoid-rich wood glycolic extract did not show any additional beneficial effect on lettuce (Fedeli, Vannini, Guarnieri, Monaci, & Loppi, 2022). Therefore, the compatibility of the different sources and the impact of bio-stimulant co-application has to be investigated thoroughly. This study evaluated moringa leaf extract and endophytic bacteria on a model crop, chilli. To our knowledge, no reports were found on the combined effects of endophytic bacteria and the methanolic, ethanolic, and aqueous extract of moringa leaves on chilli growth. These findings could be guidelines for a practical and efficient bio-stimulant formulation for chilli.

5.3 Conclusion

This study has revealed that apart from methanol and ethanol extraction, water extraction, which is a cheap and feasible method of moringa leaf extraction, could be used to produce botanical bio-stimulant. Moringa leaves were rich with antioxidant contents and could enhance the growth of chilli root. Among aqueous extraction methods, maceration of dry leaf, decoction of fresh leaf, and decoction of dry leaf showed 47.39 µg/mL, 43.22 µg/mL, and 44.50 µg/mL of DPPH scavenging activities, respectively. For phenolic content, the decoction method of dry leaf, maceration method of fresh leaf, and decoction method of fresh leaf produced 0.52, 0.32, and 0.31 of gallic acid equivalent (GAE) mg/L, respectively. Flavonoid content results exhibited that maceration of dry leaf, a decoction of fresh leaf, and maceration of fresh leaf produced 74.89, 70.78, and 69.82 of quercetin equivalent mg/mL, respectively. Overall, the decoction of fresh leaf and maceration of dry leaf showed comparable phytochemical content, especially flavonoid content, compared to extraction using polar solvents.

Furthermore, it was revealed that although dry moringa leaf extracts enhanced plant growth and yield, a high concentration of dry moringa leaf was incompatible with endophytic bacteria, inhibiting the endophytic bacterial growth and colony size. However, a 1:100 dilution of moringa leaf extract with water has increased the compatibility of the extract and bacteria. No superior extraction method was found to have a positive effect on all vegetative growth assessments in the chilli plant. Dry leaf decoction extract significantly promoted

vegetative growth, especially root and shoot biomass of chilli plants. On the other hand, dry leaf maceration extracts alone or along with *Bacillus* increased the chilli yield compared to other treatments.

Aqueous extraction increased the relative chlorophyll content, the height of seedlings, root length, and shoot biomass of chilli seedlings. Fresh moringa leaf extract obtained via maceration along with *Bacillus* sp. (FM + MR3) showed the highest chilli root length and chili seedlings' largest root surface area. On the other hand, chilli plants treated with dry moringa leaf extract obtained via decoction method and endophytic bacteria showed the most significant aboveground and root biomass of chilli plants.

Therefore, dry leaf extraction via maceration and decoction methods was used for further study. Remarkably, dry leaf decoction extract was suggested to be applied throughout the plant's vegetative growth. In contrast, dry leaf maceration extract was recommended to be used during the flowering period as both moringa extracts exhibited different efficiency at different stages of chilli development. Generally, bio-stimulants may have target effects on specific phenological phases of crops. For example, plants need strong rooting systems to absorb more nutrients as critical nutrient absorption occurs at the early stage of plant development. At the same time, root colonization of beneficial bacteria at the early growth stage can prevent root pathogen’s infestation and increase the immunity of plants. On the other hand, late application of bio-stimulants can improve fruit set quality. Knowing the target effect of bio-stimulant can be helpful in tailoring the application methods to different growth phases of crops as plants undergo distinct need at different phenological stages.

CHAPTER 6
CHARACTERIZATION OF NANO-ENCAPSULATED ENDOPHYTIC BACTERIA AND MORINGA LEAF EXTRACT

6.0 Introduction

Plant bio-stimulants depend not on their nutrient content but on stimulating their natural physiological processes related to plant growth or the plant's defense system (Roberta Bulgari, Franzoni, & Ferrante, 2019; Chanda et al., 2019). They may contain various active substances, including beneficial microorganisms that work together to trigger the plant's physiological system, stress tolerance, and improve production quality.

Bio-stimulant products containing PGPR are often reported to have low cell density and reduced longevity during product storage and upon application to soil, leading to reduced efficiency. (Bhatia et al., 2021) elucidated that maintaining stable colonization of applied PGPR strains in the rhizosphere has been a major challenge. Consequently, PGPR-based bio-stimulants are considered unstable and inefficient, producing inconsistent results in laboratory and field experiments. For example, the bioactive compounds of microalgae bio-stimulant were susceptible to degradation over time (Stirk et al., 2021). Long-term storage of liquid formulations under ambient temperature conditions, which could provide prolonged survival of microorganisms in the final product and after an application, is crucial for commercialization (Elsakhawy et al., 2021).

New formulations are essential in bio-stimulant production to enhance the efficiency and longevity of the microorganisms in the product and growth medium. Nano-encapsulation is an emerging technology based on nano-formulations. A technique in which homogenous or heterogenous functional compounds are entrapped by a polymeric material, and confines them from the external environment is known as encapsulation. For example, probiotic bacteria were successfully formulated into nano-encapsulation of poly (vinyl alcohol)

and sodium alginate nanofibers to delay the microbial growth in the fish fillets to avoid contamination by mesophilic bacteria. There were numerous reports on the feasibility of calcium-alginate microcapsules used as a delivery method for microbial bio-fertilizers or plant growth-promoting bacteria (Y. He et al., 2015; Wu et al., 2011).

Nano-emulsions are fine oil-in-water dispersions with droplet sizes ranging from 100-500 nm. It exhibits better penetration efficacy of the active ingredients due to the emulsion's large surface area and low surface tension. It was found from the previous experimental chapter that dry moringa leaf extract obtained via decoction and maceration methods showed incompatibility with endophytic bacteria. Thus, it has to be diluted in a ratio of 1:1000 to ensure that bacterial growth is not inhibited. However, properly formulating the two bio-stimulants to encapsulate the active ingredients separately would be very beneficial for the co-application of bio-stimulant. Encapsulation of endophytic bacteria using alginate nano-gel and nano-emulsion of moringa leaf extract in the aqueous alginate phase was expected to increase the stability of the product during storage. The encapsulation was also expected to stabilize the bacterial population and increase the shelf life during storage.

This chapter aims to formulate the bacterial and plant extract bio-stimulants separately in nano-formulations, determine their shelf life, and characterize their physical and chemical structures during storage. It is a pioneer study reporting the characterization of the nano-encapsulated bio-stimulant formulation.

6.1 Materials and Methods

6.1.1 Encapsulation of Endophytic Bacteria and Moringa Leaf Extract

Dry moringa leaf powder extracted via aqueous solvent was used as the active ingredient of moringa leaf. Endophytic bacteria, *Bacillus* sp., was used as a model for the alginate polymer encapsulation technique. Encapsulation was prepared using the ionic gelation method to produce nano-gels for *Bacillus* culture and oil-in-water nano-emulsion for moringa leaf extract.

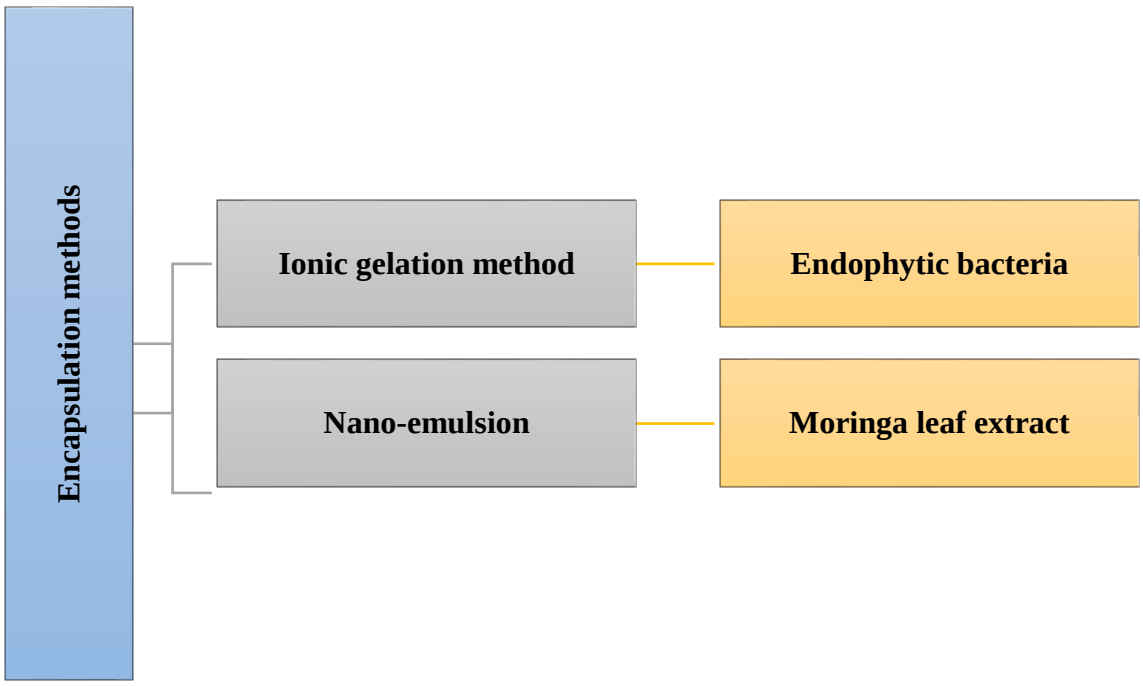


Fig. 6.1 Encapsulation methods for plant extract and bacteria

6.1.2 Effect of Homogenization on Bulking Material

The bulking materials that were tested to be incorporated in the nano-gel were sodium alginate (HiMedia, Pvt.Ltd, India), zeolite (Merck, Germany), skimmed milk (HiMedia, Pvt.Ltd, India), and modified starch. Probe sonication method was used to reduce the size of the bulking materials. The suitable nanomaterials was selected based on their ability to form nano-size with uniform polydispersity index value.

6.1.2.1 Effect of Homogenization Process on the Size and Polydispersity Index of Bulking Materials

Every component of nano-formulation was sonicated for 15 min at 10,000 rpm using an ultrasonic homogenizer (Silverson, L5M-A series, Faculty of Science and Engineering, University of Nottingham, Malaysia). The size and polydispersity index of bulking agents such as sodium alginate, zeolite, skimmed

milk, and modified starch was measured before and after ultrasonication. Each sample was diluted with distilled water with a ratio of 1:100 before taking the measurement using a zeta sizer (Nano-ZS, Malvern. USA, Faculty of Engineering, University of Nottingham, Malaysia). Particle size distribution and zeta potential were determined according to the method described by (Chawla, Kumar, Kaushik, & Dhull, 2019).

6.1.2.2 Evaluation of the Image of Bulking Materials and Plant Extract using Field Emission Scanning Electron Microscopy (FESEM) Analysis

The samples, such as nano-emulsion droplets, nano-gel, moringa leaf extract, and other bulking materials, were air-dried on double-sided conductive carbon tape placed on a stub. Then, the sample was sputtered with platinum for 30 seconds before viewing in a field emission scanning electron microscope (FEI, Quanta 400F/Oxford FESEM, Faculty and Science and Engineering, University of Nottingham, Malaysia). The bulking materials include samples before and after the ultrasonication step.

6.1.3 Nano-encapsulation Formulations of Bio-stimulants

Nano-gel containing *Bacillus* sp. was formulated using the ionic gelation method. Moringa leaf extract was encapsulated in oil-in-water droplets by using the nano-emulsion technique.

6.1.3.1 Nano-gel Formulation of Endophytic Bacteria

Bacillus sp. was freshly grown on potato dextrose agar (PDA, Merck, Germany) for the nano-gel formulation. One loop-full of each bacterial colony was transferred into 500 mL of tryptic soy broth (TSB, Merck, Germany) separately and incubated in an incubator shaker (Wisecube, Faculty of Science & Engineering, University of Nottingham, Malaysia) at room temperature for three days at 90 rpm. The stock culture was diluted to ensure that the density of endophytic bacteria was within the range of 10^7-10^9 CFU/ml (bacterial density was checked using the total plate count method).

Sodium alginate was prepared at 0.5% (w/v) in one liter of distilled water and autoclaved at 121°C for 30 minutes. Modified cornstarch (0.1 g), zeolite (0.1 g), and skimmed milk (0.1 g) were added to the sodium alginate solution before autoclaving. Calcium chloride dihydrate, 0.5 M, was autoclaved separately and kept at 10°C until use.

The stock bacterial culture was mixed with sodium alginate at 10% concentration using a magnetic stirrer for 10 min. The plastic syringe extruded the bacterial-alginate mixture into a calcium chloride solution under stirring conditions. The encapsulated bacteria in alginate gel were left overnight at room temperature for a stable gel formation. The nano-gel was washed thrice with distilled water. Then, excess water was removed by straining the gel, and the nano-gel was collected in a clean container before storing it at 10°C.

6.1.3.2 Nano-emulsion Formulation of Moringa Leaf Extract

In this study, moringa leaf extract was prepared using an oil-in-water nano-emulsion system with a high-energy method, high speed homogenizer. Two immiscible phases, oil and aqueous phases, were mixed with a surfactant with an HLB value of 10 to prepare oil-in-water nano-emulsions according to the method described by (Hong et al., 2018). Hydrophillic-Lipophillic Balance (HLB) value was set as 10. Span 80 (HLB 4.3) and Tween 80 (HLB 15) were used to prepare the surfactant with HLB value 10 using the formula below.

$$A\ (\%)\ = 100\ x\ \frac{(X - HLBb))}{(HLBa - HLBb)}$$

A (%) = 100 (X-HLBb / (HLBa-HLBb)
a= Tween 80; b= Span 80; X= required HLB of the surfactant.

Based on the calculation above, 53.2% of Tween 80 and 46.8% of Span 80 were mixed in a magnetic stirrer under low heat at 40°C for 20 minutes. Corn oil was used as the oil component for nano-emulsion preparation. The ratio of oil and

surfactant was determined by mixing various ratios at 70°C in a magnetic stirrer, and the mixture was left at room temperature for a week. The nano-emulsion formation was observed when a milky solution was formed and remained stable for at least two weeks without causing coalescence, flocculation, creaming, Ostwald ripening, or sedimentation, as mentioned in (Romes et al., 2021).

For nano-emulsion containing moringa leaf extract, 10% (w/v) of dry moringa leaf powder was macerated with sterile distilled water in a blender and left at room temperature overnight. The extract was collected by filtering the extract using a muslin cloth. The extract was diluted ten times and mixed with corn oil at a ratio of 1:5 (oil: moringa extract). The oil phase was combined in a magnetic stirrer at 50 °C for 15 min. Then, 1% of the surfactant with an HLB value of 10 was added dropwise while stirring for 30 min. The oil phase-surfactant mixture was homogenized at 10 000 rpm for 2 min and diluted with 90% of the aqueous phase consisting of distilled water.

6.1.4 Characterization of Nano-formulations

Nano-emulsion characterization was done based on the physical appearance, size, polydispersity index, zeta potential value, and structural image. Only the physical and structural characterization was done for nano-gel as the bulking materials used for nano-gel preparation were characterized before the ionic gelation method. The nano-gel was used as a nano-carrier for the endophytic bacteria.

6.1.4.1 Physical Appearance of Nano-gel and Nano-emulsion

The nanoemulsion was observed for visual appearances such as phase separation, creaming, flocculation, coalescence, and sedimentation two weeks after the preparation before proceeding to physicochemical properties analysis.

6.1.4.2 Physico-chemical Properties of Nano-emulsion

The size, polydispersity index, and zeta potential value of nano-emulsions were measured after two weeks of nanoemulsion preparation. Each sample was diluted with deionized water with a ratio of 1:100 before taking the measurement using a zeta sizer (Nano-ZS, Malvern. USA, Faculty of Engineering, University of Nottingham, Malaysia). Particle size distribution and zeta potential were determined according to the method described by (Chawla et al., 2019).

6.1.4.3 Structural Image of Nano-gel and Nanoemulsion using Field Emission Scanning Electron Microscopy (FESEM) Analysis

The method described in **section 6.1.2.2** was adopted to observe nano-encapsulation structures using FESEM.

6.1.5 Assessment of the Stability of Nano-gel and Nano-emulsion Formulation during Storage

The stability of the nano-encapsulated bio-stimulant formulation before and after six months of storage at 28°C was determined. Nano-gel was assessed for its resilience to hold bacterial population for six months and its efficiency in enhancing chilli seed germination and the length of the radicle. During the storage time, nanoemulsion samples were assessed for their physical appearance, physical-chemical properties such as zeta potential, polydispersity index and particle size, functional compounds, and their efficiency in enhancing chilli seed germination and the length of the radicle.

6.1.5.1 Physical Appearance of Nano-emulsion after the Storage period

The nano-emulsion was observed for visual changes in appearance, such as phase separation, creaming, flocculation, coalescence, and sedimentation during a storage time of six months.

6.1.5.2 Physico-chemical Properties of Nano-emulsion and Nano-gel after the Storage Period

The size, polydispersity index, and zeta potential value of nano-emulsions were measured before and after six months of storage. Each sample was diluted with deionized water with a ratio of 1:100 before taking the measurement using a zeta sizer (Nano-ZS, Malvern. USA, Faculty of Engineering, University of Nottingham, Malaysia).

6.1.5.3 Functional Groups Detection in Raw Moringa Leaf Extract and Nano-emulsion of Moringa Leaf during the Storage Period

Fourier Transform Infrared Spectroscopy (FTIR) (PerkinElmer, FT-IR/FIR Spectrometer Frontier, School of Biosciences, University of Nottingham, Malaysia) was used to determine the functional groups of the nano-gel and nano-emulsion. The optical absorption spectra were recorded in the rapid scan mode on an FTIR spectrometer (PerkinElmer, FT-IR/FIR Spectrometer Frontier) coupled with a photoacoustic detection cell. Each measurement was carried out in the spectral range between 400 and 4000 cm^{-1}, resolution of 4 cm^{-1}, and a data interval of 0.5 cm^{-1}. Each spectrum was obtained from an average of 7200 scans. The data was recorded as each sample's transmittance value (T %).

6.1.5.4 Shelf life of Encapsulated Endophytic Bacteria in Nano-gel

The alginate nano-gel containing endophytic bacteria, *Bacillus* sp., was kept at room temperature in a clean falcon tube. One gram or milliliter of nano-gel was incubated with 1 ml of 0.5 M phosphate buffer for 24 hours and diluted with sterile distilled water before plating on potato dextrose agar. The total plate count was determined on the day of preparation and 180 days after storage.

6.1.6 Effect of Nano-encapsulation Formulation on Chilli Seed Germination and Root Length

The effect of nano-encapsulation formulations was evaluated on seed germination, particularly nano-gel and moringa leaf nano-emulsion. Chilli seeds (SAKATA 461) were soaked in 5 mL of nano-gel containing *Bacillus* sp. and 5 mL of nano-emulsion of moringa leaf extract for one hour at room temperature after adding 5 mL of distilled water in each solution. Then, ten seeds were placed in a petri dish layered with double filter paper moistened with distilled water. Petri dishes were sealed with parafilm and covered with aluminium foil. The plates were kept at room temperature in a dark place and observed for germination for one week. Later, the length of the radicle was measured for each germinated seed.

6.2 Results and Discussion

6.2.1 Effect of the Sonication Process on the Size and Polydispersity Index and Image of Bulking Materials

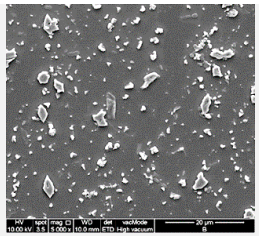
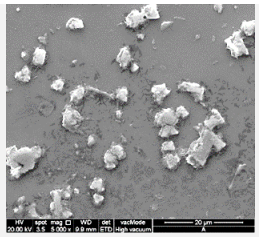
The size and polydispersity index of bio-stimulant components before and after the sonication process was demonstrated in **Table 6.1**. The extraction method plays an essential role in determining the size of dry moringa leaf extract nanoparticles. Moringa leaf extracted using the maceration method (DM) exhibited larger particle size (1115.0±19.9 d.nm) as compared to leaf extract obtained via the decoction (DB) method (567.0±55.0). After the sonication step, these sizes were significantly reduced to 155.2±1.1 d.nm and 94.5±1.2 d.nm. The polydispersity values were reduced because of the sonication process. However, no difference was observed in the size of 0.5% (w/v) sodium alginate and 0.1% (w/v) skimmed milk after sonication. Thus, sodium alginate (0.5%) and skimmed milk (0.1%) could be used directly without the sonication process, as the size of the original materials were 150 nm and 360 nm, respectively. Zeolite and modified starch were reduced in size after the sonication process. For instance, the size of zeolite was 1142 nm before sonication, and it was reduced to 611 nm after sonication. The size of the modified starch was

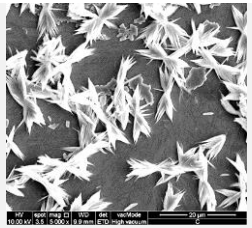
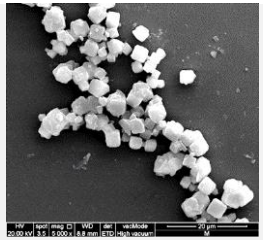
decreased from 946 nm to 599 nm after sonication. The polydispersity index of zeolite was reduced from 0.81 to 0.48, while the polydispersity index value of modified starch remained the same after the sonication process. The polydispersity index (PDI) reflects the width of particle size distribution, and a low PDI value (<0.3) demonstrates that the nanoparticles have a uniform and narrow size distribution (Lin et al., 2019).

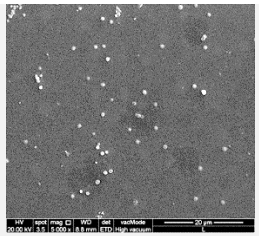
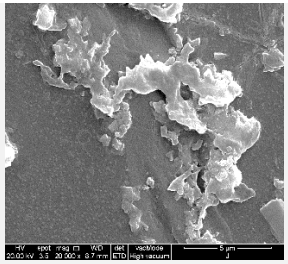
Sonication is a common technique used in nano-formulation to reduce the size of nanoparticles and improve their stability. It involves high-frequency sound waves to break down larger particles into smaller ones, typically from a few nanometers to several hundred nanometers. High-pressure homogenization is a technique utilized to generate nano-emulsions with tiny droplet sizes. The process involves using a piston or high-pressure homogenizer, where various forces, such as intense turbulence, hydraulic shear, and cavitation, work together to produce the nanoemulsion. The resultant product may be re-subjected to high-pressure homogenization until the desired polydispersity index and droplet size are attained (Antil & Gupta, 2021). Ultrasonication is a sonication mechanism that decreases the droplet size of nano-emulsions or microemulsions. This method is only suitable for small batches and is not applicable for large batches. The ultrasonication technique, specifically a probe sonicator, generates nano-emulsions. The duration of sonication is a crucial factor influencing the effectiveness of ultrasonication (Antil & Gupta, 2021).

Nano-scale materials can influence plant growth, development, and metabolic activity. The properties of nanomaterials, such as high surface area to volume ratio, may allow for efficient nutrient uptake by crops to maximize yield (Giri et al., 2023).

Table 6. 1 The effect of sonication on moringa leaf extract and bulking materials based on zetasizer and FESEM analyses

Moringa Extract and the Bulking Materials	Size (d.nm)	Polydispersity Index (PDI)	FESEM Image
Dry leaf extract by maceration (DM) before sonication	1115.0±19.9[a]	0.5390±0.03[ab]	
Dry leaf extract by maceration (DM) after sonication	155.2±1.1[b]	0.4787±0.06[ab]	
Dry leaf extract by decoction (DB) before sonication	567.0±55.0[a]	0.5663±0.04[a]	

Dry leaf extract by decoction (DB) after sonication	94.5±1.2[b]	0.4250±0.02[b]	
Sodium alginate 0.5% (w/v) before sonication	146±13.9	0.53±0.04	
Sodium alginate 0.5 % (w/v) after sonication	157±6.79	0.51±0.02	
Zeolite 0.3% before sonication	1142±320	0.819±0.18	
Zeolite 0.3% after sonication	611±212	0.48±0.05	

Modified starch 0.3 % w/v) before sonication	946±580	0.711±0.18	
Modified starch 0.3 % (w/v) after sonication	529±13.5	0.775±0.04	
Skimmed milk 0.1 % before sonication	357±26.5	0.38±0.05	
Skimmed milk 0.1 % after sonication	371±31.0	0.636±0.14	

6.2.2 Characterization of Nano-gel and Nano-emulsion Formulation

The nano-emulsions were characterized using physical, chemical, and structural methods: physical appearance, size, polydispersity index (PDI), zeta potential value, structural analysis using FESEM, and functional compounds using FTIR analysis. Nano-gel was characterized using physical appearance and structural analysis using FESEM.

6.2.2.1 Physical Appearance of Nano-emulsion and Nano-gel

Nano-gel-containing *Bacillus* culture looks like a soft gel due to the low concentration of sodium alginate. The physical appearance of nano-gel is slurry-like (**Fig. 6.2**). Nano-emulsion looked like a milk solution. The high-quality biological, structural, and mechanical characteristics of alginate biopolymer make it an appropriate component in the encapsulation of bacterial strains (Ghorbani & Maryam, 2021).

Only nano-emulsions that visually looked well dispersed and homogenous were subjected to zeta sizer analysis for various physicochemical parameters such as size, polydispersity index, zeta potential, and conductivity (**Fig. 6.3**). Nano-emulsions are emulsion systems that have a size range between 50 to 200 nanometers and appear transparent. They exhibit a milky appearance when the size range is up to 500 nanometers. While nano-emulsions are kinetically stable (meaning that they can remain stable for a relatively long time under standard conditions), they are thermodynamically unstable. This means the system tends to undergo destabilization phenomena over time, such as creaming, sedimentation, flocculation, coalescence, and Ostwald ripening (Romes et al., 2021). Nano-emulsions experience destabilization phenomena such as creaming, sedimentation, flocculation, coalescence, and Ostwald ripening, eventually causing phase separation (Nisha Raj, Anooj, Rajendran, & Vallinayagam, 2021). Creaming occurs when the droplets in the emulsion rise or settle due to density differences between the dispersed and continuous phases (**Fig. 6.3**). This can be slowed down by increasing the viscosity of the continuous phase or by using smaller droplets. Sedimentation occurs when the droplets settle due to gravity

(**Fig. 6.3**). This can also be slowed down by increasing the viscosity of the continuous phase or using denser droplets (Mustafa & Hussein, 2020). Flocculation occurs when droplets come into contact and stick together; forming larger clusters (**Fig. 6.3**). Emulsifiers that create a robust physical barrier around the droplets, preventing them from coming into contact, can mitigate this. Coalescence occurs when droplets merge, forming larger droplets. This can be prevented by using emulsifiers that adsorb tightly to the surface of the droplets, preventing them from coming into contact and merging. To prevent these destabilization phenomena and ensure the long-term stability of the nano-emulsion, careful selection of emulsifiers and optimization of the formulation is necessary. As shown in (**Fig. 6.3**), a homogenous milky solution indicates an optimized formulation for nano-emulsion.



Fig. 6.2 Nano-gel containing *Bacillus* sp. culture

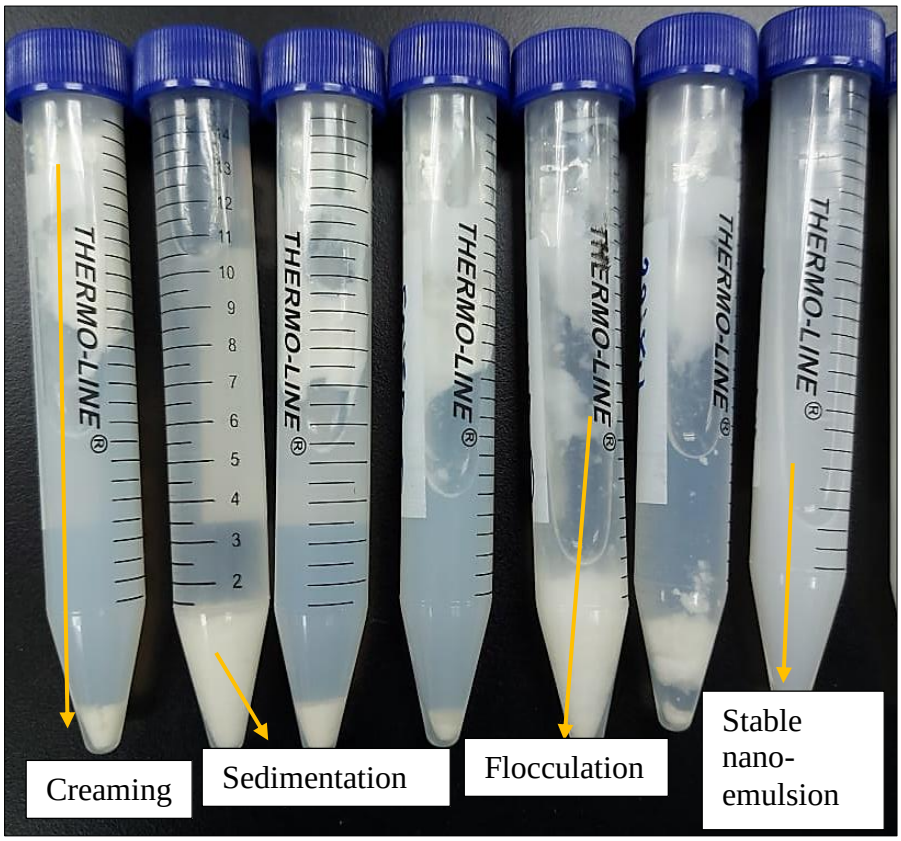


Fig. 6.3 Physical appearances that show the stability of the nano-emulsion system.

6.2.2.2 Physico-chemical Properties of Nano-emulsion Formulations

The physicochemical characterization was done for nano-emulsion formulation only and not for nano-gel. Nano-gel was formulated using sonicated raw materials in nano-size, and the endophytic bacteria were encapsulated into the nano-gel via the ionic gelation method.

Moringa leaf nano-emulsion recorded a polydispersity index below 0.5 for most formulations, which is considered uniform and stable. The range of the nano-emulsion size was between 113 d.nm and 283 d.nm. The zeta potential value ranged between 8 mV and 27 mV, while the conductivity ranged between 0.01 mS/cm and 0.4 mS/cm (**Table A11**) (**Appendix**). The nano-emulsions' polydispersity value of more than 0.5 and size value of more than 500 nm were discarded from the study.

Based on the physical appearances and physicochemical properties results, nano-formulation was selected from **Table A11** (**Appendix**) to be viewed using FESEM. The physicochemical properties of the selected formulation can be found in **Table 6.2**. The size distribution graph and zeta potential distribution graph chosen randomly for one of the samples is shown in **Fig. 6.4**.

Zeta potential value indicates the stability of the nano-emulsion formulation. Negative charge potential distribution on nano-emulsion could be due to the highly anionic behavior of sodium alginate and the least molecular weight of the non-ionic surfactant (Chawla et al., 2019). A negative zeta value induces repulsive forces more significant than the attraction forces among droplets (Mustafa & Hussein, 2020).

In sonication, an ultrasonic probe placed in a mixture of oil, water, and emulsifier generates intense, disruptive forces that form microbubbles within the mix due to cavitation effects (McClements, Das, Dhar, Nanda, & Chatterjee, 2021). In this technique, ultrasonic agitation breaks coarse droplets into nano-emulsions with good loading and release efficiency (Aswathanarayan & Vittal, 2019). The high-intensity ultrasonic waves caused rapid pressure fluctuations, which mixed the oil and water phases and converted large droplets into smaller ones (McClements et al., 2021). These nanoparticles can have improved properties, such as increased surface area and higher bioavailability, making them ideal for bio-stimulant applications. The size reduction has resulted in a larger surface area, enabling the accumulation and release of the active ingredients more effectively than their counterparts (Mustafa & Hussein, 2020). The incorporation of the active ingredient into the nanoemulsion formulations has contributed to better kinetic stability and enhances the solubility, low surface tension, and wettability that facilitates highly improved foliage adhesion where the active ingredients will be able to stay longer on leaves or other parts of the plant (Mustafa & Hussein, 2020).

Table 6.2 Physico-chemical properties of moringa leaf extract nano-emulsion

Bio-stimulant component	Size (d.nm)	Polydispersity Index (PDI)	Zeta potential (mV)
Nanoemulsion of dry moringa leaf extract (DM)	226±8.70	0.386±0.03	-38.9±1.49

*The data shown is an average of three independent readings with fifteen replications of each reading

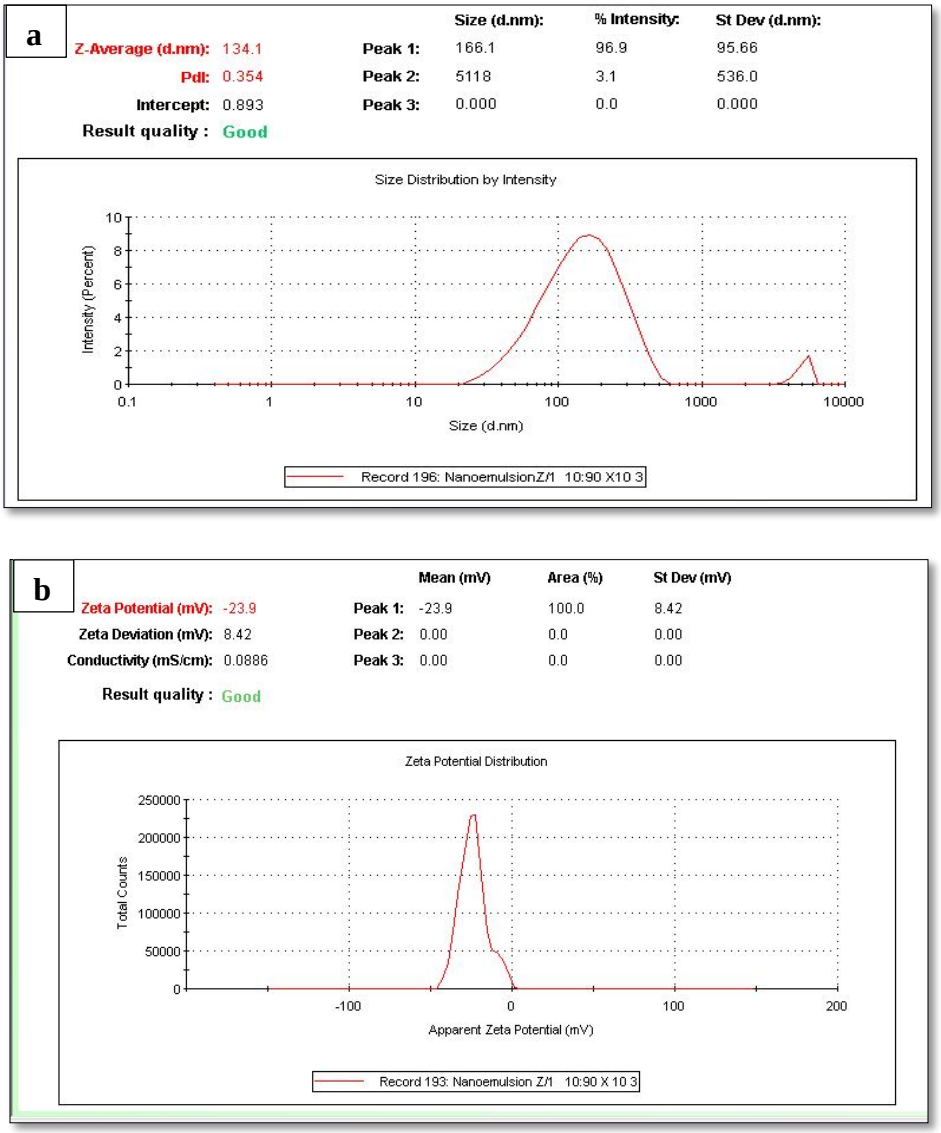


Fig. 6.4 Characterization of moringa leaf nano-emulsion. This is an example of one of the zetasizer analysis that shows (a) Size distribution graph and (b) Zeta potential distribution

6.2.2.3 Structural Image of Nano-gel and Nano-emulsion using Field Emission Scanning Electron Microscopy (FESEM) Analysis

Field Emission Scanning Electron Microscopy (FESEM) analysis revealed the distribution of bacterial cells in one drop of nano-gel (**Fig. 6.5**). Sodium alginate solution and endophytic bacteria were pre-mixed before the ionic gelation using calcium chloride solution. Thus, the bacterial cells have established throughout the nano-gel. The images depict the distribution of bacterial cells within and at the outer part of the nano-gel. Sodium alginate is a food-grade polymer widely used for encapsulating bacteria (Ghorbani & Maryam, 2021). The nano-scaled alginate could positively affect the plant and plant growth-promoting bacterial system via various mechanisms. One of the mechanisms is by increasing cell viability and bacterial growth rate by providing a high surface area and protection of inoculants in the field when applied as the carrier (de Moraes, Ribeiro, de Camargo, & Lacava, 2021).

The FESEM images showed that the moringa leaf extract could be well incorporated into a nano-formulation (**Fig. 6.5 and 6.6**). The difference in nanoemulsion formulation can be observed in FESEM images. For example, **Fig. 6.5** showed a well-dispersed oil-in-water droplets when oil and moringa leaf extract was mixed in a ratio of 1:10 in the oil phase, and 10% (v/v) oil phase was mixed with 90% (v/v) aqueous phase containing 5% (v/v) emulsifier. The nanoemulsion droplets looked clumped together and formed clusters when the 10% (v/v) oil phase was mixed with the 80% (v/v) aqueous phase containing 10% (v/v) emulsifier (**Fig. 6.6**).

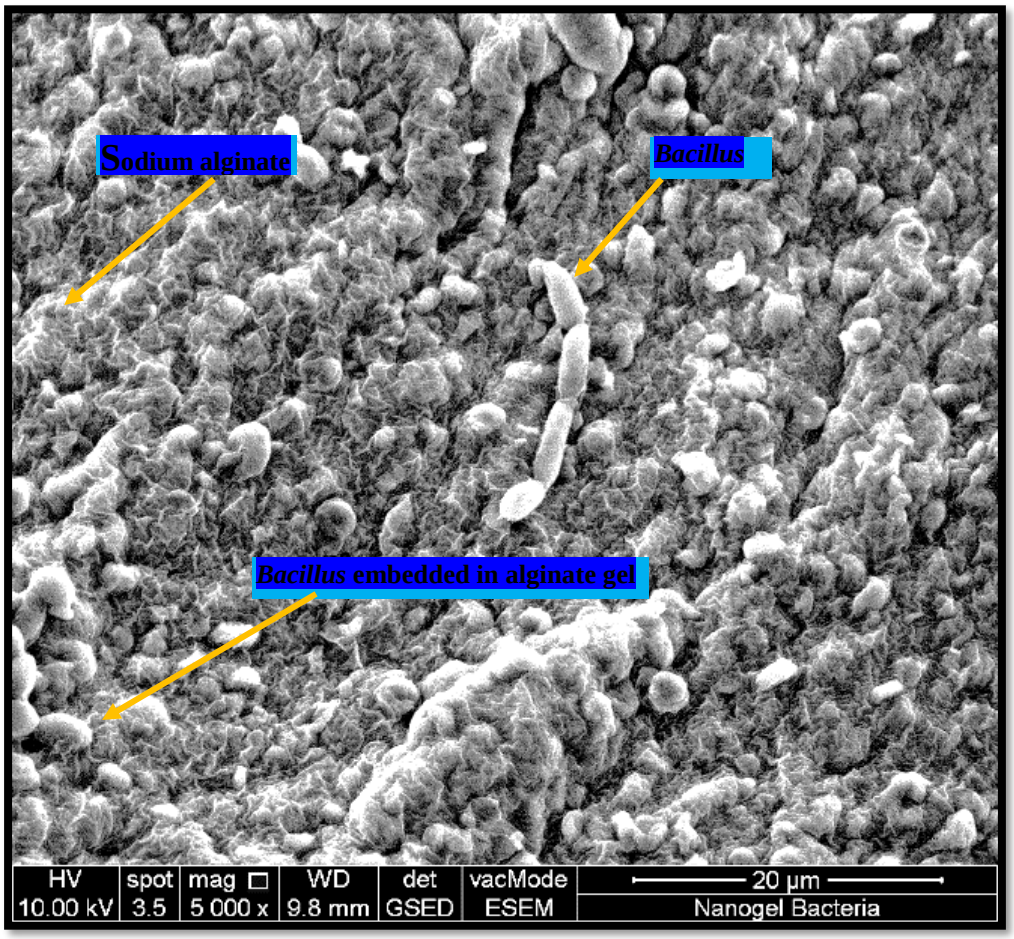


Fig. 6.5 Distribution of *Bacillus* cells on nano-gel using FESEM imaging (5000 x magnification). The bacterial cells were at the surface of the nano-gel and covered by the alginate polymer.

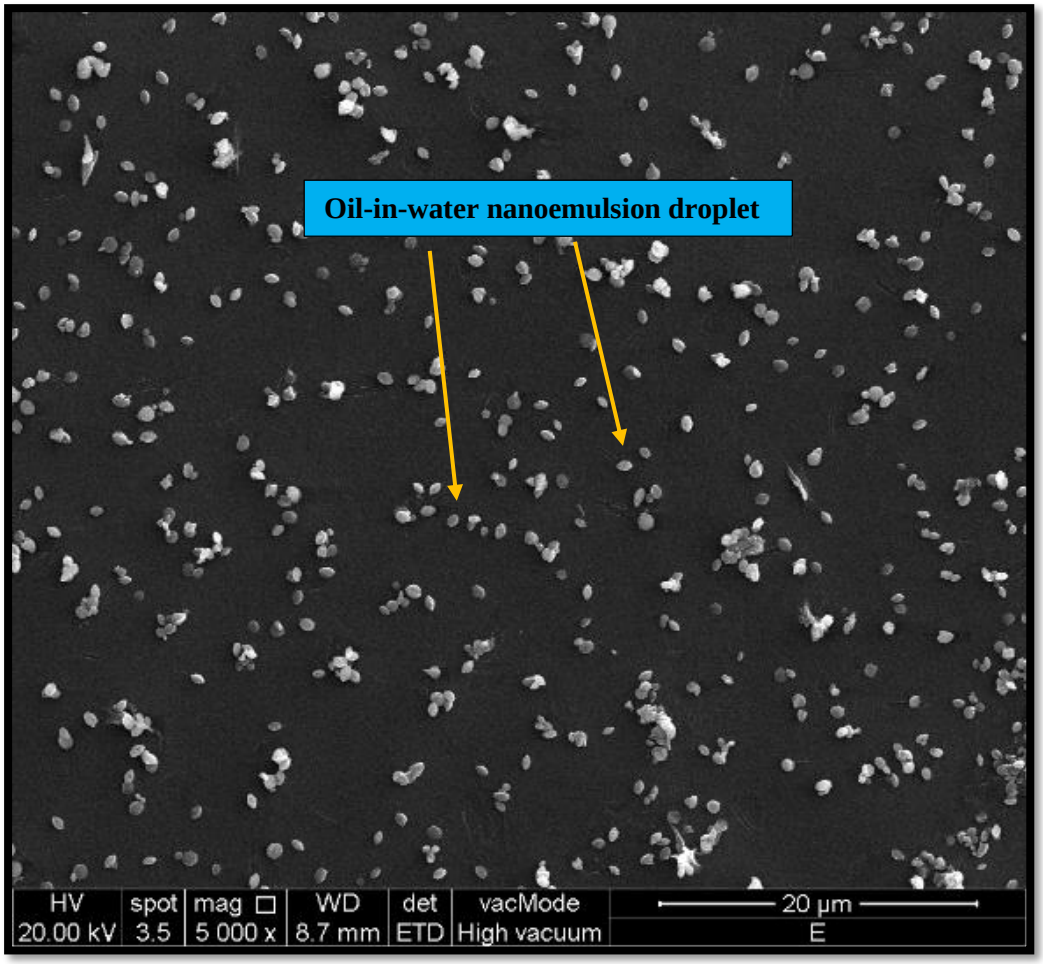


Fig. 6.6 A well-dispersed oil-in-water nano-emulsion of moringa leaf extract (5,000 x magnification)

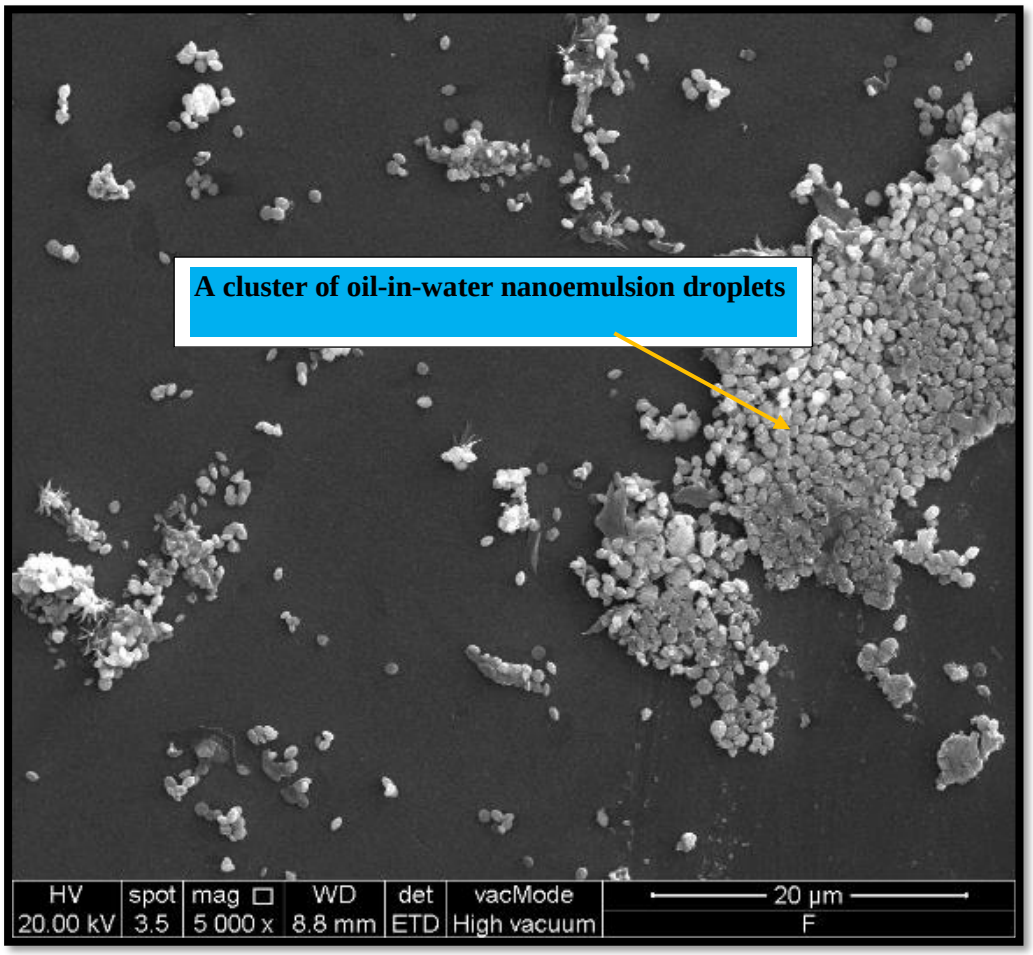


Fig. 6.7 Oil-in-water nano-emulsion of moringa leaf extract formed clusters (5,000 x magnification)

6.2.2.4 Functional Compounds Detection in Nano-emulsion

The changes in the chemical composition or structure of the emulsion were determined by the changes in the Fourier-transform infrared spectroscopy (FT-IR) analysis. **Table 6.3** shows the changes in the absorption frequencies of the functional groups of moringa leaf extract in the raw and nanoemulsion forms. **Fig. 6.8** shows the FT-IR spectrum that depicts the functional component changes from raw moringa leaf extract to nanoemulsion of moringa leaf.

The most intense absorption Amide I band is the primary band in proteins. It is associated with the C=O stretching vibrations of the peptide bonds. Its frequency was found in the range between 1600 and 1700 cm^{-1}. The characteristic 3550–

3200 cm^{-1} peak was due to O–H stretching. The region between 600 cm^{-1} and 1500 cm^{-1} is known as the fingerprint region, which contains many unique peaks (Vaishnavi and Preetha, 2021). Herein, a huge peak at 3339 cm^{-1} confirmed the vibrational stretching of the nano-emulsion's polymeric–OH group, mainly attributed to the water phase and phenolic groups present in the nano-emulsion (Chawla et al., 2019).

Table 6.3 Fourier-Transform Infrared Spectroscopy (FTIR) analysis on the changes in the functional groups of moringa leaf extract and nano-emulsion of moringa leaf extract

Dry moringa leaf extract			**Dry moringa leaf nano-emulsion**		
Absorption frequency (cm^{-1})	**Transmittance (%)**	**Functional group**	**Absorption frequency (cm^{-1})**	**Transmittance (%)**	**Functional group**
3261.44	42.39	Amines (N-H stretch)	3289.04	91.09	Amines (N-H stretch)
2177.17	95.97	Alkynes (C≡C stretch)	3009.57	93.42	Alkenes (C-H stretch)
2157.69	95.58	Alkynes (C≡C stretch)	2923.17	72.12	Alkanes (C-H stretch)
2137.89	96.25	Alkynes (C≡C stretch)	2853.82	79	Alkanes (C-H stretch)C-H
2117.28	96.45	Alkynes (C≡C stretch)	1743.57	71.39	Ketones (C=O stretch)
2036.13	96.18	Alkynes (C≡C stretch)	1648.82	94.96	Amides (C=O stretch)
1634.39	68.39	Flavanoids (C=O stretch)	1461.77	88.23	Aromatic compounds (c=c stretch)
1391.52	93.92	C-H bending vibrations in alkanes	1378.93	94.81	C-H bending vibration in alkanes
1306.86	94.17	C-O stretching vibrations in alcohols and phenols	1241.41	89.98	C-C stretching vibrations in aromatic compounds
631.68	81.76	Sulfur containing compounds (S-H	1160.26	78.17	Silicon-containing compounds (Si-O-Si

		stretch, C-S stretch)			stretch, Si-O-C stretch)
612.18	81.11	Phosphorus-containing compounds (P-O stretch, P-C stretch)	1098.84	87.04	Silicon-containing compounds (Si-O-Si stretch, Si-O-C stretch)
593.15	80.64	Phosphorus-containing compounds (P-O stretch, P-C stretch)	720.62	80.89	C-H bending vibrations in alkynes (C≡C)
573.64	81.15	Phosphorus-containing compounds (P-O stretch, P-C stretch)	613.51	91.32	Phosphorus-containing compounds (P-O stretch, P-C stretch)

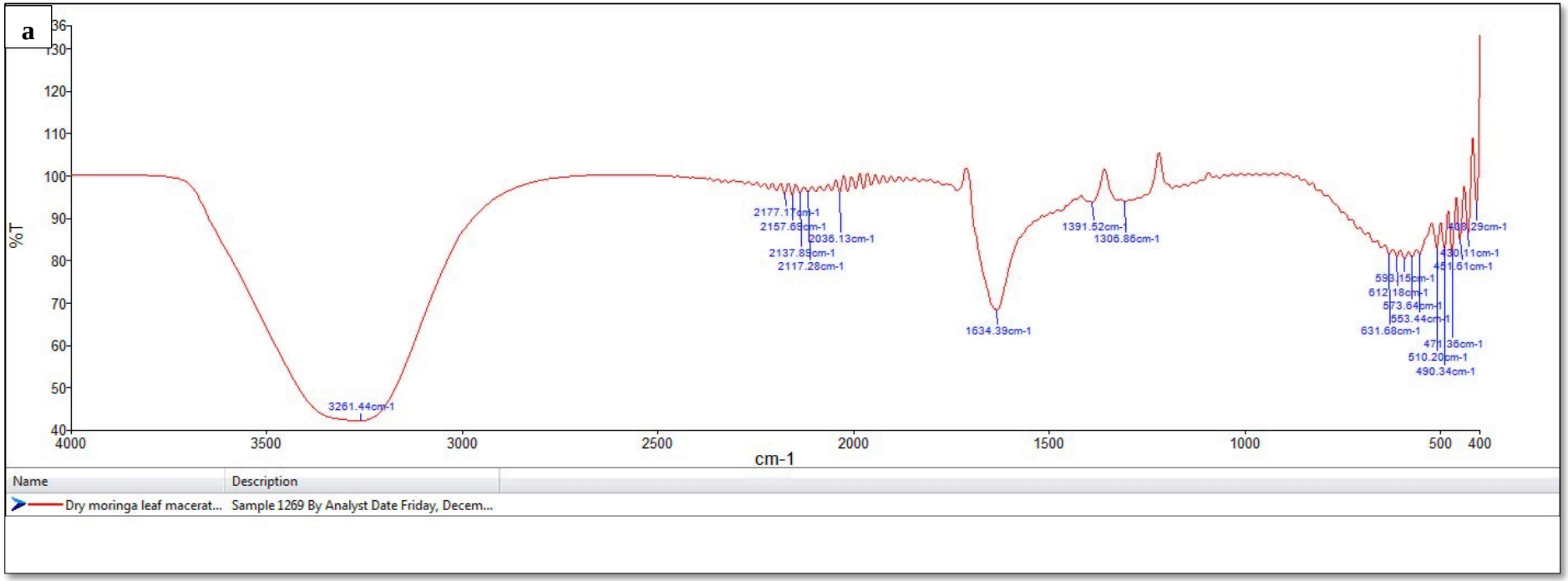


Fig. 6.8 FTIR spectra shows the difference in the functional groups of raw moringa leaf extract and nano-emulsion of moringa leaf extract (a) moringa leaf extract by maceration (DM) and (b) nano-emulsion of moringa leaf extract

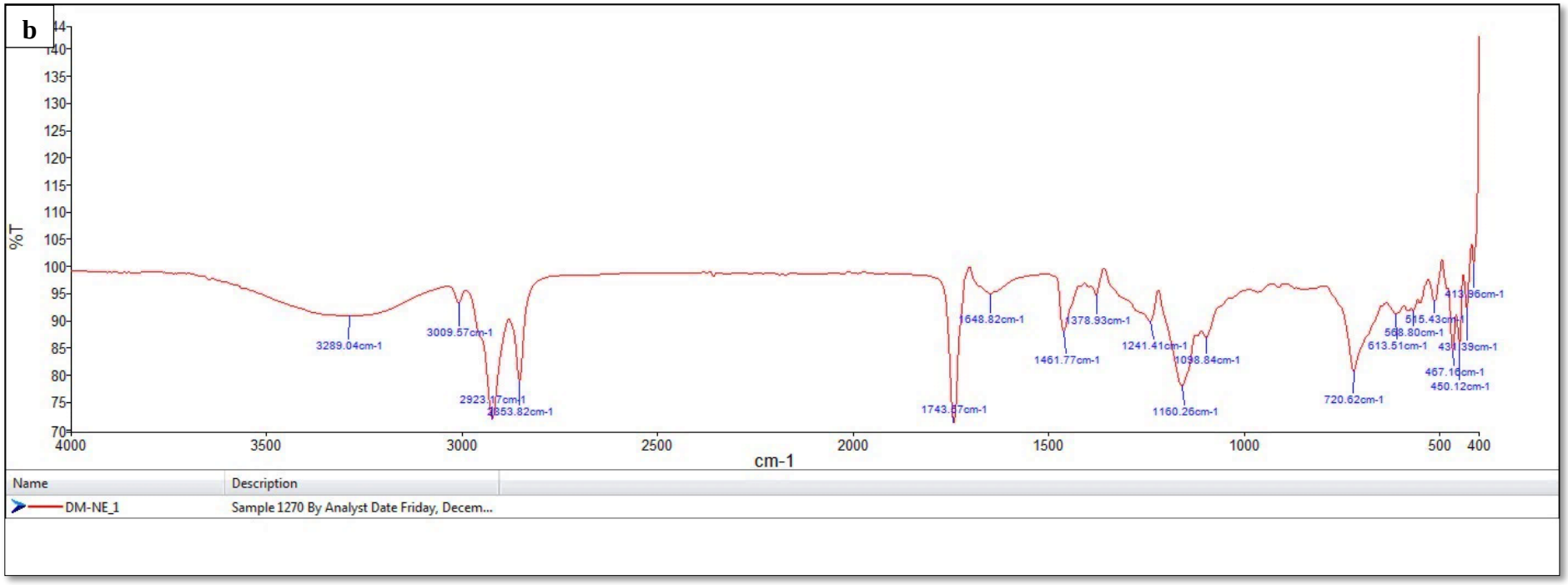


Fig. 6.8 FTIR spectra shows the difference in the functional groups of raw moringa leaf extract and nano-emulsion of moringa leaf extract (a) moringa leaf extract by maceration (DM) and (b) nano-emulsion of moringa leaf extract

6.2.3 Assessment of the Stability of Nano-gel and Nano-emulsion Formulation during Storage

The stability of nano-gel was assessed based on the shelf life of bacteria encapsulated in nano-gel over six months and their efficiency in enhancing chilli seed germination and the length of the radicles.

Nanoemulsion stability was assessed based on the physical appearance, physicochemical properties, functional compounds, and the effect to enhance chilli seed germination and the length of the radicles.

6.2.3.1 Physico-chemical Properties of Nano-encapsulation Formulations during Storage

Physico-chemical properties were evaluated for moringa leaf extract (raw plant extract), nanoemulsion of moringa leaf extract (day 0 and day 60), and moringa leaf nanoemulsion mixed with *Bacillus* sp. (day 0 and day 60) (**Table 6.4**).

The raw moringa leaf extract size was 1115 nm and was stable for 60 days, where the size was 1197 nm. The polydispersity index and zeta potential value were also stable for 60 days.

The size of the moringa leaf nanoemulsion slightly reduced from 134 nm to 119 nm after 60 days. The polydispersity index showed that the nanoemulsion system became uniform and stable over time, where the PDI value dropped from 0.35 to 0.18 in 60 days storage period. Zeta potential value increased from -23.9 mV to -33.9 mV, which indicates a stable nanoemulsion system. A similar observation was reported where the zeta potential value changed from -30 mV to -40 mV over 90 days, demonstrating the optimal nanoemulsion stability to overcome destabilization phenomena through repulsive forces formed between negatively charged nano-sized water droplets (Nisha Raj et al., 2021). Another comparable result was reported by (Vaishanavi & Preetha, 2021) where the size of the nanoemulsion gradually increased over 40 days, and the surface charge increased from ±32 mV to ±40mV. A higher magnitude of zeta potential

indicates stronger electrostatic repulsion, essential for maintaining nano-emulsions' stability. The electrical charge present on the surface of the emulsion droplets plays a significant role in determining their interaction and, consequently, the stability of the nano-emulsion. Nano-emulsions with a high zeta potential, typically exceeding 30 and with either a negative or a positive charge, are considered electrically stable. Conversely, nano-emulsions with a low zeta potential tend to undergo coagulation (Vaishanavi & Preetha, 2021). Zeta potential value is related to the ionic charges produced by the substance.

Table 6.4 Physico-chemical stability of nano-emulsion formulations

Formulation	Size (d.nm) (1st day)	Size (d.nm) (60 days)	PDI (0 day)	PDI (60 days)	Zeta potential (mV) (1st day)	Zeta potential (mV) (60 days)
Raw moringa leaf extract	1115±16.3	1197±19.2	0.539±0.02	0.50±0.02	-23.5±0.69	-25.9±0.32
Moringa leaf nano-emulsion	134±1.97	119±0.73	0.35±0.00	0.189±0.01	-23.9±0.20	-33.9±0.68

6.2.3.2 Functional Groups Determination in Nano-emulsion After Six Months of Storage

The stability of the nano-emulsion was also verified after two months of shelf life. **Fig. 6.9** depicts the FTIR spectrum of fresh moringa leaf nano-emulsion after two months of shelf life. No changes were observed in the fresh moringa leaf nano-emulsion functional groups after two months of shelf life. The major functional groups, such as amines (3271 cm^{-1}), alkynes (2115-2124 cm^{-1}), and flavonoids (1635-1637 cm^{-1}), remained the same.

The most intense absorption Amide I band is the primary band in proteins. It is associated with the C=O stretching vibrations of the peptide bonds. Its frequency was found in the range between 1600 and 1700 cm^{-1}. The characteristic 3550–3200 cm^{-1} peak was due to O–H stretching. The region between 600 cm^{-1} and 1500 cm^{-1} is known as the fingerprint region, which contains many unique peaks (Vaishanavi & Preetha, 2021). Herein, a huge peak at 3339 cm^{-1} confirmed the vibrational stretching of the polymeric–OH group of the nano-emulsion which was mainly attributed to the water phase and phenolic groups present in the nanoemulsion (Chawla et al., 2019).

Moringa leaf contain various organic compounds, including flavonoids, alkaloids, phenolic acids, terpenoids, and glycosides. Fourier transform infrared spectroscopy (FTIR) is a proper analytical technique for analyzing the composition and structure of nano-emulsions. FTIR spectroscopy analysis identified the functional groups present in the water extract of moringa leaves. It could provide information on the chemical composition and stability of the nanoemulsion.

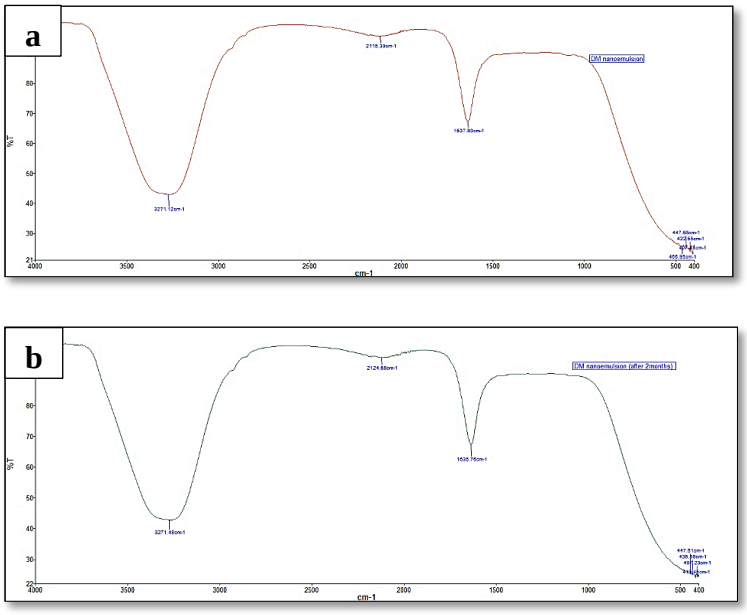


Fig. 6.9 FTIR spectra show the stability of moringa leaf nano-emulsion after two months of storage (a) Moringa leaf extract (b) Nano-emulsion of moringa leaf

6.2.3.3 Shelf-life of Encapsulated Endophytic Bacteria in Nano-gel

The shelf life of bacteria in nano-gel after six months of storage was determined using total plate count. It was found that nano-gel formulation could sustain high and stable bacterial density, which is about 1 x 10^8 cfu/ml (**Table 6.5**). The bacterial shelf life was proven durable with the formulation in alginate nano-gel. The formulation of a product is crucial in maintaining the microbial populations and determining its efficacy and marketability. It emphasizes the significance of production and storage conditions in fertilizer production technology and biocontrol agents. Developing new practical formulations is vital in advancing agriculture (Saberi-Riseh & Moradi-Pour, 2021).

Plant growth-promoting bacteria that have been commercialized may not coincide with the expected performance of plants due to problems concerning their stability in soil, field application, during storage, transport, and delivery systems (de Moraes et al., 2021). Bio-fertilizers with short shelf life possess the risk of product deterioration (Basu et al., 2021). Encapsulation of bacteria has

emerged as an effective formulation that is gaining recognition across various scientific fields, including agriculture. This formulation involves trapping the microbial agents, which are then released slowly, enhancing their chances of survival even in harsh environmental conditions. Consequently, this increases their effectiveness by boosting the colonization percentage. There are various methods of encapsulation, including extrusion, emulsion, and spray drying, each employing different polymers for multiple applications, such as alginate, gelatin, gum arabic, xanthan, gellan gum, whey protein, chitosan, and starch, among others (Saberi-Riseh et al., 2021). A recent study has demonstrated that hydrotalcite and alginate served as excellent packaging to keep the bacteria alive, providing nutrients and controlling their delivery (Didier et al., 2022).

6.2.3.4 Effect of Nano-encapsulation Formulation on Chilli Seed Germination and the Length of Root

The effect of nano-gel and nano-emulsion formulations was observed in chilli seed germination to ensure the process does not cause inhibition to seed germination (**Table 6.5**). Both moringa leaf extract and *Bacillus* sp. could enhance seed germination 100% before and after nano-formulation. However, the radicle growth showed various effects on the nano-formulation. Generally, moringa leaf extract could enhance the radicle growth better than the endophytic bacteria due to the high flavonoid content found in the moringa leaf extract. Both moringa leaf extract and endophytic bacteria were effective in enhancing the radicle growth compared to the control, which was treated with water. The nano-formulations of moringa leaf and endophytic bacteria showed a better effect on the radicle growth than pure bacterial culture and raw moringa leaf extract. Nano-gel could enhance the development of radicles better than the pure culture of bacteria as it was formulated with alginate gel, modified starch, skimmed milk, and zeolite, which could have contributed to the enhanced growth of radicles. On the other hand, moringa leaf extract tested on the chilli seeds was prepared fresh and could show better enhancement of the radicle growth. In actual circumstances, the active ingredients of moringa leaf could degrade over time due to high temperatures. Therefore, the nano-emulsion of moringa leaf extract could preserve the phytochemical activity and protect the active

ingredient from rapid degradation. Moringa leaves have a high content of carotenoids such as lutein, zeaxanthin, and β-carotene (Batra et al., 2017). The leaf extract, rich in antioxidant compounds, phytohormones, and nutrients, must be packaged in a nanoemulsion system for enhanced stability and longer shelf life.

Table 6.5 Shelf life of the nano- formulation and its efficiency in stimulating seed germination and the root length of chilli

Formulation	Seed germination (%)	Root length (cm)	TPC (cfu/mL) (Day 1)	TPC (cfu/mL) (180 days)
Moringa leaf extract	100	5.75±0.5	N/A	N/A
Moringa leaf nano-emulsion	100	6.4±1.31	N/A	N/A
***Bacillus* sp.**	100	4.85 ±0.39	$1x10^{8}$	$1x10^{8}$
Nano-gel containing *Bacillus* culture with x 100 dilution	100	5.25 ±0.25	$1x10^{8}$	$1x10^{8}$
Control (Distilled water)	100	4.55±0.85	N/A	N/A

6.3 Conclusion

A suitable formulation is essential to sustain the density and viability of bacteria. At the same time, bio-stimulant containing plant extract and endophytic bacteria could not be applied together if it has no compatibility, which will cause low efficiency in field application. In this study, bio-stimulant containing endophytic bacteria and moringa leaf extract were proven to promote the growth and yield of chilli. However, with a suitable formulation, the viability and efficiency of the bacteria and plant extract could be maintained. Furthermore, a combined application of these two bio-stimulants via the drenching method has to be encapsulated to minimize interaction that could reduce the efficiency of the bio-stimulants. Thus, this chapter focuses on nano-formulation that is not only expected to protect the active ingredients from degradation and environmental loss but also increase the absorption by plant cells.

Two nano-formulations were selected from the experiments to deliver moringa leaf extract and endophytic bacteria into the soilless medium, cocopeat, for the enhanced growth and yield of chilli. Endophytic bacteria were prepared in nano-gel, whereas moringa leaf extract was prepared in nano-emulsion. Both formulations were proven to be non-toxic for seed germination and could enhance the radicle growth of chilli. Nano-gel was also ideal for sustaining a high bacterial population for six months. Both nano-formulations were prepared using cheap and natural materials with simplified processes to ensure a practical and economic agronomic practice in the future.

CHAPTER 7
EFFECT OF NANO-ENCAPSULATED BIO-STIMULANTS ON YIELD AND POST-HARVEST QUALITY OF CHILLI

7.0 Introduction

Incorporating nano-encapsulated bio-stimulants, which enhance crop health and productivity, has emerged as a promising biotechnological strategy for crop cultivation in a soilless medium. The conventional methods using soil as the planting medium have caused many soil-borne diseases and declined soil fertility due to heavy metal toxicity, drought, salinity, and acidity. In recent years, soilless agriculture is grown in popularity among commercial farmers, where hydroponics involves the direct provision of nutrient solutions in a liquid medium. In contrast, the solid medium includes growing plants in peat, perlite, vermiculite, coco peat, rock wool, sand, and sawdust enriched with nutrient solutions (Azizoglu et al., 2021). By manipulating the microbial community in the rhizosphere of the soilless systems, plant nutrition could be increased in addition to reducing the plant stress response and pathogen attack (Edmonds et al., 2020). One of the standard methods is by using plant-growth-promoting rhizobacteria (PGPR). Root colonizing PGPR have been used in soil and soilless crop cultivation systems due to their positive effects on nutrient uptake, plant stress control, and antagonism with pathogens (Balliu et al., 2021). Likewise, the application of bio-stimulant made from plant extracts could boost the growth and yield of crops. Moringa leaf extract was regarded as plant-based and a cheap source of natural plant growth enhancers (I. Afzal et al., 2020; D. S. Arora & Kaur, 2019; Refai et al., 2023).

Bio-stimulants can improve the quality of crops by promoting the accumulation of essential nutrients, such as vitamins and minerals, and enhancing the flavor and aroma of fruits and vegetables. This can improve the overall quality of crops, making them more desirable to consumers. Associating bio-stimulant application with crop productivity has been a common way of evaluating the performance of bio-stimulant on various crops under different management practices. Very few studies have investigated bio-stimulants efficiency on fruit

quality and the influence of bio-stimulants on the post-harvest quality of fruits (Rodrigues et al., 2020). Bio-stimulants actively promote final crop quality traits and the concentration of secondary metabolites such as phenols, flavonoids, and ascorbic acids (Karabulut et al., 2021). Chilli has high phytochemicals, including carotenoids, capsaicinoids, polyphenols, and ascorbic acid, at the red ripening stage (Pola et al., 2020). The carotenoid content in chilli could be analyzed to determine the effectiveness of bio-stimulant as carotenoids have excellent scavenging activity for reactive oxygen species.

A considerable knowledge gap must be addressed to facilitate the use of PGPR for sustainable soilless agriculture, as there need to be more understanding of the interaction between soilless agriculture and PGPR (Azizoglu et al., 2021). Furthermore, no studies have been done to investigate the effect of the co-application of nano-encapsulated microbial and botanical bio-stimulants on the yield and post-harvest quality of the product, especially crops grown in a soilless medium. This study also has focussed on the microbiome changes that occurred within the chilli roots due to the co-application of nano-encapsulated bio-stimulant application, particularly nano-gel containing *Bacillus* and *Streptomyces* and nano-emulsion of moringa leaf extract that were developed as described in the previous chapter. The objectives of this study are as follows.

i) Evaluation of the synergistic effects of nano-encapsulated bio-stimulants on the vegetative growth and yield of chilli cultivated in soilless growth medium
ii) Evaluation of the synergistic effects of nano-encapsulated bio-stimulants on the post-harvest quality of chilli fruits
iii) Evaluation of the synergistic effects of nano-encapsulated bio-stimulants on chilli root microbiome
iv) Evaluation of the synergistic effects of nano-encapsulated bio-stimulants on nutrient absorption by chilli plants

7.1 Material and Methods

7.1.1 Nano-encapsulated Bio-stimulant Preparation

Bacillus and *Streptomyces* (MR3 and MR13) were freshly grown in potato dextrose agar (PDA, Merck, Germany). One loop-full of each bacterial colony was transferred into 500 mL of tryptic soy broth (TSB, Merck, Germany) separately and incubated in an incubator shaker (Wisecube, Faculty of Science & Engineering, University of Nottingham, Malaysia) at room temperature for three days at 90 rpm. *Streptomyces* was grown on PDA for more than two weeks to produce spores. The spores were collected by scrapping the agar medium using 0.1% Tween 80. The spores were collected in 1 L 0.1% (w/v) of Tween 80 in distilled water. *Bacillus* and *Streptomyces* grown in tryptic soy agar (TSA, Merck Germany) were centrifuged at 4,000 rpm for 15 min, and the cell pellets collected from 1L of TSA were mixed in 1 L sterile distilled water that was used as the stock culture. The stock culture was diluted to ensure that the density of endophytic bacteria was within the range of 10^7-10^9 CFU/ml (bacterial density was checked using the total plate count method). Sodium alginate (Sisco Research Laboratories, Pvt. Ltd., India) was prepared at 0.5% (w/v) in one liter of distilled water and autoclaved at 121°C for 30 minutes. Modified cornstarch (0.1 g), zeolite (0.1 g), and skimmed milk (0.1 g) were added to the sodium alginate solution before autoclaving. Calcium chloride dihydrate, 0.5 M, was autoclaved separately and kept at 10°C until use. The stock bacterial culture and spore solution was mixed with sodium alginate at 10% concentration using a magnetic stirrer for 10 min. A plastic syringe was used to extrude the bacterial-alginate mixture into a calcium chloride solution under stirring conditions. The encapsulated bacteria and spores in alginate gel were left overnight at room temperature for a stable gel formation. The nano-gel was washed thrice with distilled water. Then, excess water was removed by straining the gel, and the nano-gel was collected in a clean container before storing it at 10°C.

For moringa leaf extract nano-emulsion, 10% (w/v) of dry moringa leaf was macerated with sterile distilled water in a blender and left at room temperature overnight. The extract was collected by filtering the extract using a muslin cloth.

The extract was diluted 10^{-1} and mixed with vegetable oil at a ratio of 1:5 (oil: moringa extract). The oil phase was combined in a magnetic stirrer at 50°C for 15 min. Then, 1% of the surfactant with an HLB value of 10 was added dropwise while stirring for 30 min. The oil phase-surfactant mixture was homogenized at 10 000 rpm for 2 min and diluted with 90% of the aqueous phase of sterile distilled water. The same process was used for dry moringa leaf extract obtained via decoction to prepare oil-in-water nanoemulsion.

7.1.2 The effect of Co-application of Nano-encapsulated Endophytic Bacteria and Moringa Leaf Extract in Soilless Cultivation System

SJ2-461, F1 hybrid hot chilli seeds were purchased from SAKATA Corp. Chilli seeds were soaked in water overnight before sowing into wet peat moss. The polybags were covered with black plastic sheets and kept at room temperature in a dark room until germination. Once the radicle was visible, the black sheets were removed, and the polybags were kept in a glasshouse under shade until true leaves emerged. Peat moss was sprayed with water every two days once or as when required. After the 14th day of sowing, fertilizer AB with an EC reading of 0.9 mS/cm was poured into a tray, and the seedling tray was placed on it for maximum absorption of nutrients. Transplanting of seedlings was done 35 days after seed germination. The planting medium, cocoa peat was used in a 16" x 16" cm polybag. A completely randomized block design was adopted for this experiment (**Fig. A12**) (**Appendix**). There were two treatments with five replications of each treatment. Each replication consists of eight plants. The polybags were arranged in a single-line design with a distance of 1 foot between polybags and 3 feet between rows. Ten milliliters of the stock nanoemulsion was diluted with 1 L water (X100 dilution) before application, while endophytic bacteria were encapsulated as alginate nano-gel.

Combined application of bio-stimulants was carried out once during seedling transplanting and every two weeks once at the rate of 50g of encapsulated bacteria (*Bacillus* and *Streptomyces*) and 100 mL of moringa leaf nanoemulsion per plant until the termination of the experiment. The nano-gel and

nanoemulsion were combined before applying them to the soilless growth medium. The details of the bio-stimulant treatment are shown in **Table 7.1**.

Table 7.1 Nano-encapsulated bio-stimulant treatments for the cultivation of chilli in soilless growth medium

Type of bio-stimulant	Components in bio-stimulant	Dosage of application	Time of application	Method of application
Moringa leaf nano-emulsion	Decoction extract of dry moringa leaf	100 mL/plant Once every two weeks	During transplanting and Vegetative growth stage (Week 1- 8 after transplanting)	Drenching of soilless growth medium
Moringa leaf nano-emulsion	Maceration extract of dry moringa leaf	100 mL/plant Once every two weeks	Flowering and fruiting stage (Week 9-12 after transplanting)	Drenching of soilless growth medium
Nano-gel of endophytic bacteria	*Bacillus* sp.	50g/plant Once every two weeks	During transplanting and throughout the experiment	Drenching of soilless growth medium
Nano-gel of endophytic bacteria	*Streptomyces* spores	50g/plant Once every two weeks	During transplanting and throughout the experiment	Drenching of soilless growth medium

The independent variable is the liquid mineral fertilizer (AB fertilizer) doses, and the dependent variable is the bio-stimulant treatment. AB contains balanced

nutrients that are water-soluble. Fertilizers A and B were dissolved in 10 L of clean water separately. Then, an equal amount of the nutrient solution was mixed with water to achieve the desired EC (mS/cm) level suggested for chilli production according to its growth stage (manual by the Department of Agriculture, Malaysia), as described in **Table A13 (Appendix)**. Chilli plants were watered via a fertigation system five times a day with a maximum water supply of 2.0 L per pot and a minimum of 900 ml per pot/per day. The water supply was adjusted following the weather conditions. To avoid pathogenic or insect infestation, neem oil (2 teaspoons) was mixed in 1 gallon of water and a few drops of soap. It was sprayed on all plants every week. The experiment was terminated 90 days after transplanting.

7.1.3 The Effect of Nano-encapsulated Bio-stimulants on the Photosynthesis Activity of Chilli Plants

The photosynthesis activity of chilli plants was measured using the dark-adapted method on live plants at 25 days, 55 days, and 85 days after transplanting. Fluorpen-100 was used to study the photosynthetic activity by measuring the quantum yield (QY) in a dark-adapted leaf (**Fig. 7.1**). Eighteen plants were selected from each treatment for this analysis. Five fully expanded leaves of each plant were covered with aluminium foil for 20 min before taking the QY reading using a Fluorpen-100.



Fig. 7.1 Dark adaptation method for quantum yield (QY) measurement to determine photosynthesis activity of chilli plants

7.1.4 The Effect of Co-application of Nano-encapsulated Bio-stimulants on the Vegetative Growth of Chilli

At 90 days after transplanting, vegetative growth analysis, such as root length, shoot length, root biomass, and aboveground biomass, were measured. Plant height was measured above the soil level until the shoot tip using a measuring tape. Then, the plants were uprooted, and the roots were separated from the plants and washed thoroughly before scanning using a root image scanner (Winrhizo) to measure the length of the roots and total root surface area. Then, the plant's leaves, roots, and stems were partitioned separately and placed in an oven for three days at 60°C.

7.1.5 The Effect of Nano-encapsulated Bio-stimulants on Chilli Length, Color, and Yield

Chilli fruits were harvested from day 65 until day 90 at a maturity index of 4, 5, and 6 to determine the total yield. The Department of Agriculture, Malaysia, has illustrated the six-maturity index of chilli as described in **Table 7.2.**

Table 7.2 Maturity index of chilli fruits

Maturity index	Color	Image
1	Light green	
2	Dark green and shiny	
3	Dual colour (Green mixed with red)	

4	Reddish and mixed with green colour	
5	Bright red	
6	Blackish red and sometimes shrinked skin	

Matured chilli fruits that have attained stage two (green color) and stage five (red color) were harvested after 19 and 27 days and washed with clean water. The collected chillies were packed separately in a zipped vacuum bag and kept at 10°C until further analysis.

The chilli length was measured, and the mean value was recorded for 50 fruits harvested simultaneously. Chilli fruits of both treatments were collected, and the weight was recorded separately from day 65-90 to calculate the total yield of chilli plants and the mean yield per plant.

The color value was measured using a colorimeter (Hunter Lab) in terms of L, a, and b values where L represents the degree of lightness, a value denotes the

degree of redness or greenness, and b value represents the degree of yellowness or blueness. The intensity and representation of color are measured by chroma (C) and are calculated by ((a*) 2 + (b*) 2) 1/2.

7.1.6 The Effect of Nano-encapsulated Bio-stimulants on Phenolic and Flavonoid Contents in Chilli Fruits

Chillies of uniform weight were sampled for other phytochemical and nutrient analyses. Fruits were separated into three parts: pericarp, placenta, and seeds, using a clean scalpel. Pericarps of five fruits were collected and made into a composite sample from each treatment and replication. Harvested fruits were dried at 50°C in an oven for 24-72h, ground into a fine powder, and kept at RT in an airtight container before extraction (Pandey, Deendayal, Pandey, Pant, & Ahmed, 2010).

Dried chilli pericarps, 0.05 g, was extracted with 4 ml of 80% ethanol in water. It was left in an ultrasonic bath at 60°C for 1 h. The samples were let to cool down and centrifuged at 8000 rpm for 5 min. The supernatant was filter sterilized with a 0.25 µm filter membrane. Total phenolics were estimated with the modified Folin–Ciocalteu colorimetric method (Kopta, Sekara, Pokluda, Ferby, & Caruso, 2020).

The supernatant (0.2 ml) was mixed with 0.2 ml of Folin–Ciocalteu's reagent (1:1 v/v) and 0.6 ml of deionized water for phenol content. After 5 min, 1 ml of sodium carbonate was added to the test tubes and kept in the dark for 1 h. The absorbance of the resulting blue color was measured at 765 nm using a UV-VIS Ultraviolet-visible spectroscopy) Helios Beta spectrophotometer (Waltham, MA, USA) against a reference solution (**Fig. 7.2**). The results were expressed as gallic acid equivalents (GAE), mg GAE per 1 g fresh weight. Standard gallic acid was prepared by mixing 1g of gallic acid with 10 ml methanol and kept in a dark vial at 4°C (**Fig. A9**) (**Appendix**).

For flavonoid analysis, 500-µl supernatant was mixed with 150 µl of 5% sodium nitrate and 2 ml of distilled water. After 5 min, 150µl of Al_2Cl_3 was added and

left for 1 min. Then 2 ml of NaOH and 1.2ml of distilled water was added to the mixture and incubated for 30 min at RT, and the absorbance was read at 510 nm (**Fig. 7.8**). The results were calculated using the standard calibration curve of quercetin in methanol and expressed as quercetin equivalents (mg/g) (**Fig. A10**) (**Appendix**).

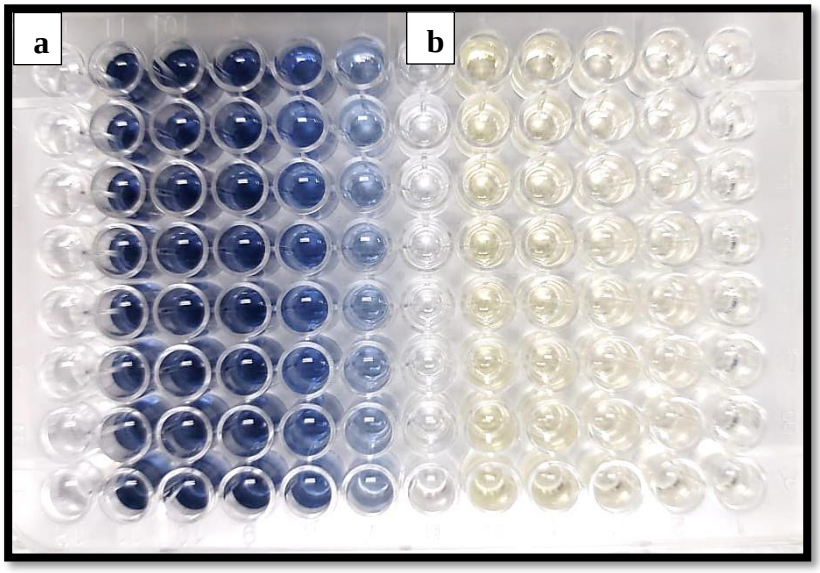


Fig. 7.2 Phytochemical analysis in chilli fruits. (a) Phenolic content;
(b) Flavonoid content

7.1.7 The Effect of Nano-encapsulated Bio-stimulant on the Capsaicin Content in Chilli Fruits

Standard capsaicin (purity 99%) was obtained from Sigma Aldrich. Acetonitrile and methanol of HPLC grade and HPLC-grade water /double distilled water were used. Other chemicals used were of analytical grade.

A 1 g powder sample was dissolved in 10 mL of acetonitrile for capsaicin extraction with two hours of shaking on a shaker. After that, the flask was left overnight at room temperature. The supernatant was filtered with Whatman filter paper No.1, followed by 0.45 µm Millipore membrane, and with a 10 mL disposable syringe into a 1 mL glass vial. The volume of injection in HPLC was taken at 20 µL.

The standard solution of capsaicin (Sigma Chemicals Co. M2028) was prepared as 50-ppm stocks in absolute ethanol and used for retention time verification and instrument calibration. A stock solution of capsaicin was made by dissolving 1 mg of standard capsaicin in 1 ml of methanol. The working solution was prepared by diluting the stock solution to the desired concentration using methanol. The calibration graphs were expressed as chromatographic peak areas of standard capsaicin versus corresponding standard concentrations in the concentration range of 50-1000 µg/ml (**Fig. A14**) (**Appendix**).

The samples were analyzed using Agilent high-performance liquid chromatography (HPLC) system equipped with an autosampler, temperature controller, and UV detector. The capsaicin was determined under uniform HPLC conditions: column temperature of 30°C, flow rate 1.5 mL min−1, and run time was 20 min. The mobile phase was isocratic with the solvent combination (Acetonitrile: water containing 1% acetic acid in 70:30), and the detection was at 280 nm.

7.1.8 The Effect of Nano-encapsulated Bio-stimulants on Ascorbic Acid of Chilli Fruits

Standard ascorbic acid (purity 99%) was obtained from Sigma Aldrich. Acetonitrile and methanol of HPLC grade and HPLC-grade water /double distilled water were used. Other chemicals used were of analytical grade. The ascorbic acid stock standard was prepared in methanol and stored in a glass-stoppered bottle at 4°C in the dark. Working standards were diluted using a mobile phase consisting of acetonitrile and 0.1% acetic acid at a ratio of 50:50. Standard solutions were filtered with 0.25 µm Millipore membrane before injection. All samples were carried out in triplicate, kept in tightly stoppered dark glass vials, and stored at 4°C for less than 1 h before analysis (**Fig. 7.3**). Calibration curve was between 10-100 µg/ml based on 6 points calibration (**Fig. A15**) **(Appendix**).

Ascorbic acid was estimated in green as well as red chilli. Five hundred milligrams of ground sample was mixed with 30 mL of the extractant solution

containing 3% metaphosphoric acid plus 10 mM EDTA and sonicated at 25 °C for 30 min. The solution was centrifuged at 2000 rpm for 10 min. The supernatant was filtered using Whatman, No.42 filter paper followed by 0.25 µm Millipore filters and stored at 4°C less than 1 h before analysis.

HPLC system equipped with a UV (ultraviolet/visible light) detector was used for ascorbic acid. The chromatographic separation was performed using C18 column (100 x 3mm x 3µm) thermostated at 25°C with the isocratic method. The isocratic method with a mobile phase consisting of acetonitrile and 0.1% acetic acid at a ratio of 50:50 was used. The flow rate was 1.2 ml/min and 5 min run time. The injection volume was 20 µl. Ascorbic acid was detected with a maximum absorption peak of 245 nm.

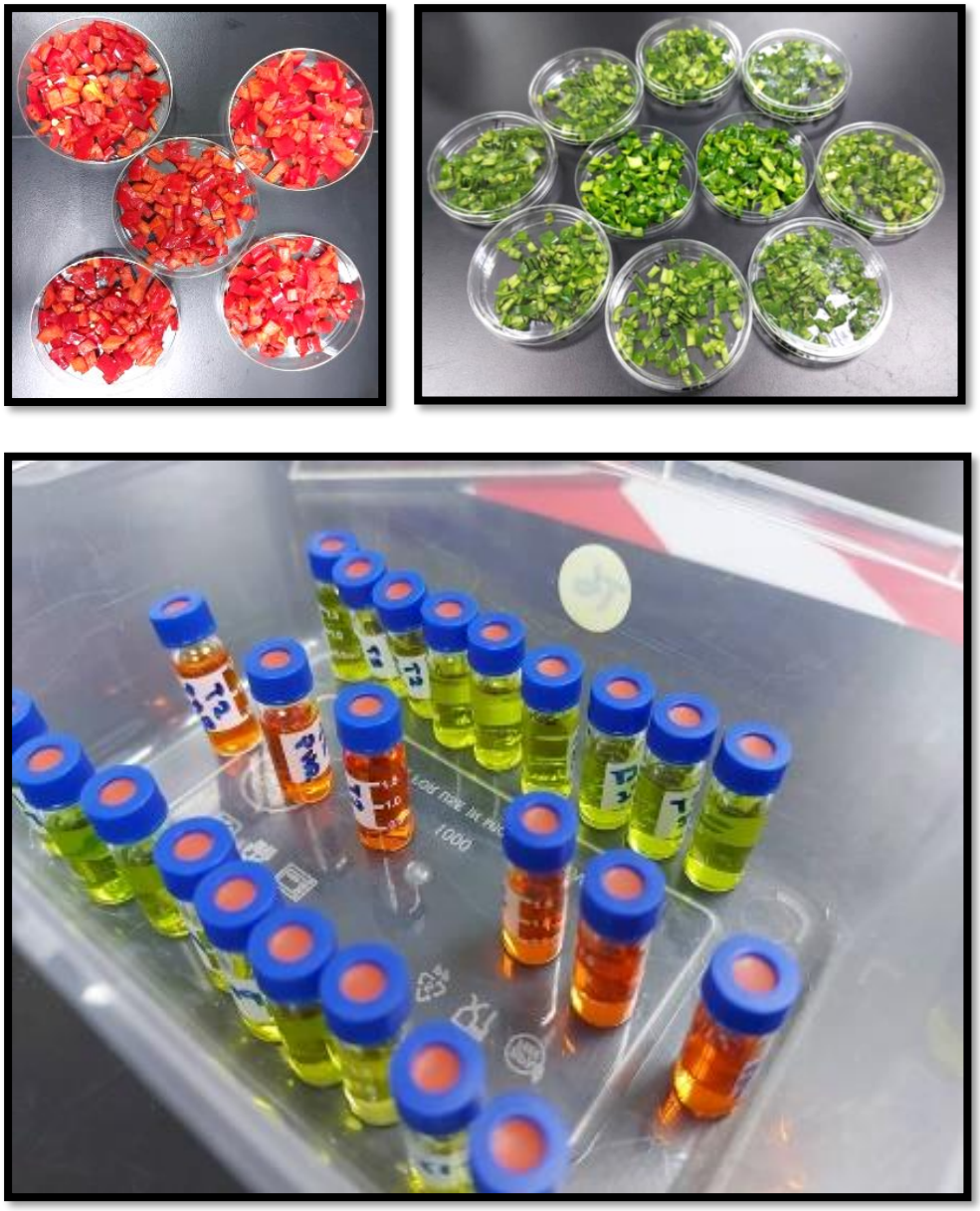


Fig 7.3 Chilli pericarps and the chilli extracts in vials for capsaicin and ascorbic acid analysis using HPLC

7.1.9 The Impact of Nano-encapsulated Bio-stimulants on the Nutrient Content in Soilless Planting Material and in Different Parts of the Chilli Plant

In this study, cocoa peat was used as the planting material. The AB fertilizer and encapsulated nano-bio-stimulant were applied to the cocoa peat throughout the experiments. Therefore, the nutrient content of cocoa peat, such as N, total P_2O_5, K_2O, MgO, and CaO, were assessed via ICP-MS analysis. For plant nutrient

analysis, chilli leaves, stems, twigs, fruits, and roots were tested for N, P, K, Mg, Ca, and ash content. A composite sample was prepared from 15 healthy chilli plants and 15 bags of planting material. The cocoa peat samples and plant parts were dried at 50°C for 3 days and ground before sending five replications of plant samples and six replications of planting materials for nutrient analysis at Applied Agriculture Resources Sdn. Bhd. Laboratory, Selangor, accredited by the Department of Standards, Malaysia (SAMM).

7.1.10 The Impact of Nano-encapsulated Treatments on Chilli Root Microbial Profiling using Next-generation Sequencing Analysis (NGS)

The change in the root microbiome of chilli plants grown on a soilless medium was investigated using large-scale sequencing technology. Two treatments were introduced to chilli plants: AB fertilizer application (T1) and AB fertilizer+Bio-stimulant (T2). A control sample was included in this analysis, where the chilli plants were only grown in soil without any fertilizer or bio-stimulant application (T3). The purpose of including T3 is to compare the native microbiome of chilli roots grown in soil.

7.1.10.1 Collection of Chilli Rhizosphere Samples and Metagenomics DNA Extraction

Five individual chilli plants from each treatment were randomly selected. Chilli roots were harvested and shaken to remove the loosely adhered planting material and cocoa peat. The adjacent cocopeat on the root's surface was manually separated and collected. Altogether, composite samples of cocopeat attached to 15 chilli roots from each treatment were collected. Four replication were prepared from each composite sample for each treatment. To isolate total bacterial community DNA, 0.25 mg of the sample was homogenized in DNA/RNA shield (DNA/RNA transport and storage medium for any biological sample), followed by DNA purification using the Zymobiomics DNA extraction kit (Zymo Research, Irvine, CA) according to the manufacturer's instructions.

7.1.10.2 Library Preparation and Sequencing

The bacterial 16S rRNA V3 hypervariable region was amplified from provided gDNA template using the primers 341F: CCTACGGGNGGCWGCAG and 518R: ATTACCGCGGCTGCTGG (Klindworth et al. 2013; García-López et al. 2020). Five additional bases of inline barcode were incorporated at the 5' end of the primers to enable inline barcoding (Glenn et al., 2019). Different samples were amplified using various forward and reverse inline primers combinations. PCR was performed using SolarBio PCR master mix (SolarBio, China) using the PCR profile of 95 °C for 3 minutes followed by 30 cycles of 95 °C for 15 s, 50 °C for the 20s, and 72 °C for 10s. The barcoded amplicons were subsequently visualized on the gel, normalized and pooled based on their intensity, and purified with 0.8 X volume of SPRI bead. The purified pooled amplicons were subsequently processed with the NEB Ultra II Library preparation kit to incorporate the Illumina adapter and dual-index barcodes. The constructed library was quantified with Denovix high-sensitivity assay and sequenced on a NovaSEQ6000 (Illumina, San Diego) for 2 x 150 paired-end sequencing.

7.1.10.3 Microbiome Data Analysis

Paired-end reads were overlapped using fast v0.21 (Chen, Zhou, Chen, & Gu, 2018). Demultiplexing and primer removal of the merged reads used cut adapt v1.18 (Martin, 2011). The demultiplexed and trimmed reads were imported into QIIME2 v.2021.4 (Bolyen et al., 2019) and denoised with dada2 (Callahan et al., 2016). Taxonomic assignment of the ASV used q2-feature-classifier (Bokulich et al., 2018) trained on the latest GTDB release (R07-RS207) 16S rRNA database (trimmed to only retain the V3 hypervariable region) that is comprised of 311,480 bacterial and 6,062 archaeal genomes (Parks et al., 2020). ASVs with the taxonomic assignment at least to the phylum level were selected for subsequent analysis. Both the ASV table and taxonomic classification table were exported using QIIME2 tools into tab-separated values (tsv format) and manually formatted to generate Microbiome Analyst-compatible input (Chong, Liu, Zhou, & Xia, 2020) that can perform SparCC co-occurrence network construction (Friedman & Alm, 2012) and statistical analysis using the linear

discriminant analysis (LDA) effect size (LEfSe) method (Segata et al., 2011). Alpha- and beta-diversity was calculated using the QIIME2 plug-ins. The filtered relative abundance table was also used as the input to generate Krona plots for intuitive exploration of relative abundances within the hierarchies of taxonomic classifications (Ondov, Bergman, & Phillippy, 2011). PiCRUSt2 analysis was also carried out to predict the function of each ASV that can be subsequently visualized and analyzed statistically in the STAMP software (Parks, Tyson, Hugenholtz, & Beiko, 2014).

7.1.11 Statistical Analysis

Statistical analysis was performed using the Univariate analysis with Tukey's test ($p < 0.05$) by the SPSS software (IBM Statistics, version 28, USA) to determine the significance of the data.

7.2 Results and Discussion

7.2.1 The Effect of Nano-encapsulated Bio-stimulants on the Photosynthesis Activity of Chilli Plants

The quantum yield measurement, which represents the photosynthesis activity, was measured on life-chilli plants for three months. It was observed that the first two months did not show significant readings among treatments (**Fig. 7.4**). Both treatments showed a quantum yield reading of 0.53 in the first month. The quantum yield value of treatment one and two were recorded as 0.621 and 0.619 respectively in the second month of the study. However, the quantum yield of plants not treated with bio-stimulant (Treatment 1) showed a decrease in the level of quantum yield during the third-month measurement. Treatment 1 showed a quantum yield value of 0.581 while treatment 2 exhibited 0.618 quantum yield in the third month of the study. The reading of quantum yield in the second month was higher than in the first month. A reduction in the quantum yield value was observed after the second month due to the pest infestation. Plants treated with bio-stimulants overcame the severity of pest attacks and started to promote plant growth. The quantum yield reading that showed a higher

value than the plants not treated with bio-stimulants proves this. At the end of the third month, chilli plants treated with bio-stimulants showed the ability to enhance photosynthesis activity. The results from the previous chapters have proven that the endophytic bacteria in the bio-stimulant could produce metabolites such as plant pigments and many carotenoids that play an essential role in the photosynthesis system. Carotenoids are pigments in the light-harvesting complexes of plants and microbes that provide photoprotection and light-absorbing functions and could be used as antioxidants (Saejung & Apaiwong, 2015). Furthermore, moringa leaf extract is rich in carotenoids and could increase the chlorophyll content and photosynthesis that, eventually promoted the overall growth and yield by seed treatment and foliar application (M. Arif et al., 2019; Rady et al., 2019).

The photosynthesis activity was determined by measuring the quantum yield using Fluorpen (FP-726).Quantum yield refers to the measurement of the efficiency of a photosynthetic system, which is defined as the ratio of the number of photons absorbed by the photosystem to the number of photons utilized in photosynthesis. In other words, quantum yield measures photosynthesis's effectiveness in converting light energy into chemical energy, which the plant uses for growth and metabolism (Liatile et al., 2022). Quantum yield is an essential parameter in plant physiology and is used to assess the health and productivity of plants under different environmental conditions. It can be used to quantify the impact of stress factors such as drought, high temperature, or pollution on plant photosynthesis, as well as to evaluate the effectiveness of various treatments or interventions aimed at improving plant growth and productivity (Liatile et al., 2022; A. Noman et al., 2018).

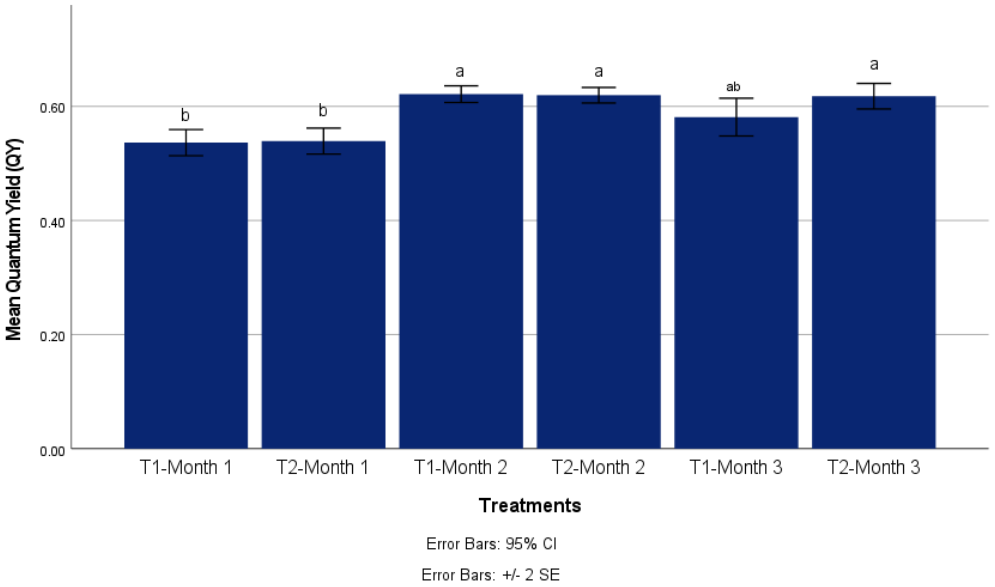


Fig.7.4 Effect of nano-encapsulated bio-stimulants on the quantum yield (QY) of chilli plants at one-month interval. Each value is the mean of forty replicates (n=40), and bars sharing the same letters are not significantly different ($p \leq 0.05$) according to Tukey's HSD. The vertical bar in the graphs indicates the standard error.

7.2.2 The Effect of Nano-encapsulated Bio-stimulants on the Vegetative Growth of Chilli Plants

The chilli plants treated with the bio-stimulant showed a significant increase in height, which accounted for 60 cm of mean value compared to the plants that were not treated with the bio-stimulant, which represents 53 cm of the mean value (**Fig. 7.5**). However, the aboveground biomass and root biomass of chilli plants did not show a significant difference between treatments (**Fig. 7.6 and 7.7**).

In **Chapter 5**, the combined effect of endophytic bacteria and moringa leaf extract positively affected the vegetative growth of chilli, such as enhanced plant height, root length, root biomass, and aboveground biomass. However, nano-encapsulated bio-stimulants were only able to increase plant height significantly. The root and aboveground biomass of chilli did not show significant differences between treatments. This could be due to the difference in the bio-stimulant concentration in both experiments. In nano-formulations, the amount of active ingredients used was reduced compared to the direct application of raw

ingredients such as moringa leaf extract and endophytic bacterial culture (**Chapter 5**). Thus, the formulation must be improved to set an optimum dosage and amount of bio-stimulants applied to crops. Bio-stimulant dose, time of application, crop cultivar and species, and environmental conditions could affect crop action (Admane et al., 2023; Paler & Alcantara, 2021). In addition to this, one of the functions of nano-formulation is the slow release of active ingredients. Thus, nano-formulations take longer time to show a significant and consistent effect on crops compared to the direct application of freshly prepared bio-stimulants.

Previous research has reported both the positive and negative effects of bio-stimulant application on crops. Enhanced plant growth, yield, and quality of products were reported as a positive response to bio-stimulant applications (Garai et al., 2021; Kapur et al., 2018; Layek et al., 2018; Yap, El-sherif, Habib, & Khattab, 2021). In contrast, the number of seeds per pod and the yield of mungbean were not significantly affected by the leaves of *Azolla* (Paler & Alcantara, 2021). No bio-stimulant effect was found on shallots' growth, yield, and nutritional value (Francke, Majkowska-Gadomska, Kaliniewicz, & Jadwisieńczak, 2022). In addition to this, it was found that the combined application of microbial-based bio-stimulant did not improve the morphological traits of pepper fruit, yield, nutrient content, and phytochemical content in fruit (Majkowska-Gadomska, Dobrowolski, Jadwisieńczak, Kaliniewicz, & Francke, 2021).

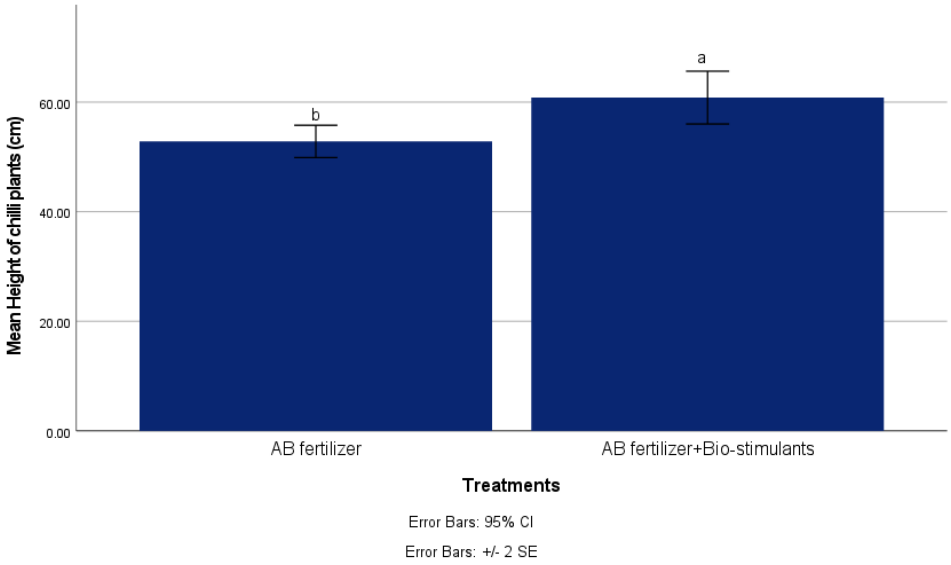


Fig. 7.5. Effect of nano-encapsulated bio-stimulants on the height of chilli plants. Each value is the mean of forty replicates (n=40), and bars sharing the same letters are not significantly different ($p \leq 0.05$) according to Tukey's HSD. The vertical bar in the graphs indicates the standard error.

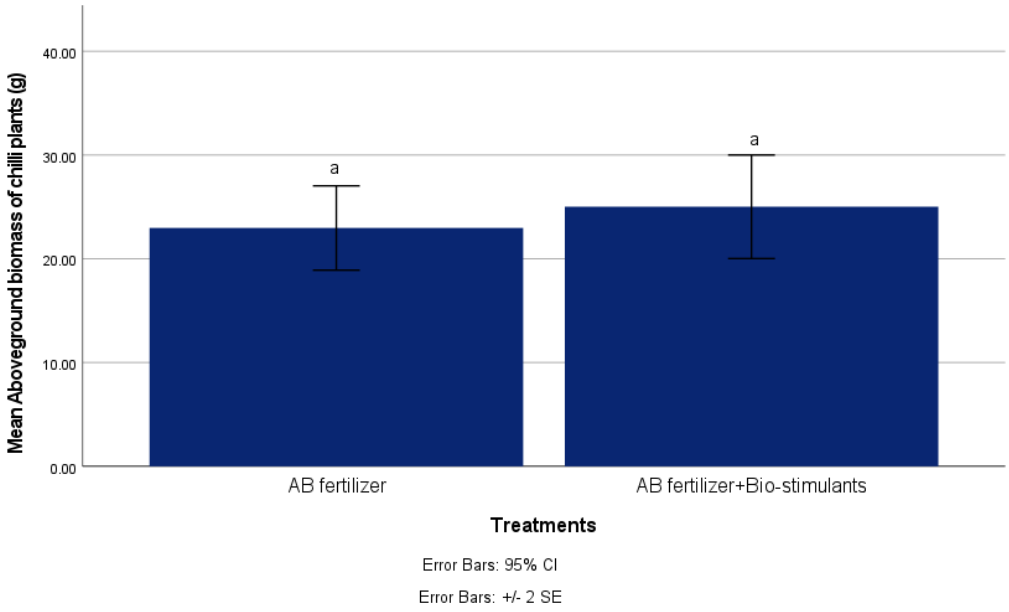


Fig. 7.6 Effect of nano-encapsulated bio-stimulants on aboveground biomass of chilli plants. Each value is the mean of forty replicates (n=40), and bars sharing the same letters are not significantly different ($p \leq 0.05$) according to Tukey's HSD. The vertical bar in the graphs indicates the standard error.

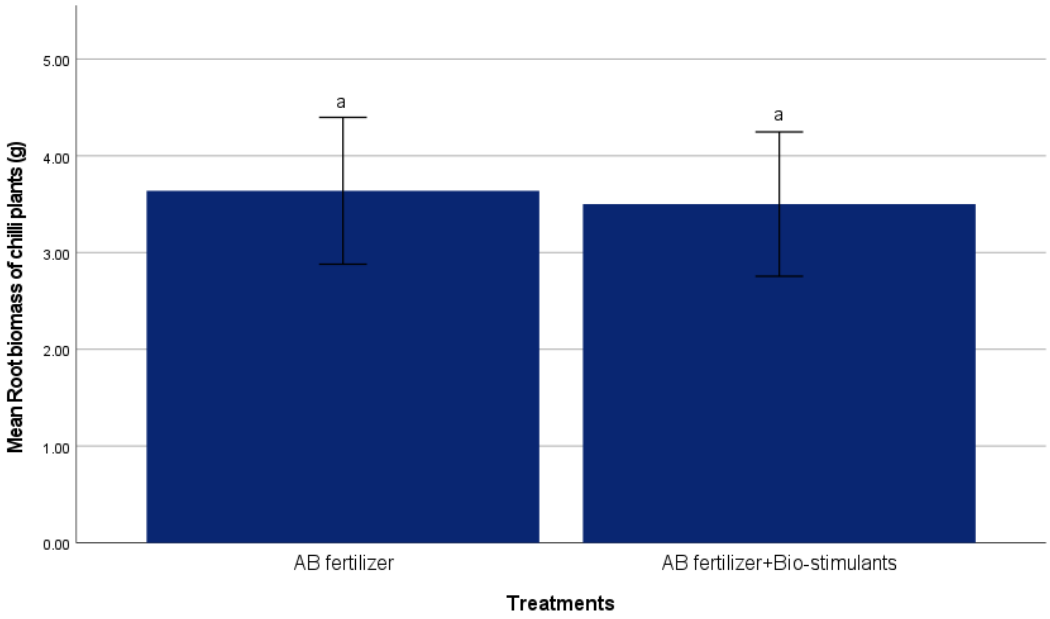


Fig. 7.7 Effect of nano-encapsulated bio-stimulants on the biomass of chilli roots. Each value is the mean of forty replicates (n=40), and bars sharing the same letters are not significantly different ($p \leq 0.05$) according to Tukey's HSD. The vertical bar in the graphs indicates the standard error.

7.2.3 The Effect of Nano-encapsulated Bio-stimulants on the Yield and Post-harvest Qualities of Chilli such as Chilli Length, and Color

The plants treated with bio-stimulant have recorded higher yields than those not treated with bio-stimulant. The total yield of chilli plants treated with bio-stimulant was 575.41 g, whereas chilli plants not treated with bio-stimulants recorded a 522.16 g yield. However, there were no significant differences in chilli fruits' mean weight and length (**Table 7.3**). Applying seaweed bio-stimulants increased micronutrient and protein concentration in rice grains and improved rice growth, yield, and quality (Layek et al., 2018). Likewise, (Mehmood et al., 2021) reported the effect of moringa leaf bio-stimulant in enhancing the growth and yield of black cumin.

Chilli plants treated with bio-stimulant showed a higher L value, -15.38±11.9 in chilli fruits compared to -8.29±7.97 of chilli plants not treated with bio-stimulants. The bio-stimulant-treated plants also proved the higher chroma value in chilli. Plants treated with bio-stimulants exhibited a chroma value of 8.77±5.56 in chilli fruits compared to the 5.30±6.81 mean chroma value found

in those of chilli plants not treated with bio-stimulants. This shows that chilli fruits showed the effect of bio-stimulant treatment on their pigmentation development compared to plants not treated with bio-stimulants. A study conducted by (Admane et al., 2023) proved that moringa leaf extract could enhance chlorophyll content and photosynthesis rate in plants. This indicates that bio-stimulant has an effect in increasing the plant pigment content in chilli plants. Carotenoids function as light-harvesting pigments, extend the range of light absorption and participate in photoprotection in photosynthetic tissues while acting as colorants and precursors for isoprenoids in non-photosynthetic tissues (Swapnil et al., 2021). This finding is in agreement with the enhanced photosynthesis activity of chilli plants treated with bio-stimulants and the metabolites produced by endophytic bacteria such as rhodopsin, rhodoxanthan, lycoxanthan, rubixanthin, echinenone, carotene, zeaxanthin astaxanthin in the experiments in chapter 4 (**Table A5**) (**Appendix**). Bio-stimulants made from other sources could also increase the chlorophyll and carotenoid content of the plant (Liatile et al., 2022).

For the colorimeter analysis, a* negative values indicates green color, positive values indicate magenta color, b* negative values indicate blue color and positive values indicate yellow color. The L value measures the lightness or darkness of a sample. A negative L value means the sample is very dark and absorbs significant light across the visible spectrum. This could be due to factors such as high pigment concentration, low reflectance, or strong light absorption by the sample. Chroma is one of the three components of the HunterLab color scale, along with L (lightness) and a (red-green axis), and b (yellow-blue axis) coordinates. Chroma is measured as the distance from the neutral axis (grey color) to the color of the sample in the a-b plane. A higher chroma value indicates that the color is more intense or saturated, while a lower chroma value suggests that the color is less severe or desaturated.

Table 7.3 The effect of nano-encapsulated bio-stimulants on the length, color and yield of chilli

Treatments	Yield per plant (g)	Weight per chilli (g)	Length of chilli (cm)	L* (Reflected lightness)	a*	b*	C* (Chroma)
AB fertilizer	522.16	17.9±1.3	6.75± 0.59	-8.29±7.97	0.54±2.94	-3.36±6.68	5.30±6.81
AB fertilizer + Bio-stimulant	575.41	17.9±1.7	6.89±0.53	-15.38±11.9	2.35±3.03	-6.27±7.44	8.77±5.56

7.2.4 The Effect of Nano-encapsulated Bio-stimulants on Phenolic and Flavonoid Contents in Chilli Fruits

The phenolic and flavonoid content was evaluated in chillies 19 days after anthesis, 27 days after anthesis, and post-harvest analysis after three days for both green and red chillies (**Table 7.4**). Phenolics and flavonoid content showed no difference between treatments during the fruit developmental stage. Both the phenolic and flavonoid contents increased as the chilli matured. Red chillies harvested from the plants that were not treated with bio-stimulants recorded the highest phenolic content (1.332±0.56 mg/L) compared to the chillies that were treated with bio-stimulants (0.8830±0.19 mg/L). In contrast, red chillies harvested from the plants treated with bio-stimulants recorded the highest flavonoid content (15.77±1.0 mg/L) than plants not treated with bio-stimulants (13.098±1.8 mg/L). Chilli fruits are rich in phenolics and flavonoids, plant compounds with antioxidant and anti-inflammatory properties. These compounds have various health benefits, such as reducing the risk of chronic diseases like cancer, cardiovascular disease, and neurodegenerative disorders. One of the bio-stimulant used in this study, moringa leaf extract, was proven to produce high content of phenolic and flavonoid compounds in the previous experiments in **Chapter 5**. In support of this,(Admane et al., 2023) reported that moringa leaf extract could improve the color, total phenolic content, and antioxidant activity of lettuce. The bio-stimulant application was also found to have different effects in producing phenolic and flavonoid content, where 37% and 74% of the increase were observed, respectively. The leaves and flowers of the yarrow showed an increase in total phenols, total flavonoids and antioxidant activity after applying a bio-stimulant of humic and fulvic acid (Bayat, Shafie, Aminifard, & Daghighi, 2021).

Table 7.4 The effect of nano-encapsulated bio-stimulants on the phenolics and flavonoid content in green and red chillies

Treatments	Time of harvest	Phenolics (mg/L)	Flavanoid (mg/L)
AB fertilizer (T1-19)	19 days after anthesis	0.8376 ± 0.04^{b}	6.1221 ± 0.7^{c}
AB fertilizer+Bio-stimulants (T2-19)	19 days after anthesis	0.7529 ± 0.04^{b}	3.9419 ± 1.0^{c}
AB fertilizer (T1-27)	27 days after anthesis	0.7349 ± 0.27^{b}	5.8314 ± 1.1^{c}
AB fertilizer+Bio-stimulants (T2-27)	27 days after anthesis	0.8163 ± 0.18^{b}	6.0930 ± 2.4^{c}
AB fertilizer (T1-PHG)	Post-harvest analysis of green chillies after 3 days	0.8236 ± 0.34^{b}	4.0581 ± 0.6^{c}
AB fertilizer+Bio-stimulants (T2-PHG)	Post-harvest analysis of green chillies after 3 days	0.9084 ± 0.23^{b}	5.5988 ± 1.5^{c}
AB fertilizer (T1-PHR)	Post-harvest analysis of red chillies after 3 days	1.332 ± 0.56^{a}	13.0988 ± 1.8^{b}
AB fertilizer+Bio-stimulants (T2-PHR)	Post-harvest analysis of red chillies after 3 days	0.8830 ± 0.19^{b}	15.7733 ± 1.0^{a}

7.2.5 The Effect of Nano-encapsulated Bio-stimulant on the Capsaicin and Ascorbic Acid Content in Chilli Fruits

Green chillies not treated with bio-stimulant exhibited significantly highest capsaicin content after harvesting, which has recorded a concentration of 74.67±11.8 mg/L (**Fig. 7.8**). Chillies treated with bio-stimulants and analyzed during the developmental stages at 19 and 27 days after the anthesis recorded 60.82±0.32 mg/L and 45.89±3.4 mg/L concentration of capsaicin respectively. The capsaicin content was generally lower in red chillies than green chillies. This shows that the bio-stimulant effect was better in increasing the capsaicin content while the fruit was still developing, but it could not be maintained three days after harvesting. Bio-stimulant treatment showed enhanced capsaicin content in the early stages of chilli development. The capsaicin content in chilli varies according to the different times of fruit development. This result was supported by a study investigating the direct metabolic response of chilli to foliar bio-stimulant to determine the synthesis of capsaicinoid response in the first 72 h of application (Zamljen, Medič, et al., 2022). It was found that capsaicin significantly increased in both the placenta and pericarp by the first hour of application and then decreased within three hours and increased after 24 hours. This finding shows that foliar application, a direct application of bio-stimulant on the fruit, could cause instability in the concentration of capsaicin in a chilli. It was also stated that nitrogen applied to leaves is taken up in the first 48 h after application (Zamljen, Medic, Hudina, Veberic, & Slatnar, 2022). In the current study, the bio-stimulants were applied via the drenching method, where the transportation of nutrients from roots to fruits might take different duration and effects on the capsaicinoids synthesis. Furthermore, capsaicinoid degradation occurs as chillies mature due to peroxidase activity (Zamljen, Medič, et al., 2022). This result indicates that the bio-stimulant application time and method greatly influence energy-consuming activity, such as capsaicinoid synthesis.

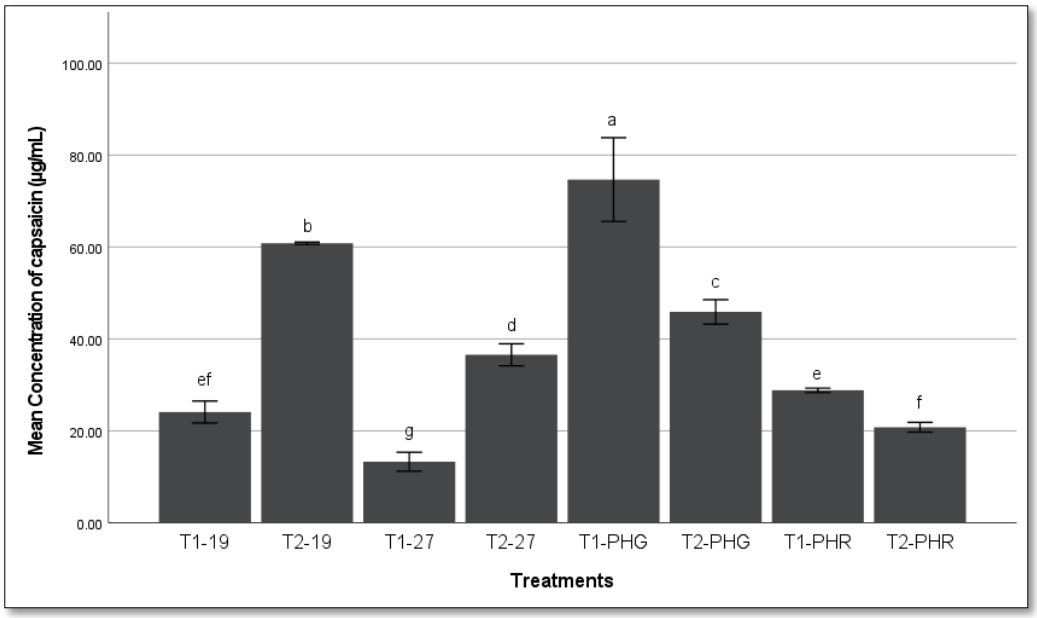


Fig. 7.8 Effect of nano-encapsulated bio-stimulants on the capsaicin content in chilli fruits. T1: AB fertilizer treatment and T2: AB fertilizer+Bio-stimulants treatment. Each value is the mean of fifty replicates (n=50), and bars sharing the same letters are not significantly different ($p \leq 0.05$) according to Tukey's HSD. The vertical bar in the graphs indicates the standard error.

The ascorbic acid content was highest in red chillies after harvesting, followed by green chillies at 27 days after anthesis (**Fig.7.9**). The concentration of ascorbic acid in chilli fruits increased significantly 27 days after the anthesis. Still, ascorbic acid concentration was very low in green chillies three days after harvesting. Chillies left to develop further until they turned red showed the highest ascorbic acid content in chillies treated with bio-stimulants (144.29±6.1 mg/L), followed by green chillies treated with bio-stimulants twenty-seven days after anthesis (132.85 ±1.67). In general, bio-stimulant treatment has positively affected the plants to produce a significantly higher ascorbic acid content in red chillies.

Ascorbic acid, commonly known as vitamin C, is a major antioxidant that protects cells from oxidative damage by reactive oxygen species and is a cofactor for the enzyme involved in photosynthesis (Kamran, Mushtaq, Arif, & Rashid, 2023). The vitamin C content in fruits was found to be increased by bio-stimulant applications of various sources such as algae extract, moringa leaf extract, plant growth-promoting bacteria, humic acid, fulvic acid, protein hydrolysates and

amino acids (Koleška et al., 2017; Paradiković et al., 2011; Valencia et al., 2018).

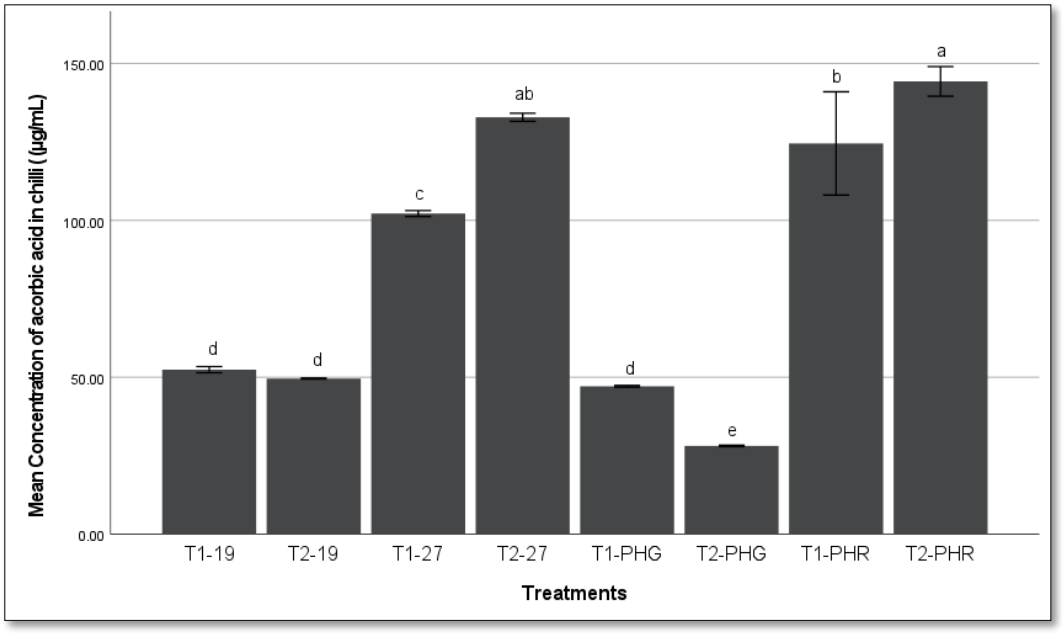


Fig.7.9 Effect of nano-encapsulated bio-stimulants on the ascorbic acid content in chilli fruits. T1: AB fertilizer treatment and T2: AB fertilizer+Bio-stimulants treatment. Each value is the mean of fifty replicates (n=50), and bars sharing the same letters are not significantly different ($p \leq 0.05$) according to Tukey's HSD. The vertical bar in the graphs indicates the standard error.

7.2.6 The Effect of Encapsulated Bio-stimulant Application via Drenching Method on the Nutrient Content of Different Parts of Chilli Plant and the Soilless Medium

The investigation aimed to determine the nutrients absorbed by the soilless medium and the amount taken up by the plants treated with a bio-stimulant. The nutrient content in the soilless medium treated with bio-stimulant showed higher nitrogen, phosphorus, and calcium content, and no difference was observed for other treatments (**Table 7.5**). This indicates that nano-encapsulated bio-stimulant could enrich the soilless growth medium with nutrients. This result supports a report stating that bio-stimulants are suitable for the soilless medium by improving nutrient use efficiency and increasing confined nutrient availability in the rhizosphere (Admane et al., 2023).

The nutrient content in chilli leaf, stem, twig, fruit, and root will indicate the amount of nutrients absorbed in different parts of plants as an effect of bio-stimulant application (**Table 7.6**). The ash and nitrogen content was higher in leaf samples compared to other parts of plants. However, no difference was observed except for the ash content in the stem of chilli plants treated with bio-stimulant. Phosphorus content was the highest in roots treated with AB fertilizer. A slight increase in the calcium content in leaves and magnesium content in the stem was observed among chilli plants treated with bio-stimulant. No changes were observed for the potassium contents of all plants under different treatments.

In the previous studies, it was observed that phosphorus and potassium levels in roots were closely related to enhanced root length. Nitrogen and chlorophyll were closely related to the dry weight of the shoot and root (Mutale-joan et al., 2020). Foliar bio-stimulant application increased the concentration of calcium and magnesium in shallots (Francke et al., 2022). This was in agreement with the current study, where only these two nutrients were higher in plants treated with bio-stimulants than in plants not. Another study has reported increased nitrogen and phosphorus in rice grains after bio-stimulant treatment (Layek et al., 2018). In general, bio-stimulants were known for their characteristics to increase nutrient uptake and nutrient use efficiency. However, the current study did not provide significant results on nutrient content in the leaf, stem, and root of chilli as a positive effect of bio-stimulant. These results support the vegetative growth results where no significant increase was observed in root length, root biomass, and aboveground biomass of chilli after treating them with nano-encapsulated bio-stimulants.

Table 7.5 Nutrient content in soilless medium (cocopeat) after uprooting chilli plants

Treatments	**N (%)**	**Total P_2O_5 (%)**	**K_2O (%)**	**CaO (%)**	**MgO (%)**
AB fertilizer	0.89±0.03	0.21±0.02	0.58±0.05	1.61±0.04	0.28±0.00
AB fertilizer + Bio-stimulant	0.94±0.01	0.24±0.02	0.53±0.10	1.97±0.11	0.27±0.01
Control	0.47±0.11	0.14±0.01	2.33±0.51	0.31±0.39	0.21±0.05

Table 7.6 Nutrient contents in different parts of chilli plants using ICP-MS

Treatments	Chilli parts	Ash (%)	N (%)	P (%)	K (%)	Ca (%)	Mg (%)
AB fertilizer	Leaves	22.87±0.29	3.99±0.16	0.37±0.01	7.1±0.00	1.90±0.14	1.28±0.05
AB fertilizer+ bio-stimulant	Leaves	22.00±0.96	3.97±0.08	0.36±0.02	6.08±0.38	2.32±0.07	1.30±0.07
AB fertilizer	Stems Twigs	16.2±0.37	2.26±0.01	0.305±0.00	5.37±0.17	0.85±0.01	0.73±0.01
AB fertilizer+bio-stimulant	Stems Twigs	18.4±0.33	2.30±0.04	0.313±0.00	6.04±0.11	1.04±0.02	0.84±0.02
AB fertilizer	Roots	11.82±0.01	2.59±0.01	0.787±0.01	2.94±0.01	1.69±0.01	0.25±0.01
AB fertilizer+ bio-stimulant	Roots	10.57±0.01	2.38±0.01	0.61±0.01	2.68±0.01	1.59±0.01	0.25±0.01
AB fertilizer	Fruits	11.86±0.01	2.68±0.01	0.437±0.01	3.14±0.01	0.12±0.01	0.22±0.01
AB fertilizer+ bio-stimulant	Fruits	11.27±0.01	2.61±0.01	0.439±0.01	3.14±0.01	0.13±0.01	0.22±0.01

7.2.7 The Effect of Nano-encapsulated Treatments on Chilli Root Microbial Profiling using Next-generation Sequencing Analysis (NGS)

This study is aimed to evaluate the differences in the bacterial community of chilli roots at the fruiting stage as a response to nano-encapsulated bio-stimulants and AB fertilizer. Three treatments were included to assess the changes in the root microbiome of chilli, namely, treatment with AB fertilizer (T1), treatment with AB fertilizer and nano-encapsulated bio-stimulants (T2), and no treatments or control (T3). In general, 4083 amplicon sequence unit (ASV) was obtained for the next-generation sequencing analysis (**Table 7.7**).

Table 7.7 Amplicon sequence unit (ASV) details of each group of treatment applied to chilli plants

X	FullDataset	T1	T2	T3
Number of Samples	12	4	4	4
Number of Raw Reads	1017446	398181	259674	359591
Average Raw Reads	84787 ± 29875	99545 ± 32443	64918 ± 32682	89897 ± 17333
Number of Filtered Reads	890949	347184	224973	318792
Average Filtered Reads	74245 ± 25909	86796 ± 27363	56243 ± 28402	79698 ± 14971
Number of ASVs	4083	1947	2236	1536
Average ASVs	1135 ± 326	1282 ± 155	1213 ± 484	910 ± 38
ace	1454.796 ± 497.210	1741.630 ± 383.785	1487.523 ± 709.219	1135.235 ± 94.323
chao1	1383.871 ± 454.310	1638.118 ± 331.529	1418.273 ± 663.905	1095.222 ± 85.152
faith_pd	81.546 ± 16.385	90.065 ± 6.216	84.595 ± 25.605	69.978 ± 1.602
goods_coverage	0.935 ± 0.028	0.918 ± 0.019	0.938 ± 0.043	0.950 ± 0.008
observed_features	1111.000 ± 322.151	1243.000 ± 140.648	1196.250 ± 512.836	893.750 ± 43.538
shannon_entropy	8.957 ± 0.629	9.225 ± 0.121	9.270 ± 0.818	8.377 ± 0.303
simpson	0.997 ± 0.005	1.000 ± 0.000	0.998 ± 0.005	0.992 ± 0.005

The chilli root microbiome analysis based on 16S rRNA was done to determine the difference in microbial taxonomy, phylogenetic diversity, and functional diversity. This is the most popular sequencing technique for analyzing bacterial diversity studies to create targeted amplicon sequences. Root microbiome, also known as the rhizospheric microbiome, refers to the dynamic community of microorganisms associated with plant roots. Signaling molecules such as root exudates influence rhizosphere microbiome composition and dynamics of the microbial community (I. Arif et al., 2020). This study is essential to demonstrate

the presence of the endophytic bacterial bio-stimulant applied to the soil, the survival of the specific bacteria in the soil, and chemical fertilizers. Furthermore, rhizosphere microbial communities can promote plant growth by enhancing adaptation to environmental stresses, improving nutrient acquisition, and improving plant metabolic functions (Q. Liu et al., 2022). The data on the root microbial biodiversity and abundance could reveal the short-term effect of nano-encapsulated bio-stimulants consisting of moringa leaf extract and endophytic bacteria. Plants allocate 27% of carbon to their roots, and roots release about 11 % of the net fixed carbon to the root zone, where the amount and type of exudates depend on plant species, ages, and root zone microorganisms and the growing environment (Balliu et al., 2021).

Sequences were clustered into operation taxonomic units (OTUs) for amplicon sequencing-based microbiota analysis. OTU is similar to the amplicon sequence unit (ASV) count. OTU generally represents sequences that are not more than 3% different from each other. In contrast, ASV based approach has a higher sensitivity in detecting the bacterial strains present, sometimes at the expense of specificity (Callahan, McMurdie, & Holmes, 2017).

A study reported that 983 OTUs were significantly correlated to concentrations of the phytohormones in the rhizosphere, including 288 negative and 695 positive correlations (Xiong & Lu, 2022). Root exudates that act as allelochemicals cause inhibitory effects on other organisms cause root growth injury, reduce nutrient absorption, and decrease photosynthesis and overall plant growth (Balliu et al., 2021). This indicates that root exudates have the potential to not only attract certain microbes to the roots but also impede the growth of certain microorganisms in the rhizosphere. Microorganisms have the potential to function as bio-stimulants that can improve plant growth and increase crop productivity under unfavorable environmental conditions caused by abiotic stress (Romano et al., 2020). The inability of microbial inoculants to effectively perform their intended functions can be attributed to issues associated with the production and formulation processes of the inoculant (Vassilev et al., 2017). The bio-stimulants applied in this study were prepared in nano-formulation as they have numerous merits due to their high surface area and solubility. Besides,

nano-encapsulation could protect the inoculated bacteria from extreme conditions in the rhizosphere. Introducing PGPR strains through inoculation is often difficult due to the resistance of the pre-existing microbial communities, which can cause the newly introduced strains to disappear quickly or fail to establish themselves (Koprivova & Kopriva, 2022). Nanoparticles, directly and indirectly, affect the interaction between plants and microbes. The direct effect involves the provision of nutrients in the rhizosphere, while the indirect impact consists of stimulating bacterial strains (S. Rai et al., 2023). There is a growing recognition of the potential of modifying the plant microbiome as a sustainable means of safeguarding plants against diseases and enhancing agricultural productivity (Gao et al., 2021). Thus, adding bio-stimulants to manipulate the root microbiome is considered an environmentally sustainable strategy.

7.2.8 Rarefaction

Rarefaction curves check the sampling efficiency within and between sample groups. The samples were rarefied to the lowest number of reads per library, and rarefaction curves of bacterial samples tended to approach the saturation plateau, indicating that the coverage of samples was good enough. Rarefaction using shannon_entropy, shao1, ace, Simpson, goods_coverage, faith_pd, and observed_features indices revealed similar results as depicted in **Table 7.7** and **Fig. 7.10**. This shows that the number of ASVs is sufficient for the taxonomy study and able to represent the root microbiome of chilli roots.

However, soil and soilless medium could promote different microbial communities. It was reported that the rhizosphere's richness two months after transplanting into the coconut fiber substrate was significantly lower than the plants grown in the soil (Anzalone et al., 2022). This shows that the microbial richness in the soil rhizosphere is far greater than in the soilless medium. The growing medium primarily influences the root-associated microbiome's microbial diversity, which plays a critical role in facilitating the interactions between the plant roots and their surrounding environment(Friesem et al., 2021).

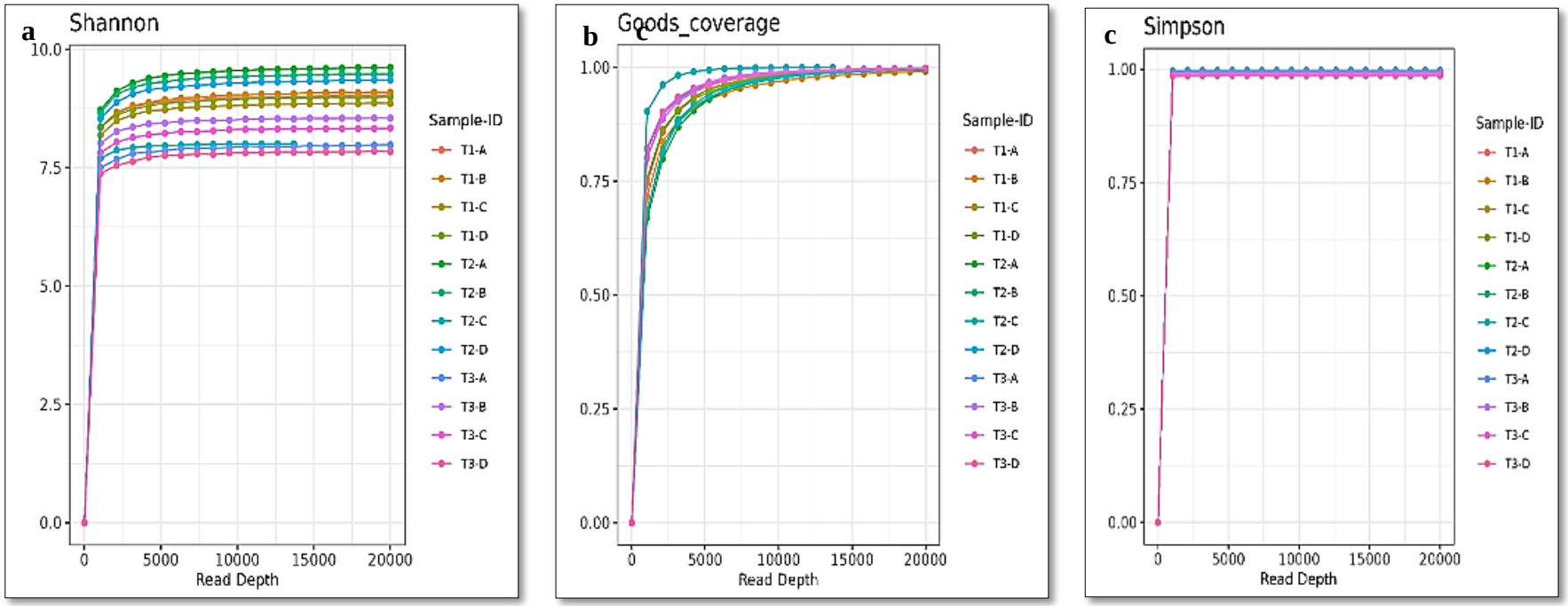


Fig. 7.10 Rarefaction curves comparing the alpha diversity of bacteria in chilli root rhizosphere (a) Shannon; (b) Goods' coverage, and (c) Simpson. Each point represents the average value of 10 random sampling at the specific sampling depth

7.2.9 Shared and Unique ASVs

The Venn diagram shows the shared and unique amplicon sequence variants of the chilli root microbiome in response to chemical fertilizer (T1), chemical fertilizer and nano-encapsulated bio-stimulant (T2), and control treatment, chilli grown in soil without any chemical fertilizer and bio-stimulant (T3) (**Fig. 7.11**). The number of shared ASVs among all three treatments was 347, which accounted for 8.5% of the total ASVs. About 647 ASVs were shared between T1 and T2. T1 and T3 shared 227 ASVs, but only 66 ASVs shared by T2 and T3. The highest number of unique ASVs belongs to T2 (1175 ASVs), which showed the highest percentage (28.8%) of all the shared and unique ASVs. This was followed by 895 unique ASVs of T3 and 725 ASVs of T1. Shared ASVs can help identify common microbial communities across different samples, while unique ASVs can provide insight into the specific microbial diversity present in each instance or environment. This result indicates that treatment 2 (Nano-encapsulated bio-stimulant treatment) has the majority of unique groups of bacteria that did not exist in other treatments.

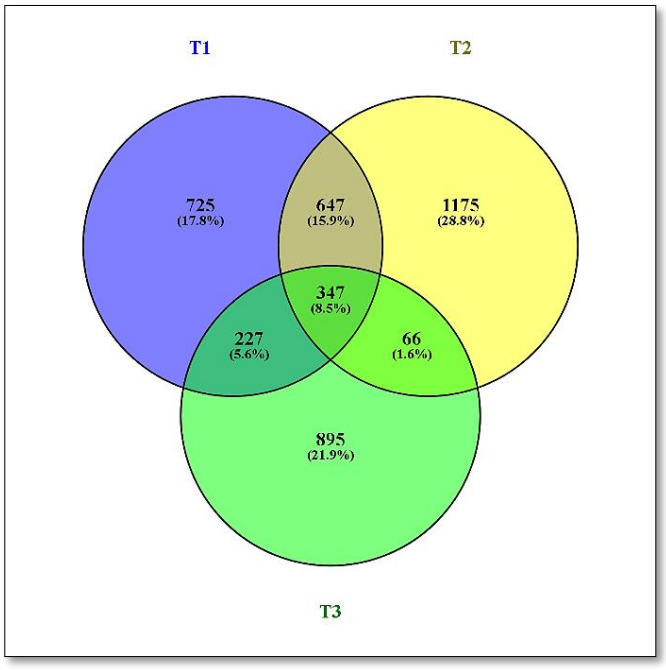


Fig. 7.11 Venn diagram of the shared and unique amplicon sequence variants of chilli root microbiome

The reason for this could be the microbial inoculant, which may have stimulated the secretion of plant roots, attracting more effective microbes (Q. Liu et al., 2022). PGPR colonizing the surface of the root system have been used in both soil and soilless culture system due to their effects on nutrient uptake, mitigating plant stress, and controlling pathogens (Balliu et al., 2021). Bacteria that live in association with plants have genetic codes that contain biosynthetic genes responsible for producing antibiotics and toxins. These substances play a crucial role in regulating the populations, variety, and spatial arrangement of other microorganisms co-existing in the same host plant (M. Noman et al., 2021). The diversity and composition of the crop microbiome are affected by plant health, growth and development stage, and the magnitude of the root exudates (S. Rai et al., 2023).

7.2.10 Alpha- and Beta- Diversity

Taxonomic diversity describes the richness and abundance of species with a particular emphasis on *Bacilli* and *Actinomycetes* class. Alpha diversity shows the diversity within a sample and can be measured with indices such as Shannon index, chao1, Ace and Simpson, or species count using rarefaction curves (**Table 7.8**). Alpha diversity was calculated based on the Bray-Curtis index using PERMANOVA statistics (**Fig. 7.12**). The results of estimators of alpha diversity did not show significant differences in the diversity and species richness of bacterial communities within treatments, although an outlier was observed within treatment 2 (T2-C). The outlier was included in the statistical analysis. Alpha diversity measurement using various indices showed similar results, indicating the reliability of the results. Treatments 1 and 2 did not show significant differences between treatments compared to control (T3). The microbial diversity within each root rhizobiome sample (T1, T2, and T3) is similar. However, this does not mean that the microbial communities in the two samples are identical. They may have different species composition or relative abundance of specific microbial taxa, but their diversity levels are similar overall.

Beta diversity described the diversity between samples and was analyzed based on Bray-Curtis dissimilarities (**Fig. 7.13**). Principle component analysis (PCA) is a statistical technique used to analyze beta diversity data and visualize differences in microbial community composition between samples. Pricipal Coordinate Analysis (PCoA) is similar to PCA, which attempts to represent the distances between samples. PCA focuses on shared variance, whereas PCoA focuses on distances.

Table 7.8 Individual alpha diversity metrics in chilli roots for every group of treatment

SampleID	ace	chao1	faith_pd	goods_coverage	observed_features	shannon_entropy	simpson
T1-A	1490.88	1433.27	88.75	0.93	1165	9.21	1.00
T1-B	2303.09	2124.96	99.17	0.89	1453	9.39	1.00
T1-C	1674.28	1571.27	86.86	0.92	1192	9.10	1.00
T1-D	1498.27	1422.97	85.48	0.93	1162	9.20	1.00
T2-A	1958.67	1862.78	99.87	0.91	1527	9.80	1.00
T2-B	1700.72	1612.39	94.34	0.93	1385	9.65	1.00
T2-C	435.64	434.46	46.34	1.00	432	8.05	0.99
T2-D	1855.06	1763.46	97.83	0.91	1441	9.58	1.00
T3-A	1224.27	1187.88	68.72	0.94	897	8.19	0.99
T3-B	1022.74	997.74	71.56	0.96	877	8.71	1.00
T3-C	1200.97	1140.37	71.15	0.95	952	8.55	0.99
T3-D	1092.96	1054.90	68.48	0.95	849	8.06	0.99

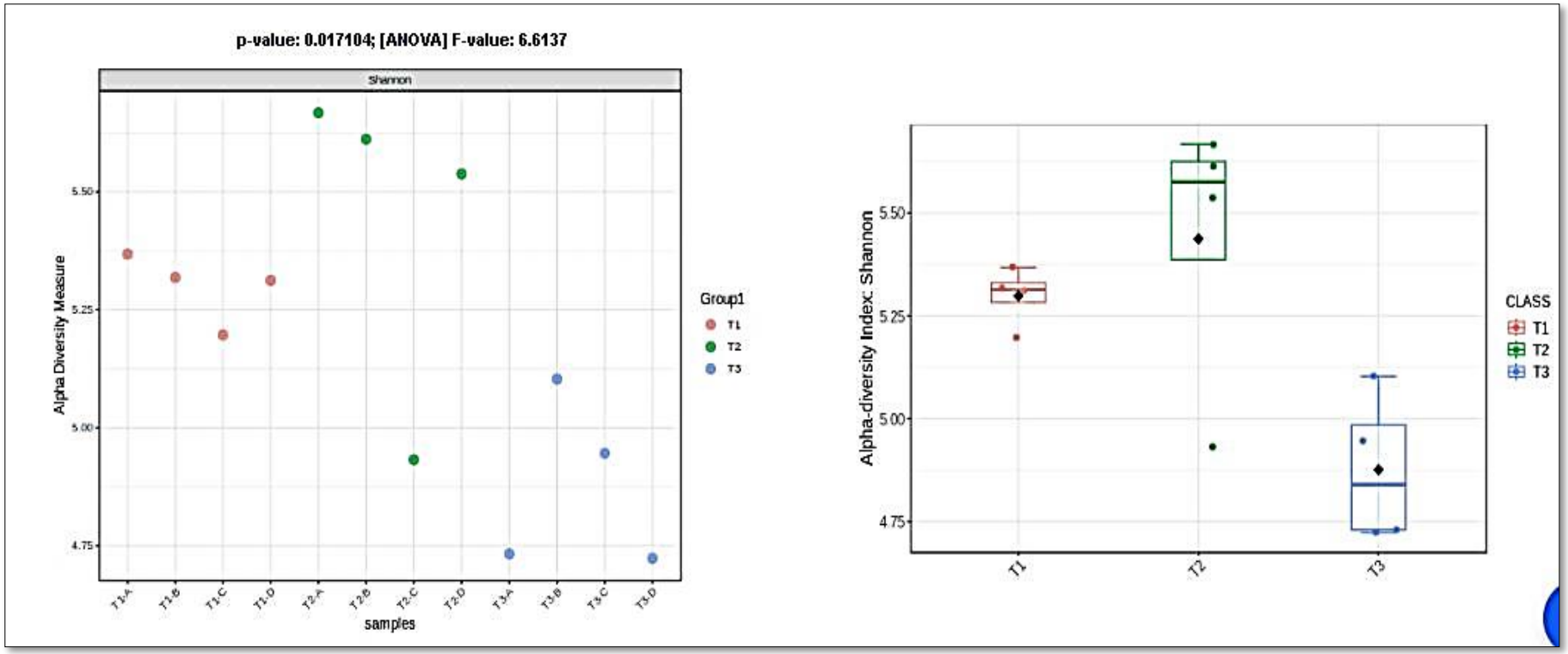


Fig. 7.12 Alpha diversity was calculated based on Bray-curtis index using PERMANOVA statistics

PCoA was used to assess differences in the bacterial community compositions of the chilli root microbiome under different treatments, such as AB fertilizer (T1), AB fertilizer + Bio-stimulant (T2), and Control (T3). All treatments caused distinct bacterial community separations in the PCoA plots. The principal variation of bacteria between treatments was assessed based on Weighted UniFrac and Bray-Curtis distances, which explained 48.11% and 44.96 % of the total observed variation (**Fig. 7.13** and **7.14**). The distance between samples on the scatterplot represents the dissimilarity in species composition between samples. The samples that are close together on the scatterplot are more similar in terms of species composition, whereas samples that are far apart are more dissimilar. It shows that every treatment has recruited different groups of bacteria that show significant differences. Previously, bio-fertilizer contributed to accumulation of more beneficial bacteria at the rhizosphere level (Q. Liu et al., 2022).

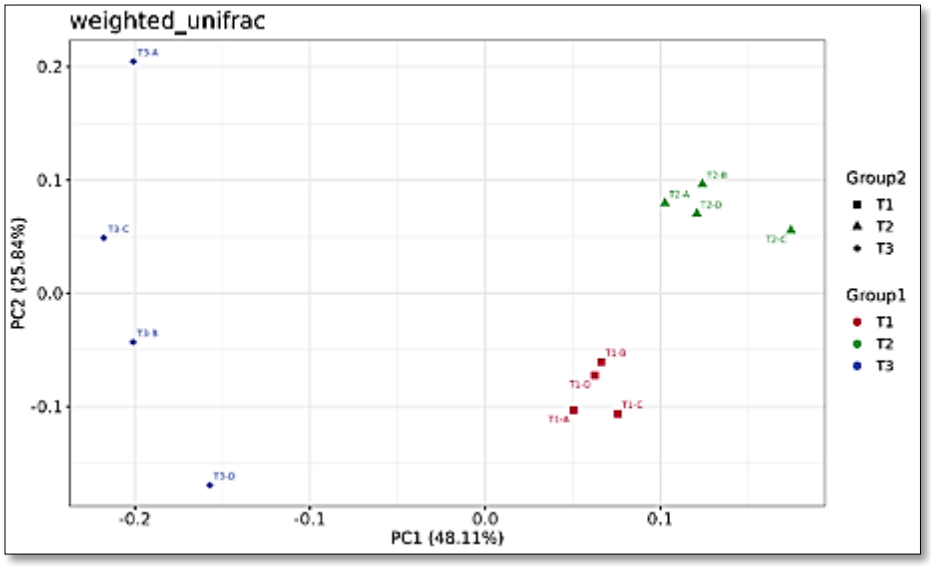


Fig. 7.13 Beta diversity represented by the Principal Coordinate Analysis (PCA) plot with distances between subjects determined by the weighted UniFrac metric. Each plot point represents a single sample and was colored and shaped by the group according to the legend. Samples clustered close together are more likely to share a similar microbial composition.

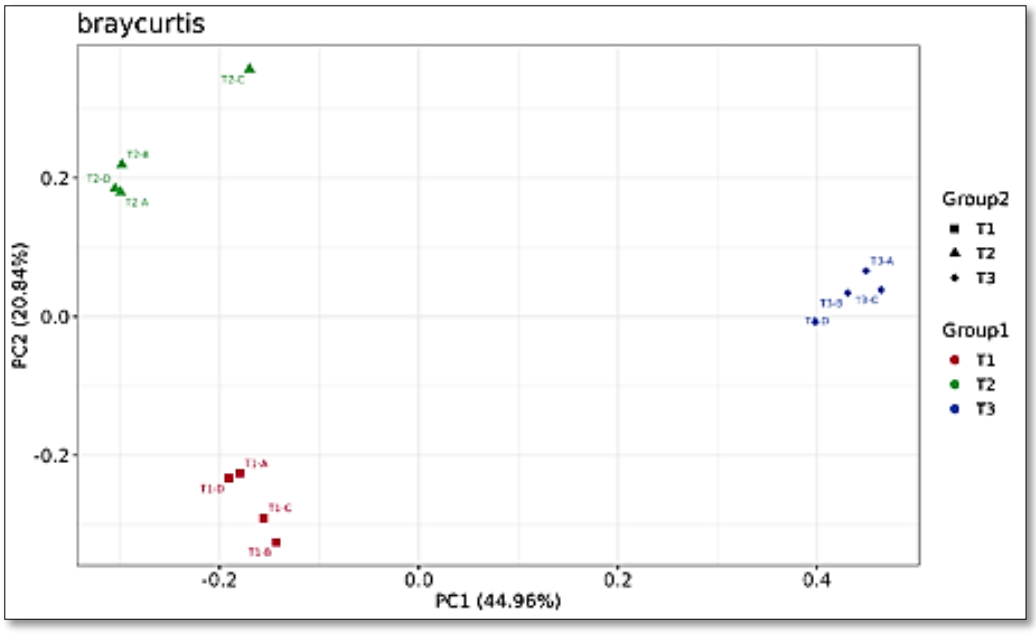


Fig. 7.14 Beta diversity represented by the Principal Coordinate Analysis (PCA) plot with distances between subjects determined by the Bray Curtis metric. Each plot point represents a single sample and was colored and shaped by the group according to the legend. Samples that clustered close together are more likely to share the similar microbial composition

7.2.11 Phylogenetic tree

Phylogenetic diversity reveals the presence of different evolutionary lineages in the chilli rhizosphere as a response to the bio-stimulant application. Dendrogram links treatments 1 and 2 as both were treated with AB fertilizer and used cocopeat is the substrate (**Fig. 7.15**). However, there was still a distinct branch between treatment 1 and treatment 2 that indicates the changes in the root microbiome diversity due to the bio-stimulant application. In contrast, treatment 3 (control), soil treated with water, reveals the natural microbiota in chilli roots and soil. Among the root exudates identified in plants, certain compounds are shared across species, while others are exclusive to particular plants (M. Noman et al., 2021). The uniqueness of the rhizosphere is measured by secreting species-specific crop root exudates that contain amino acids, organic acids, fatty acids, sugars, nucleotides, phytohormones, putrescine, terpenoids, sterols, and vitamins which communicate signals in the microbiome for active colonization, uptake of nutrients and water (S. Rai et al., 2023).

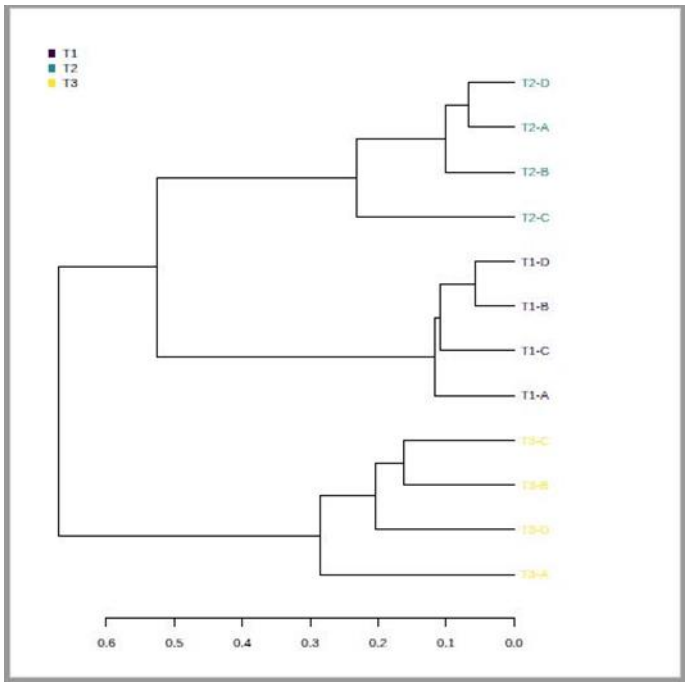


Fig. 7.15 Dendogram shows linkage between bacterial populations in chilli roots of different treatments. (T1: AB fertilizer; T2: AB fertilizer + Bio-stimulants and T3: Control)

7.2.12 Composition of Microbial Community in Chilli Root

A total of 389 phyla, 2150 orders, 880 classes, 3675 families, 6206 genera, and 7009 species were identified in all three treatments. The detail of the microbial community composition in each treatment is described in **Table 7.9**. Chemotaxis is a chemical process employed by microorganisms to detect and respond to plant-derived signals exuded from plant roots to initiate the microbial colonization step (M. Noman et al., 2021).

Table 7.9 The composition of microbial community between different treatments of chilli roots

Microbial community composition	T1	T2	T3	Total number
Species	2535	2562	1912	7009
Genus	2252	2255	1699	6206
Family	1337	1301	1037	3675
Class	311	319	250	880
Order	776	766	608	2150
Phylum	138	142	109	389

*T1= AB fertilizer, T2=AB fertilizer+ Bio-stimulant and T3= Control (No fertilizer or bio-stimulant)

The pie chart shows the top 15 phyla that showed significant dominance in all three treatments (**Fig. 7.16**). The sequences were assigned to 389 phyla predominated by Proteobacteria, which accounted for 48% of the bacterial sequences, followed by 9% of Actinobacteria. In a different study, among the top 14 phyla identified in the soil rhizosphere, the dominant phyla across the treatments were Proteobacteria (Olasupo et al., 2022). As previously reported, many substrate transporters in the Firmicutes and Proteobacteria phyla promote the adaptation of microbial communities to the nutrient-rich surroundings of plants (M. Noman et al., 2021). Proteobacteria was also found to be an important phylum in the global C, N, and S cycling and serves as biological control agents (Hu, Shao, Gao, Long, & Rengel, 2022). At the order level, Rhizobiales, Burkholderiales, Alphaproteobacteria, Saccharimonadales, Caulobacterales, Steroidobacterales, and Chitinophagales have dominated the chilli roots (**Fig. A16**) (**Appendix**). T1 and T2 exhibited more species than T3, as AB fertilizers were included in T1 and T2. Fertilizer addition significantly affected the diversity and species composition of the microbial community in the rhizosphere (Q. Liu et al., 2022).

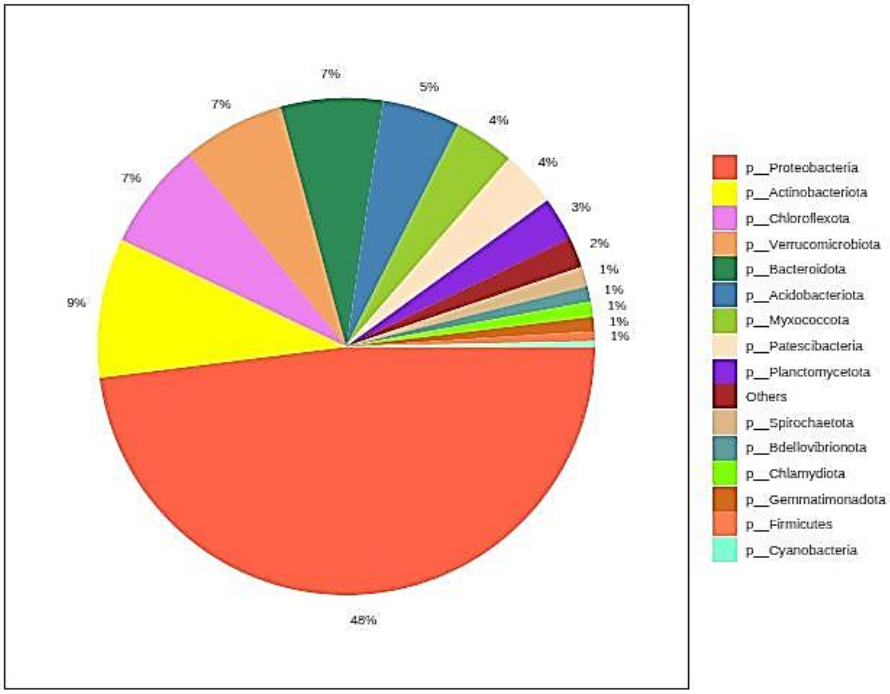


Fig. 7.16 Pie chart shows the main phyla commonly found in

chilli roots of all three treatments

At the class level, Alphaproteobacteria, Gammaproteobacteria, and Verrucomicrobium were identified as the most dominating class of bacteria in chilli roots (**Fig. A.17**) (**Appendix**). At the family level, Xanthobacteraceae, Beijerinkia, Chitrophagacea, Steroidbactericeae, Rhizobiales, and Gammaproteobacteria were dominant (**Fig. A.18**) (**Appendix**). At the genus level, Pseudolabrys, Rhizomicrobium, Hyphomicrobium, and Methylosinus dominated chilli root rhizosphere (**Fig. A.19**) (**Appendix**). All three treatments detected many unknown species in the rhizosphere samples (**Fig.7.17**). This justifies the observation in the Venn diagram that every treatment has shared a unique amplicon sequences, meaning the samples contain similar and specific bacterial species where most could be new or unreported. Therefore, a comparison was made between treatments to calculate the percentage of *Bacillus* and *Streptomyces* genus and species in the root microbiome based on the ASV identification (**Fig. 7.18**). This comparison aims to evaluate whether using a bio-stimulant in treatment 2 can augment the populations of the *Bacillus* and *Streptomyces* genera within the roots of chilli plants. The results indicates that an increase in the percentage of *Baciilus* and *Streptomyces* was observed in treatment 2, which was added with nano-gel containing the endophytic bacteria. The added inoculum in the form of nano gel was able to colonize the root rhizosphere efficiently. It, therefore, could increase the abundance of these genera, which are already present in the chilli root microbiome. *Bacillus* produces stress-tolerant spores, ACC, siderophores, and exopolysaccharides, while *Streptomyces* produces bioactive natural products and activates the nitrogenase enzymes (Bhat et al., 2022).

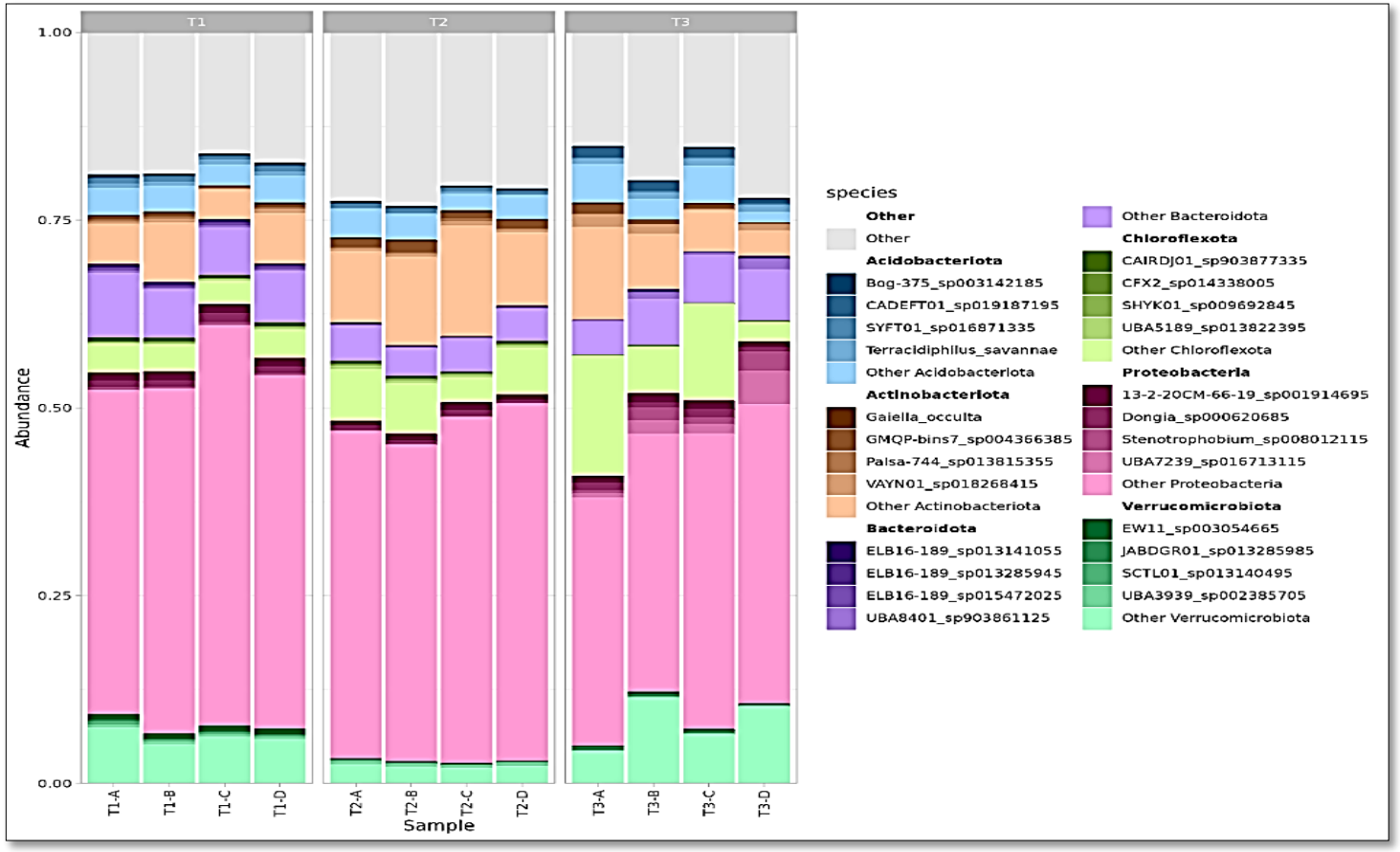


Fig. 7.17 Species-level identification of each chilli root sample in all the treatments

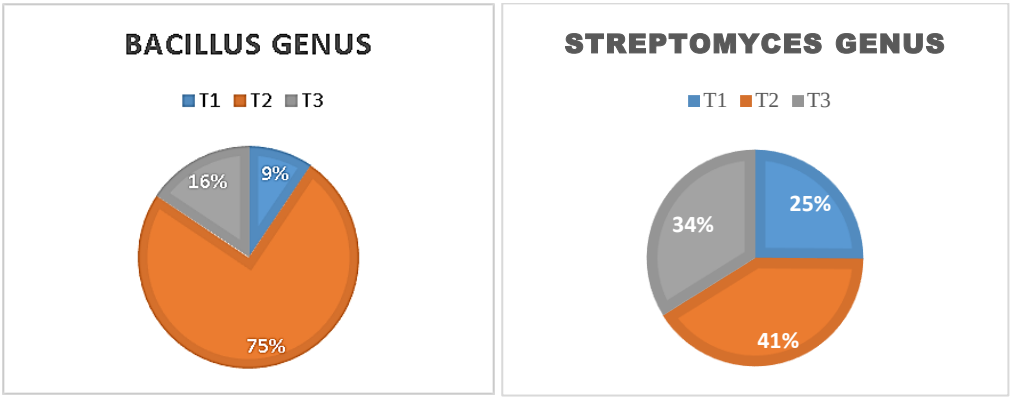


Fig. 7.18 Percentage of *Bacillus* and *Streptomycetes* genus in the root microbiome of all three treatments

7.2.13 Correlation of the Chilli Root Microbiome

The correlation data shows that Firmicutes A and G in Treatment 2 were positively correlated with each other (**Fig.7.19**). The P-value threshold was 0.05, and the correlation threshold was 0.8. Correlation analysis was done based on Sparse Partial Correlation Coefficient (SPARCC), a method for inferring the correlation structure among multiple variables in large data sets. It is a statistical method used to examine the relationship between two or more variables to determine if there is a relationship between the variables and the nature of the relationship. Firmicutes phylum was abundant in treatment 2 (AB fertilizer + Bio-stimulant). *Bacillus* belongs to the Firmicutes phylum, a group of gram-positive endophytic bacteria that was added as nano-gel in treatment 2 of this study. Firmicutes play a role in plant growth and health by forming symbiotic relationships with plant roots. Some species of Firmicutes include symbiotic associations with plant roots, known as root-associated microbiomes or rhizospheres. These bacteria can play several vital roles in plant growth, including providing the plant with essential nutrients, suppressing harmful pathogens, and promoting the overall health and development of the plant.

In particular, Firmicutes can help plants absorb nutrients from the soil, such as nitrogen, phosphorus, and sulfur, and can improve nutrient uptake efficiency. They can also protect plants from phytopathogens by producing antibiotics and

other secondary metabolites and outcompeting harmful microbes for nutrients. Additionally, Firmicutes have been shown to positively affect plant growth and yield by producing plant growth-promoting compounds such as auxins, gibberellins, and cytokinins. Some species of Firmicutes can also help improve soil structure by producing exopolysaccharides, which will enhance the stability and fertility of the soil.

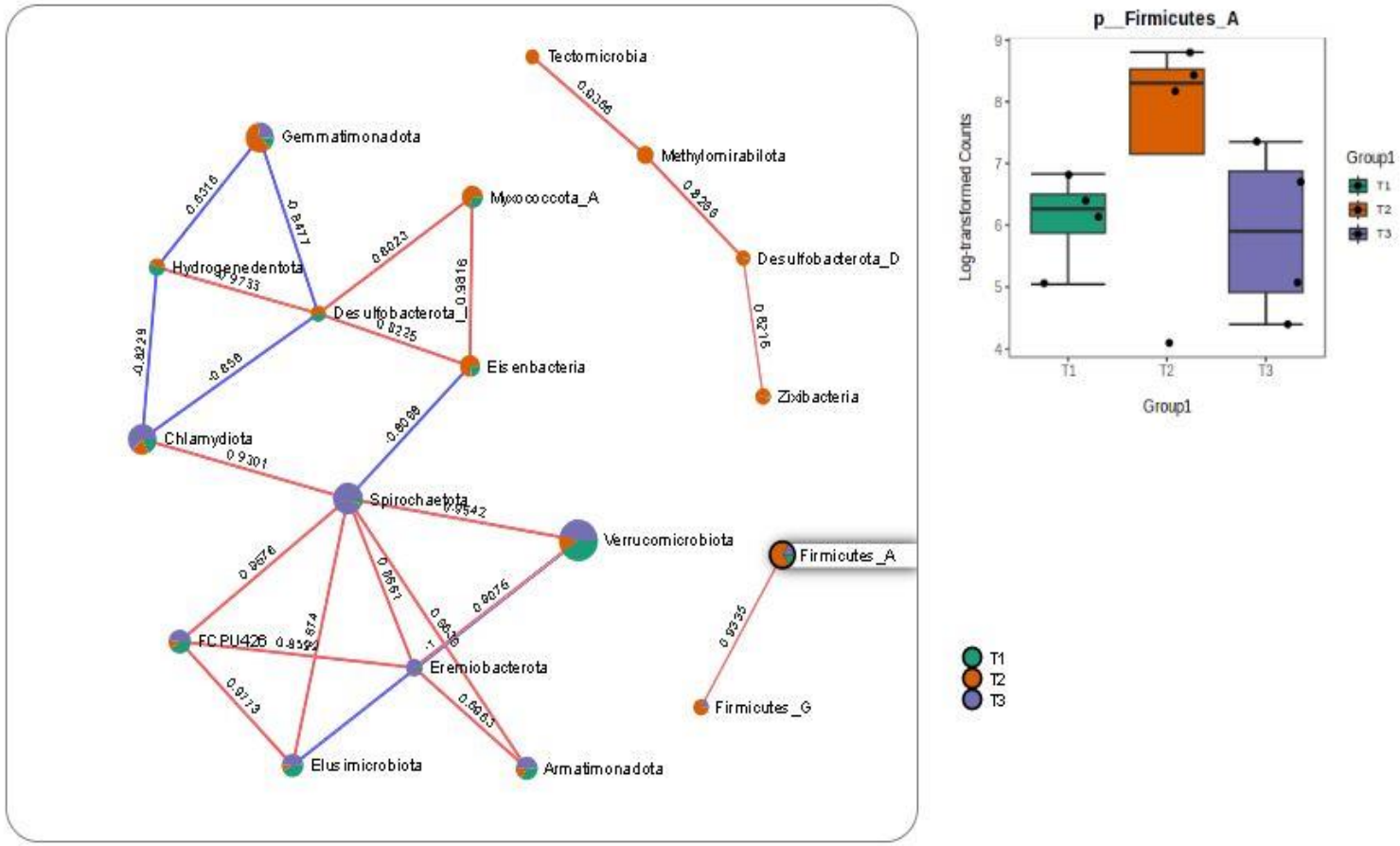


Fig. 7.19 Correlation between bacterial genus in chilli roots using Sparse Partial Correlation Coefficient (SPARCC)

7.2.14 Linear Discriminant Analysis Effect Size (Biomarker identifier)

At the phylum level, Firmicutes were found to be abundant in treatment 2 (**Fig. 7.20**). This is again proving the endophytic *Bacillus* that was added as the nano-gel bio-stimulant has played an essential role in increasing the abundance of the Firmicutes phylum in treatment two as compared to other treatments. Apart from the Firmicutes phylum, other phyla that were listed as the biomarker of the microbiome in chilli roots of treatment 2 include Myxococcota, Gemmatimonadota, Bdellovibrionota, Desulfobacterota_B, Nitrospirota, and Eisenbacteria.

Linear Discriminant Analysis (LDA) is a statistical technique used in microbiome studies to identify the main difference between microbial communities or groups of individuals. It is used to determine the most informative microbial profiles for distinguishing microbial communities. It is used to predict the class of a given sample based on its microbial composition. LEfSe (Linear Discriminant Analysis Effect Size) is a bioinformatics tool to identify biomarkers from high-dimensional biological data. A computational method uses linear discriminant analysis (LDA) to detect differences in the relative abundance of taxa (such as bacteria, viruses, or fungi) between two or more groups of samples. LEfSe aims to identify the taxa that are most responsible for the observed differences between the groups. LEfSe works by comparing the relative abundance of each taxon in each group of samples and quantifying the effect size of each taxon. It then applies a statistical test to determine the significance of the effect size. It generates a cladogram that visualizes the taxa most responsible for the observed group differences. The cladogram provides a hierarchical view of the taxa and allows researchers to identify the discriminatory taxa at different levels of resolution.

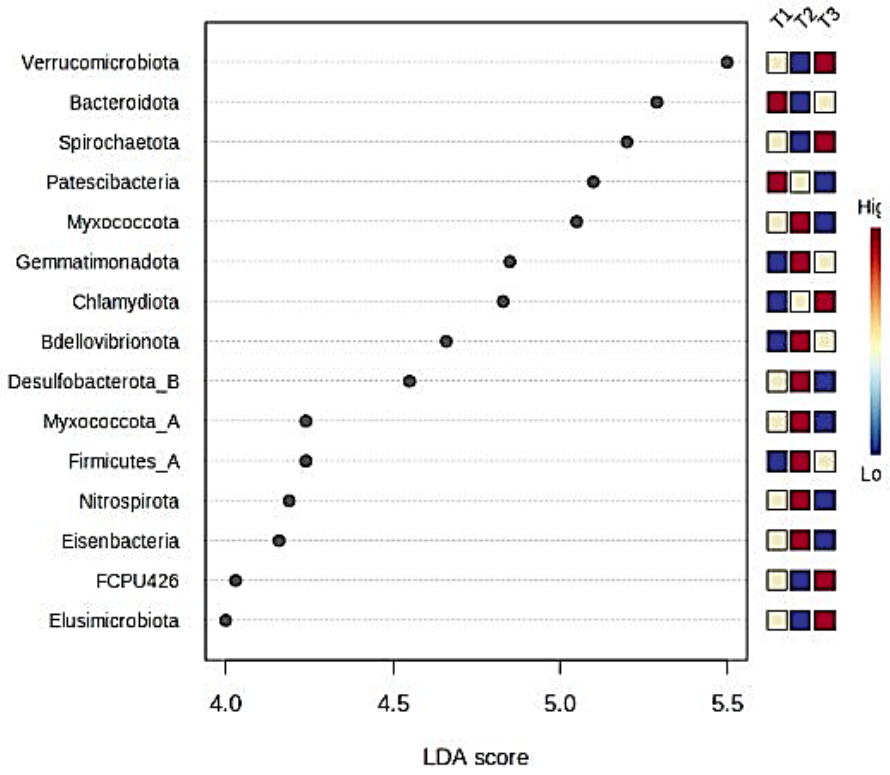


Fig. 7.20 Linear Discriminant Analysis (LDA) scores of differentially abundant species among individuals in each treatment. The top 15 LDA scores represent the effect size of each large phylum. Phyla enriched in each group with an LDA score >2 is considered.

One of the biomarkers identified at the class level was Actinomycetia, which was abundant in treatment 2 (**Fig. 7.21**). The actinomycetes class was not plentiful in treatments 1(AB fertilizer) and 3 (Control). This reveals the impact of applying nano gel-containing *Streptomyces* in treatment 2 (AB fertilizer + Bio-stimulant). Other classes of bacteria, such as Ktedonobacteria and Verrucomicrobiae, were the natural microbiome present in chilli roots and function as the biomarker for the indigenous microbiome in chilli roots.

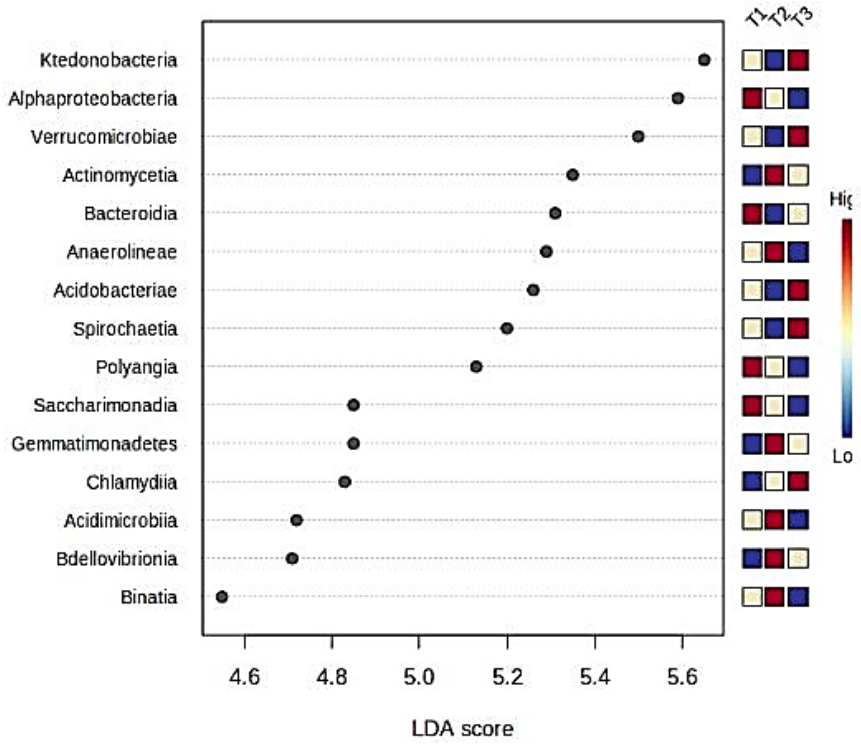


Fig. 7.21 Linear Discriminant Analysis (LDA) scores of differentially abundant species among individuals in each treatment. The top 15 LDA scores represent the effect size of each abundant class. Classes enriched in each group with an LDA score >2 are considered.

The LDA scores revealed that certain bacterial families had predominated the chilli roots under different treatments. For example, the native microbiome found in chilli root is Ktedonobacteraceae, the most abundant microbial family in the control samples (T3) not treated with AB fertilizer or bio-stimulant. AB fertilizer treatment alone (T1) has favored the significant growth of Opitutaceae, Chitinophagaceae, Beijerinckiaceae, and Polyangiaceae families. Nano-encapsulated bio-stimulant and AB fertilizer (T2) might have triggered the plant roots to secrete root exudates and recruit distinct bacterial phylum to dominate chilli roots, especially Verrucomicrobiota, Bacteroida and Spirochaetota and Ktedonobacteria (**Fig. 7.20** and **7.21**).

Verrucomicrobiota phylum is a slow-growing but highly abundant bacteria in complex root microbiota. It was estimated to account for up to 25% of rice root

endophytes that play a hidden role in the root interactome (Bünger, Jiang, Müller, Hurek, & Reinhold-Hurek, 2020). The interactome is the whole set of molecular interactions in a particular cell. Verrucomicrobiota is highly diverse in metabolic capabilities and possesses great potential to play an essential role in various global geochemical cycles (Chiang et al., 2018). Bacteroidota is ecologically crucial for the proper functioning of the soil as they represent a versatile group of bacteria with many unique molecular mechanisms (Kruczyńska et al., 2023). Spirochaetota is chemoheterotrophs that can survive in anaerobic or micro-aerobic environments and effectively degrade pectin, phosphate, and cellulose (A. Liu et al., 2022). Ktedonobacteraceae are a diverse group of bacteria commonly found in the rhizosphere and the soil surrounding plant roots, and they play an essential role in soil ecology and nutrient cycling. They are known for their ability to fix nitrogen, converting atmospheric nitrogen into a form that plants can take up. Ktedonobacteraceae is a family of bacteria within the order Rhizobiales, a group of nitrogen-fixing bacteria that form symbiotic relationships with plants, providing them with essential nitrogen and other nutrients in exchange for carbon compounds (Erlacher et al., 2015).

7.2.15 Association among Chilli Root Microbial Profiling, Bio-stimulant Treatment and Nutrient Content in Soilless Medium

Heatmaps display correlations or patterns in large data sets, allowing for quick and easy identification of areas of high or low values. This study examined the correlation between Actinobacteriota and Firmicutes phyla-containing bacterial bio-stimulant used in three treatments and the nutrient content of soilless media (**Fig. 7.22**). The intensity of the color corresponds to the magnitude of the value. Actinobacteria are a diverse group of microorganisms with varying physiological and metabolic characteristics. Some Actinobacteria are known to be efficient at mobilizing phosphorus, while others may not be able to utilize phosphorus as effectively. Soilless medium conditions such as pH and moisture can also influence the relationship between soilless medium nutrient content and bacteria. Both treatments 1 and 2 were applied with AB fertilizer, which significantly affects the pH of the soilless medium, while T3 was not applied with AB fertilizer. Therefore, it could have a desirable pH for the growth of

Actinobacteria. It is important to note that the pH requirements for Actinobacteria can vary depending on specific environmental conditions, nutrient availability, and other factors. The Actinobacteriota group in T3 was positively correlated with the nutrient contents such as P_2O_5, K_2O, and MgO. The Firmicutes phylum in T1 was positively correlated with N, CaO, P_2O_5, K_2O, and MgO in the soilless medium in T1. In contrast to these positive correlations, Actinobacteria and Firmicutes in treatment two mainly were negatively correlated with the nutrient content of the soilless medium. This indicates that the bio-stimulant containing endophytic bacteria could either utilize the nutrients for its consumption or involve in biological activities to mobilize the nutrients for plant uptake. Actinobacteria are often considered oligotrophic bacteria, meaning they thrive in nutrient-poor environments. A negative correlation between nutrients and Actinobacteria indicates that as the abundance of Actinobacteria increases, the nutrient availability in the soilless medium may be low. Actinobacteria are known to efficiently consume or mobilize nutrients, such as phosphorus, in the growth medium. As a result, an increase in the abundance of Actinobacteria can lead to a depletion of available nutrients in the soilless medium. Only treatment 2 shows a neutral to negative correlation for all the nutrients in the soilless medium for Actinonobacteriota and Firmicutes group, indicating these two phyla's role in mobilizing nutrients for plant uptake. A similar observation was reported when the relative abundance of Firmicutes and Actinobacteria in the tomato rhizosphere was negatively associated with the incidence of bacterial wilt disease (Xiong & Lu, 2022).



Fig. 7.22 Heatmap shows the correlation between Actinobacteriota and Firmicutes groups in different treatments and the nutrient content in the soilless medium. (+1 indicates positive correlation and-1 indicates negative correlation)

7.2.16 Association among Chilli Root Microbial Profiling, Bio-stimulant Treatment and Nutrient Content in Different Parts of the Chilli Plant

The relationship between chilli root microbiome, especially Actinobacteriota and Firmicutes, and nutrient uptake by chilli plants was investigated (**Fig. 7.23**). The nutrient uptake could be related to nutrient accumulation at different parts of the chilli plant, such as leaves, stems, fruits, and roots. Only treatments 1 and 2 were compared to assess the relationship between bacterial bio-stimulant and nutrient content in various parts of the chilli plant.

High nutrient content in chilli leaves was positively correlated with the abundance of Actionabacteriota found in chilli roots in T1 (AB fertilizer). The positive correlation between nutrient content in chilli leaf and the quantity of Actionabacteriota in chilli roots may suggest that these bacteria were involved in the plant's uptake and utilization of nutrients. However, this might not be true, as the same amount of nutrient content was found in the leaf of chilli plants treated with AB fertilizer and bio-stimulants (T2). Still, it correlated negatively with the abundance of Firmicutes and Actinobacteria group. The high nutrient content in chilli leaf could be related to the direct nutrient absorption from the fertigation liquid fertilizer. Past research has stated that phosphorus and potassium content in roots were closely associated with the crop's enhanced root length, indicating that improvised nutrient uptake was observed in plants treated with seaweed extracts (Mutale-joan et al., 2020). Based on **Table 7.5**, the nutrient contents in different parts of chilli plants treated with T1 (AB fertilizer) and T2 (AB fertilizer+ Bio-stimulant) were not significantly different. This shows that bio-stimulant application did not cause any difference in nutrient uptake or nutrient accumulation in different parts of chilli plants. Other bacteria in the root microbiome, apart from these two groups, could have contributed to the increase in plant growth promotion regardless of the treatments. For example, the native microbiome found in chilli root is Ktedonobacteraceae, known for its ability to fix nitrogen and could have contributed to supplying N to chilli leaf. Likewise, Verrucomicrobiota is another bacterium phylum found in abundance in chilli roots that degrade complex carbohydrates and release nutrients necessary for plant growth and development. In addition, these bacteria were

found to produce hormones and other signaling molecules that can influence plant growth and stress responses. Past study has indicated that different exudate compounds secreted by the roots may act synergistically in selecting the root-associated bacterial communities (P. Wang et al., 2022). This explains plant root exudates' complexity in shaping root microbiomes. It was also reported that soil microbial community structure and activity of soil microbes could be altered by fertilization, where Proteobacteria were enriched under N application. At the same time, Actinobacteria and Acidobacteria were found under low or medium N application (S. Yang, Xiao, Liang, He, & Tan, 2021). This shows that the effect of fertilization or bio-stimulant application could be observed in the composition of the soil bacterial community as whole and dominant soil bacterial phyla. Nano-encapsulated bio-stimulants consist of moringa leaf extract, *Streptomyces* sp., and *Bacillus* sp. Thus, the change in the microbial community could be related to the synergistic effect of nano-encapsulated endophytic bacteria and moringa leaf extract. Therefore, assessing the total microbial community change could be a more practical approach than the targeted bacterial phylum, as complex interactions occur in the root microbiome due to the presence of root metabolites and external metabolites provided by the bio-stimulant components.

Probably the increased abundance of Actinobacteriota and Firmicutes due to the application of bacterial bio-stimulant in T2 might have outcompeted the Ktedonobacteraceae, Alphaproteobacteria, and Verrucomiota groups of bacteria that dominated chilli roots. The interaction between these dominant groups and inoculated endophytic bacteria is unpredictable. The positive correlations observed for Treatment 2 include root nutrient content, Actinobacteriota in roots, stem nutrient content, and Firmicutes in roots. This shows that the nutrient content in chilli leaf and chilli fruits are not directly correlated with the abundance of endophytic bio-stimulant in chilli roots. The endophytic bio-stimulant could have reduced the indigenous beneficial bacteria in chilli roots as it has robust colonization and antibacterial potential. It is also possible that the nano-emulsion of moringa leaf act as bio-control to inhibit the growth of the indigenous beneficial bacteria in roots. It was also previously mentioned that inoculated PGPR triggers biochemical changes in the plant root to attract other

specific bacteria (Ortiz-Liébana, Zotti, Barquero, & González-Andrés, 2022). These results have given a remarkable insight that the beneficial bacteria or bio-stimulant added as inoculum might inhibit the proliferation of naturally beneficial bacteria in plant roots. The naturally useful bacteria might not be culturable on agar media, as some require specific growth conditions or interactions with other microorganisms to thrive in the rhizosphere. It was also reported that a single PGPR strain, no matter how effective it might be in facilitating plant growth under laboratory conditions, is extremely unlikely to be able to meet all of a plant's needs in the natural environment occupied by thousands of different indigenous microorganisms (Glick & Gamalero, 2021). Therefore, studying the interaction of indigenous and inoculated microbes is crucial to better understand the application method and dosage. Besides bacteria, fungi, protists, and algae also regarded as the potential members of the crop microbiome, which can protect crop health and yield (Xiong & Lu, 2022).

Chilli leaves	p__*Actinobacteriota* (T1)	p__*Actinobacteriota* (T2)	p__*Firmicutes* (T1)	p__*Firmicutes* (T2)
Ash (%)	0.95	0.06	-0.14	-0.76
N (%)	0.96	0.00	0.14	-0.40
P (%)	0.86	0.19	-0.29	-0.64
K (%)	-0.70	0.34	-0.12	-0.60
Ca (%)	0.94	0.73	-0.21	-0.03
Mg (%)	0.94	-0.15	-0.22	-0.71
Chilli roots				
Ash (%)	0.62	0.72	-0.12	0.12
N (%)	0.39	0.83	-0.12	0.51
P (%)	0.25	0.83	-0.51	-0.12
K (%)	0.83	0.83	-0.89	0.12
Ca (%)	0.89	0.83	-0.12	0.51
Mg (%)	0.95	0.83	0.51	-0.12
Chilli fruits				
Ash (%)	0.25	-0.83	0.51	0.12
N (%)	0.39	-0.83	0.51	0.12
P (%)	0.83	0.20	0.51	0.51
K (%)	-0.64	-0.72	-0.12	0.12
Ca (%)	0.83	-0.72	0.51	-0.12
Mg (%)	0.72	0.83	-0.89	-0.39
Chilli stems & twigs				
Ash (%)	-0.21	0.16	-0.37	0.36
N (%)	-0.72	0.17	-0.12	0.36
P (%)	0.43	0.09	-0.07	0.33
K (%)	-0.09	0.09	-0.33	0.33
Ca (%)	0.09	0.83	0.33	0.51
Mg (%)	-0.09	-0.17	-0.33	0.21

Fig. 7.23 Heatmap shows the correlation between Actinobacteriota and Firmicutes groups in different treatments and the nutrient content in different parts of chilli plant.
(+1 indicates positive correlation and indicates negative correlation)

7.3 Conclusion

The addition of nano-encapsulated bio-stimulant that promotes crop health and productivity has emerged as a promising biotechnological approach towards achieving sustainable food production and security. It was proven in this study that nano-encapsulated bio-stimulant applied via a soilless growing medium could enhance the height of chilli. However, root and aboveground biomass did not show significant differences between treatments. The photosynthesis of plants treated with bio-stimulant was higher than in other treatments. Bio-stimulant significantly increased the carotenoid content, yield, and length of chilli. Post-harvest analysis revealed that bio-stimulant positively affects the phenolic content in green chillies, flavonoid content in green and red chillies, and ascorbic content in red chillies.

In general, nano-encapsulated bio-stimulant could positively contribute to plant growth. A shift in the chilli root microbiome revealed the capability of the bio-stimulant to cause changes in improving the bacterial diversity of soilless medium than soil. This indicates that nano-encapsulated bio-stimulant could be used successfully in the soilless medium for better microbial establishment. Next-generation sequencing revealed the colonization potential of the nano-encapsulated endophytic bacteria as the *Bacillus* and *Streptomyces* genus were found to dominate chilli roots treated with nano gel-containing endophytic bacteria. The bio-stimulant application changed the taxonomy of the root microbiome as it exhibited the highest unique amplicon sequences. The alpha and beta diversity also showed a significant difference between treatments. According to the biomarker analysis, the use of bio-stimulant resulted in a shift in the predominant groups in the chilli root rhizosphere, and this was associated with a negative correlation with nutrient absorption by various parts of the chilli plant.

CHAPTER 8
GENERAL CONCLUSION

The main objective of this study was to assess the impact of nano-encapsulated bio-stimulant on a soilless medium. This study can be regarded as pioneering research in the use of bio-stimulant for soilless medium, which has the potential to enhance nutrient absorption by plants, ultimately leading to improved plant growth, yield, and quality. The primary limitation of bio-stimulant products is their short shelf life and decreased efficacy caused by the rapid degradation of active ingredients exposed to environmental stress. The concentration and viability of microorganisms in the liquid culture during storage can also be reduced, further reducing the product's effectiveness. The present study involves the development of a nano-encapsulated bio-stimulant aimed at enhancing its absorption, extending its shelf life, and assessing its efficacy in chilli cultivation via a fertigation system. In this study, a novel approach was used to evaluate the combined application of a nano-gel containing endophytic bacteria (*Bacillus* sp. and *Streptomyces* sp.) and a nanoemulsion of moringa leaf (aqueous extract) in a soilless medium. This approach had not been previously used in other studies, making it unique and innovative. The study aimed to investigate the potential benefits of using this combination of techniques to improve plant growth and yield. The results of this study may provide valuable insights into the use of nanotechnology in agriculture and its potential to enhance crop productivity.

Chapter 3 demonstrated the selection process of plant growth-promoting endophytes derived from medicinal plants to produce a microbial bio-stimulant. The qualitative and quantitative assays revealed that most bacterial endophytes from moringa roots exhibited the most beneficial traits, followed by those from chilli roots. Seven of the 70 endophytic bacteria studied were chosen for further experiments based on their display of multiple and more robust plant growth-promoting beneficial traits. The results of chapter 3 indicate that endophytic bacteria from moringa roots can be regarded as potential candidates for bio-stimulant development.

Chapter 4 identified endophytes with beneficial traits belong to the *Streptomyces, Bacillus*, and *Enterobacter* groups. Seven endophytic isolates were found to enhance chili seed germination and seedling growth, with mixed cultures displaying better root development during the seedling stage than single cultures. The study demonstrated that non-native endophytic bacteria from moringa roots could improve the vegetative growth of chilli seedlings. While the *Enterobacter* group was an efficient bio-stimulating agent for chilli seedlings, it was not recommended for use in field experiments due to bio-safety concerns. MR3 (*Bacillus subtilis*) and MR13 (*Streptomyces panaciradicis*) were identified as potential endophytes for bio-stimulant formulation. Metabolite production by endophytes revealed that these endophytic bacteria could produce several essential and beneficial active compounds for agricultural use, such as carotenoids and antioxidants. Furthermore, mixed cultures of *Streptomyces* and *Bacillus* exhibited a bio-stimulating effect in increasing the length, root biomass, aboveground biomass, and yield of chilli. This chapter suggests that non-native endophytic bacteria could be effective in colonizing and exhibiting beneficial effects in different crops.

Chapter 5 has shown that water extraction of moringa leaf is a cost-effective method for producing organic bio-stimulant for chilli. Moringa leaf contain antioxidants that enhance chilli's health and root growth for better nutrient use efficiency. Dry moringa leaf decoction extract was effective in significantly promoting vegetative growth, especially the root and shoot biomass of chilli plants, when applied with dry moringa leaf maceration extract, alone or with *Bacillus*. Thus, dry leaf decoction extract was applied throughout the plant's vegetative growth. In addition, dry leaf maceration extract was applied during the flowering period in a shade-house experiment, as both moringa extracts demonstrated different efficiencies during different crop development stages. These findings suggest that moringa leaf bio-stimulants could be customized according to their potential function at a specific phenological stage of the crop.

A suitable formulation is crucial to maintain the viability and density of bacteria in a bio-stimulant. Moreover, the compatibility of the plant extract and endophytic bacteria in a bio-stimulant is also vital to ensure efficient field

application. To address these issues, the focus of **Chapter 6** was on nano-formulations that can protect the active ingredients from environmental degradation and enhance their absorption by plant cells. The results suggest that the nano-emulsion method could be used to encapsulate moringa leaf extract while nano-gel can be used for endophytic bacteria to extend the shelf-life of active ingredients.

In **Chapter 7**, the effect of nano-encapsulated bio-stimulants that were applied by drenching the soilless medium was assessed on their potential to enhance the vegetative growth, quality, and yield of chilli. The results showed that the application of bio-stimulants had a notable impact on the vegetative growth, carotenoid content, yield, and length of the chilli plants. Furthermore, the post-harvest analysis demonstrated that the bio-stimulants positively influenced the phenolic content in green chillies, flavonoid content in both green and red chillies, and ascorbic content, specifically in red chillies. Through next-generation sequencing, it was discovered that the use of nano-gel containing endophytic bacteria could colonize chilli roots, with the *Bacillus* and *Streptomyces* genus being the most prevalent. Additionally, the application of the bio-stimulant resulted in a shift in the taxonomy of the root microbiome, with the highest number of distinct amplicon sequences observed. The analysis of biomarkers indicated that the application of the bio-stimulant caused a change in the dominant groups present in the rhizosphere of chilli roots, which was found to be negatively correlated with nutrient uptake by different parts of the chilli plant. The results of chapter 7 suggest that nano-encapsulated bio-stimulant consisting of endophtic bacteria and moringa leaf extract could be applied to fruity vegetables as it has the potential to increase the carotenoid and ascorbic acid content of the fruits. It also has the potential to increase the photosynthesis activity in the plant and the antioxidant activity of the fruits.

Overall, encapsulated bio-stimulant led a notable impact on the vegetative growth, length, yield, phenolic, flavonoid and ascorbic acid content of chilli. Endophytic bacteria, *Bacillus* and *Streptomyces* produced many pigmentation compounds which could have increased the carotenoid contents in chilli leaves and fruits. Carotenoid contents could be related to the enhanced photosynthesis,

increased yield and improved color and flavour of chilli fruits. High carotenoid content also could be associated with high antioxidant content and improved plant immunity. On the other hand, moringa leaf extract rich in flavonoid and phenolic acids also could have improved pigmentation compound production in chilli plants. Flavonoids also regulate cell growth of chilli plants that could directly affect the vegetative growth promotion.

The feasibility of utilizing nano-encapsulated bio-stimulants to enhance the growth, yield, and quality of chilli has been established in this study, especially in promoting yield and the quality of chilli fruits. A significant positive response of combined application of moringa leaf and endophytic bacterial bio-stimulants on the vegetative growth before formulating it indicates that the dosage and application method must be optimized to maximize the crop's benefits.

Future studies shall focus on the most efficient method and time of bio-stimulant application methods including seed bio-priming, root drenching, foliar application, and drenching of planting material. The best time to apply bio-stimulants has to be determined by evaluating the growth and yield of crops treated with bio-stimulants at various phenological stages of crop, such as seed bio-priming, seedling growth, transplanting, vegetative growth, flowering, and fruiting stages. Further advancements could be made to this technology for its implementation in fertigation systems and soilless mediums to enhance crop cultivation in the future. Encapsulated bio-stimulants such as nano-emulsions and nano-gels can be diluted and applied to plants via fertigation systems for various crops. This study also simplified the application method, such as using water for moringa leaf extraction and low concentrations of alginate and other bulking materials, considering the cost when produced on a large scale. However, the challenge of this study is the economic feasibility, where the actual price and profit analysis need to be done based on in situ productivity in vegetable farms. The efficiency and user-friendly characteristics of the product must be tested in real farmers' plots to ensure that the growers will accept and use the encapsulated bio-stimulant products.

Below is the list of conference papers and journal manuscripts from this study.

Peer-reviewed and published journal article

- Stella, M., Asgar, A. and Yasmeen, S. (2022). Plant Bio-stimulant: Prospective, Safe and Natural Resources. Journal of Soil Science and Plant Nutrition. Volume 22, Issue 2, Pages 2570 – 2586. https://doi.10.1007/s42729-022-00828-6

  This review highlights studies conducted on both the bulk materials and nanoparticles as bio-stimulant, and the article was published in a Q1 (Springer) journal with an impact factor of 3.6.

Published and peer-reviewed conference papers

- Stella, M., Asgar, A. and Yasmeen, S. (2022). Efficacy of endophytic bio-stimulant for root growth enhancement in chili. International Conference for Sustainable Agriculture Volume: 7th ASIAN PGPR International Conference. 23 - 26 August 2022 Universiti Putra Malaysia. This oral presentation demonstrates the efficiency of beneficial bacteia to stimulate the root growth of chilli. The extended abstract was published in the conference proceedings.

- Stella, M., Asgar, A., Yasmeen, S., Christina, V.S. (2022). Encapsulated Actinobacteria: Promising phyto-stimulant. 8th Plant Genomics and Gene Editing Congress: ASIA Co-located with the 3rd Microbiome for Agriculture Congress: Asia. 15th-16th November 2022. Kuala Lumpur, Malaysia.

  https://www.global-engage.com/event/microbiome-agriculture-asia/.

  The poster presented at this conference was selected as the best in the agriculture category, and I was given a 30-minute slot for an oral presentation during the conference.

- Stella, M., Asgar, A. and Yasmeen, S. (2022). Organic plant bio-stimulant for early, enhanced, and healthy growth of chilli seedlings. Chemistry proceedings. Volume 4, p.1-4. https://doi.org/10.3390/xxxxxwww.mdpi.com/journal/chemproc. The extended abstract was published in the e-proceedings.

Submitted articles under-review

- The research article "Green Agriculture: Bacterial Plant Bio-stimulant for *Capsicum annuum* Growth Promotion" is under review by the Journal of Plant Growth Regulation (Q1 Springer journal with an impact factor of 4.64). This research article demonstrates the endophytic bacterial bio-stimulants effectiveness in enhancing seed germination, root growth, and colonization of chilli.

- Review paper entitled "Enhancing and Evaluating the Effectiveness of Bio-Stimulant Products: Current Practices and Future Directions for Sustainable Food Production". -Ready for submission to a high-impact journal. The primary aim of this review is to emphasize the importance of enhancing the value of plant bio-stimulants while considering their impact on the environment and food security.

- The research article entitled "The interplay of nano-encapsulated bio-stimulant treatment and chilli root microbiome grown in the soilless medium." -Submitted to the supervisor. This study demonstrates the efficacy of nano-encapsulated bio-stimulants in improving the vegetative growth of chilli plants. It examines the changes in the root microbiome of chilli plants treated with nano-encapsulated bio-stimulant.

- The research article entitled "Synergistic effect of *Moringa oleifera* leaf extract and endophytic bacteria bio-stimulant on the growth and yield of *Capsicum annuum*." -Submitted to the supervisor. This study determined the efficiency of the co-application of aqueous extract of moringa leaves

and plant growth-promoting endophytic bacteria in promoting the growth and yield of chilli.

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Appendix

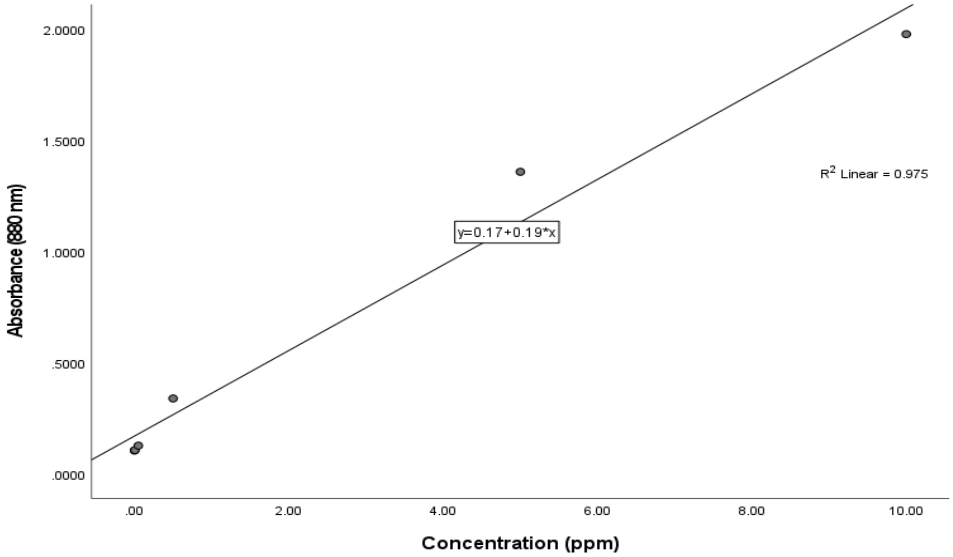


Fig. A1. Standard graph for soluble phosphorus (orthophosphate). There was a statistically significant, strong positive correlation between concentration of soluble P and absorbance at 880 nm, r (46) =.885, $p < .0005$ (IBM SPSS statistics, version 25).

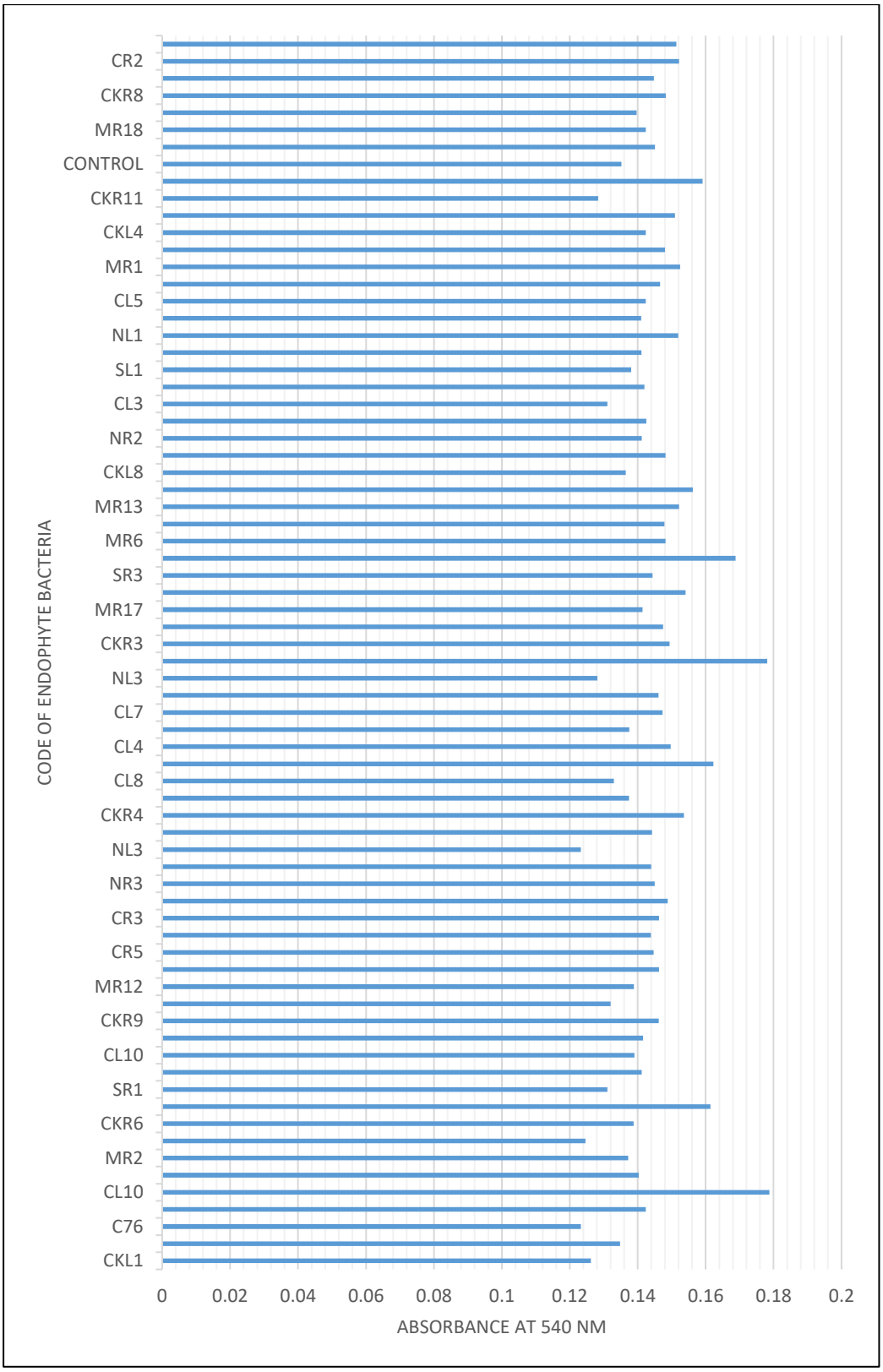


Fig. A2. Qualitative screening of ACC deaminase producing endophytes. Values are means of eight independent readings

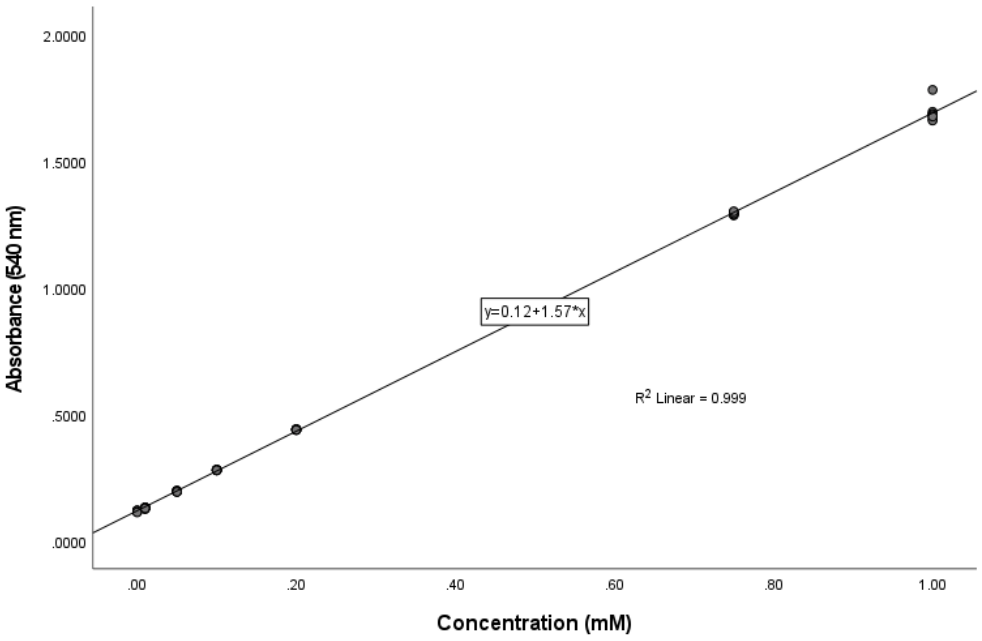


Fig. A3. Alpha ketoglutaric standard calibration curve. There was a statistically significant, strong positive correlation between concentration of α-ketoglutaric and absorbance at 540 nm, r (46) =0.999p < .0005 (IBM SPSS statistics, version 25)

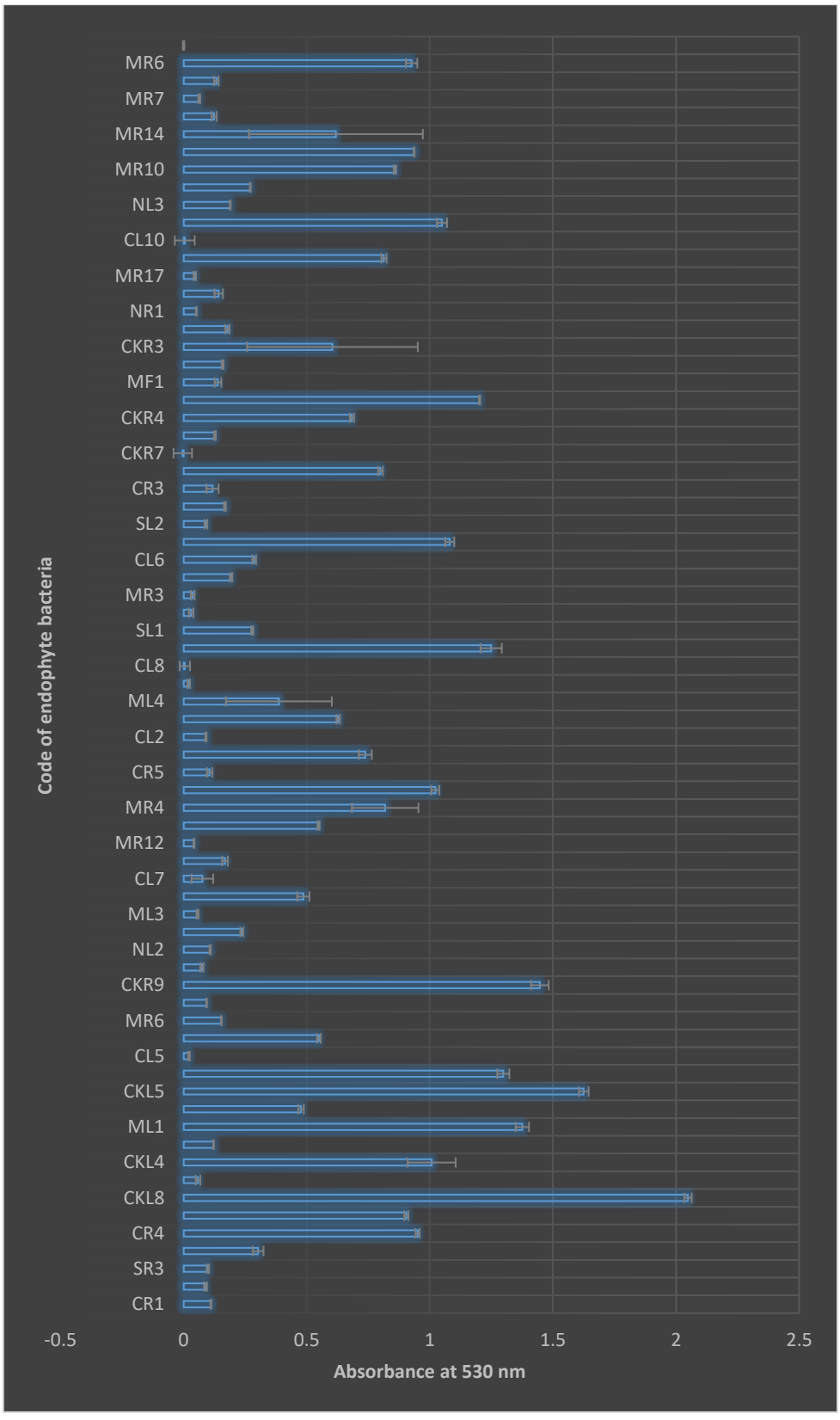


Fig. A4 Pre-liminary screening of indole acetic acid-producing endophytes. Values are means of eight independent readings

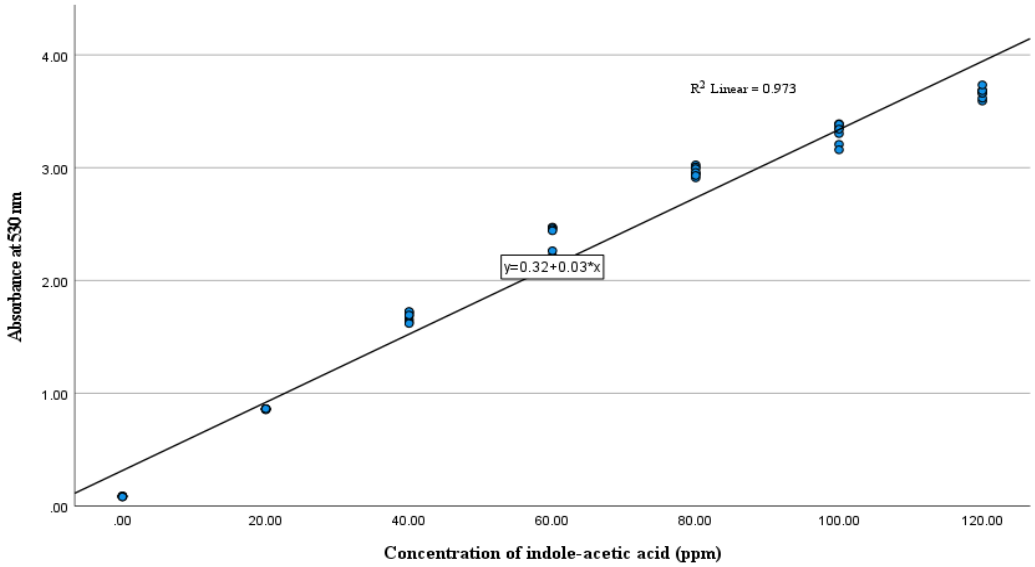


Fig. A5. Indole-acetic acid standard calibration curve. There was a statistically significant, strong positive correlation between concentration of indole-acetic acid and absorbance at 530 nm, r (46) =0.973p < .0005 (IBM SPSS statistics, version 25).

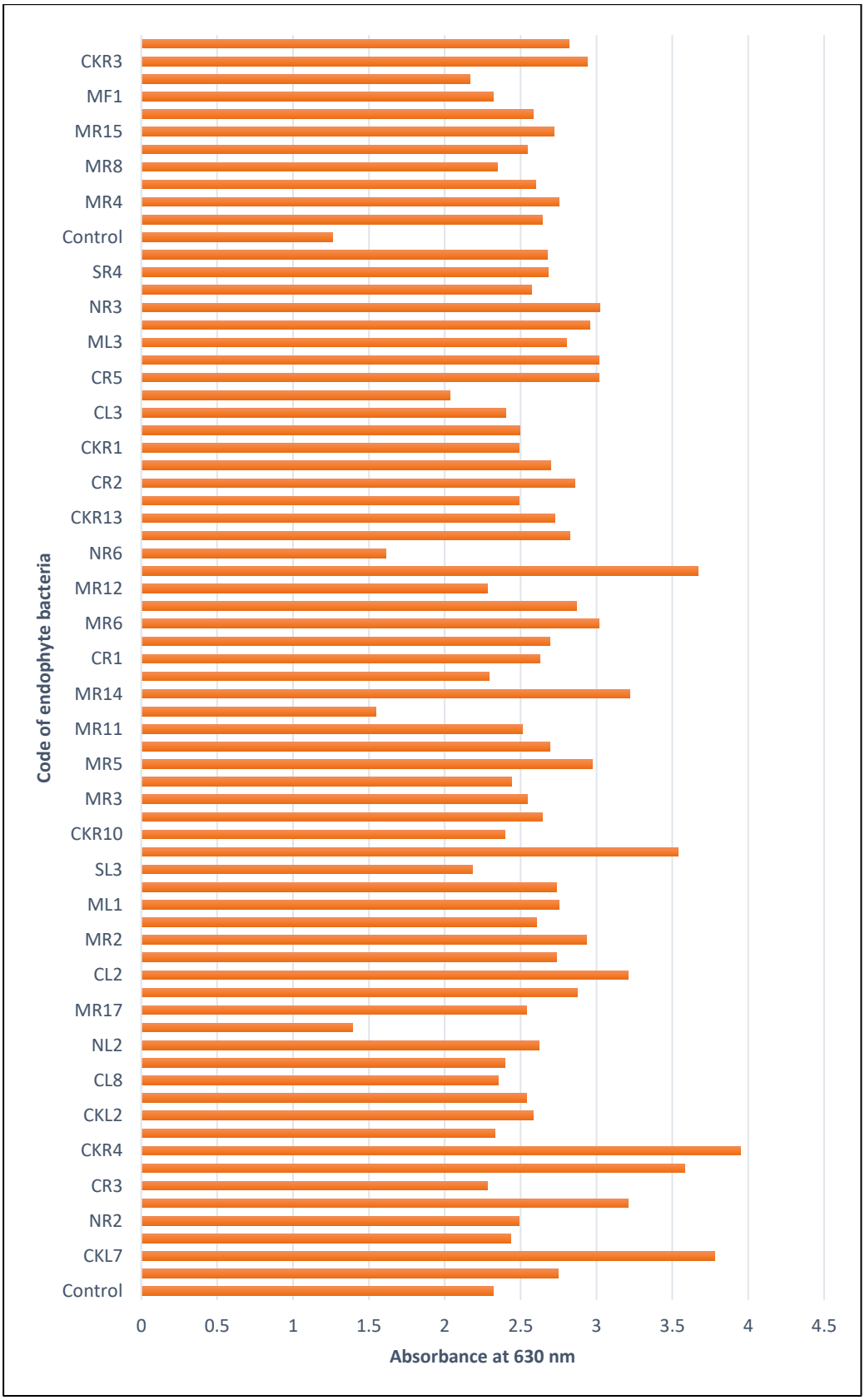
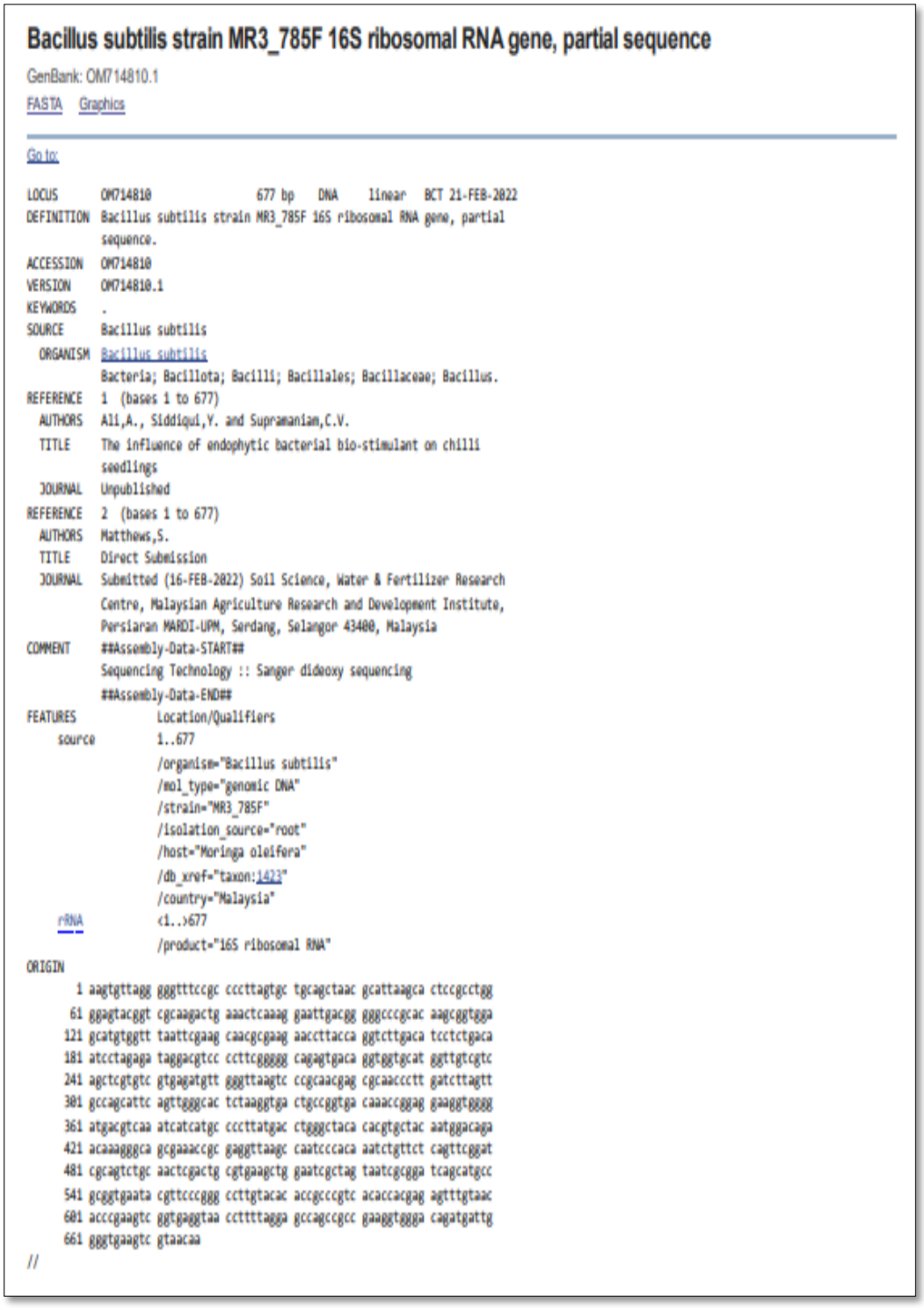


Fig. A6. Screening of siderophore producing endophytes. Values are means of eight independent readings

Table A4. Logistic regression predicting likelihood of seed germination based on treatments

Treatments	B	SE	Wald	df	Sig.	Exp (B)	95% CL for EXP (B) Lower Upper	
Water (Control)	-22.519		8.065	11	.707			
MR13	-22.456	5684.178	.000	1	.997	.000	.000	.
CKR8	-22.256	5684.178	.000	1	.997	.000	.000	.
MR10	-22.221	5684.178	.000	1	.997	.000	.000	.
MR3	-22.221	5684.178	.000	1	.997	.000	.000	.
MR1	-22.164	5684.178	.000	1	.997	.000	.000	.
CKL7	-22.030	5684.178	.000	1	.997	.000	.000	.
ML1	-21.864	5684.178	.000	1	.997	.000	.000	.
Moringa leaves extract	-23.218	5684.178	.000	1	.997	.000	.000	.
Chitosan (LMW)	.000	5684.178	.000	1	.997	.000	.000	.
Chitosan (MMW)	.000	8038.618	.000	1	1.000	1.000	.000	.
Constant	21.203	8038.618	.000	1	1.000	1.000	.000	.



Bacillus subtilis strain MR3_785F 16S ribosomal RNA gene, partial sequence

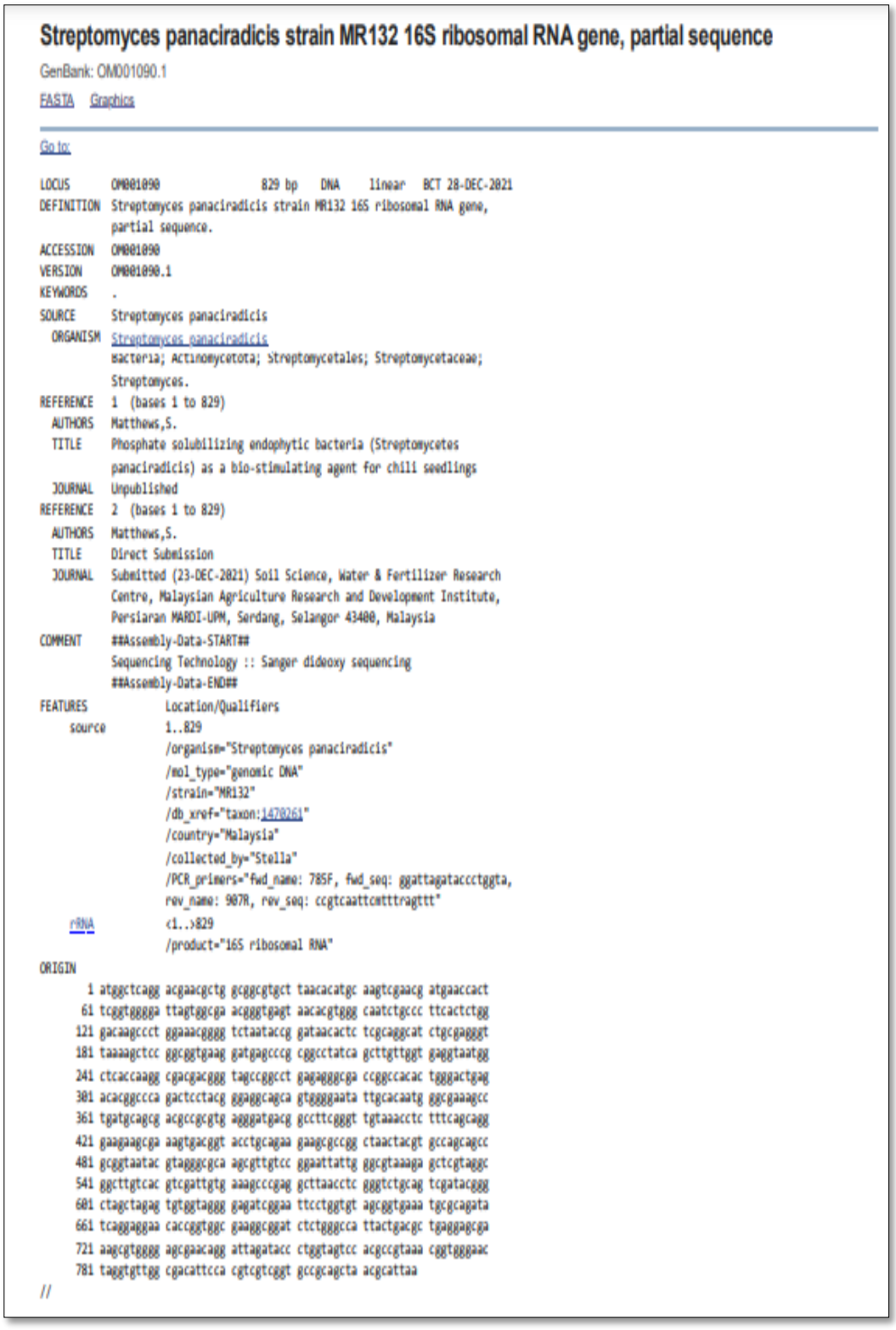
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Fig. A7. The DNA sequences of *Bacillus subtilis* (MR3) deposited in the NCBI database under the accession numbers OM714810



Streptomyces panaciradicis strain MR132 16S ribosomal RNA gene, partial sequence

GenBank: OM001090.1

FASTA Graphics

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            Streptomyces.
REFERENCE   1  (bases 1 to 829)
  AUTHORS   Matthews,S.
  TITLE     Phosphate solubilizing endophytic bacteria (Streptomycetes
            panaciradicis) as a bio-stimulating agent for chili seedlings
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 829)
  AUTHORS   Matthews,S.
  TITLE     Direct Submission
  JOURNAL   Submitted (23-DEC-2021) Soil Science, Water & Fertilizer Research
            Centre, Malaysian Agriculture Research and Development Institute,
            Persiaran MARDI-UPM, Serdang, Selangor 43400, Malaysia
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Fig. A8. The DNA sequences of *Streptomyces panaciradicis* (MR13) deposited in the NCBI database under the accession numbers OM001090

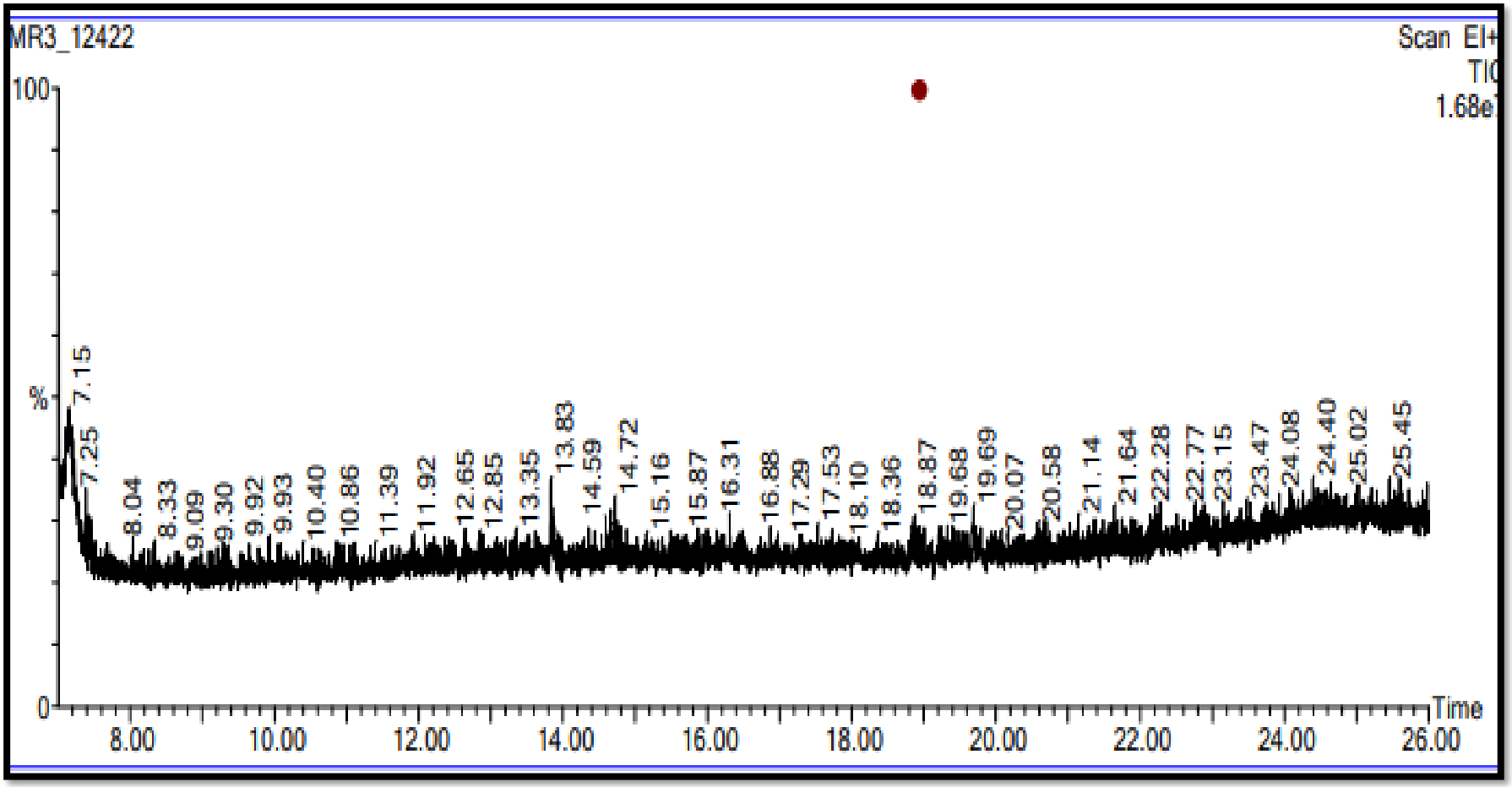


Fig. A9. GC-MS chromatogram of *Bacillus* sp. (MR3)

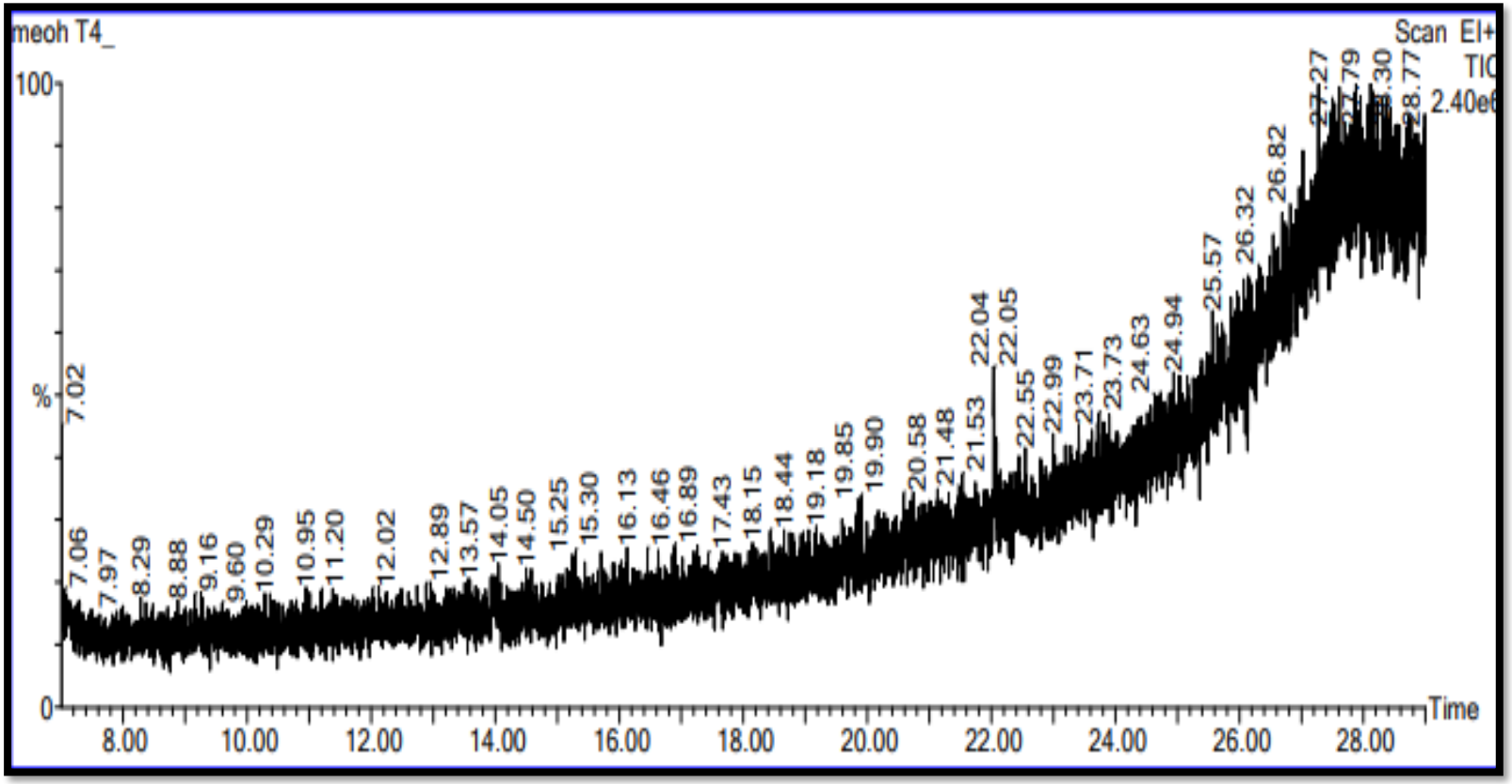


Fig. A10. GC-MS chromatogram of *Streptomyces* sp. (MR13)

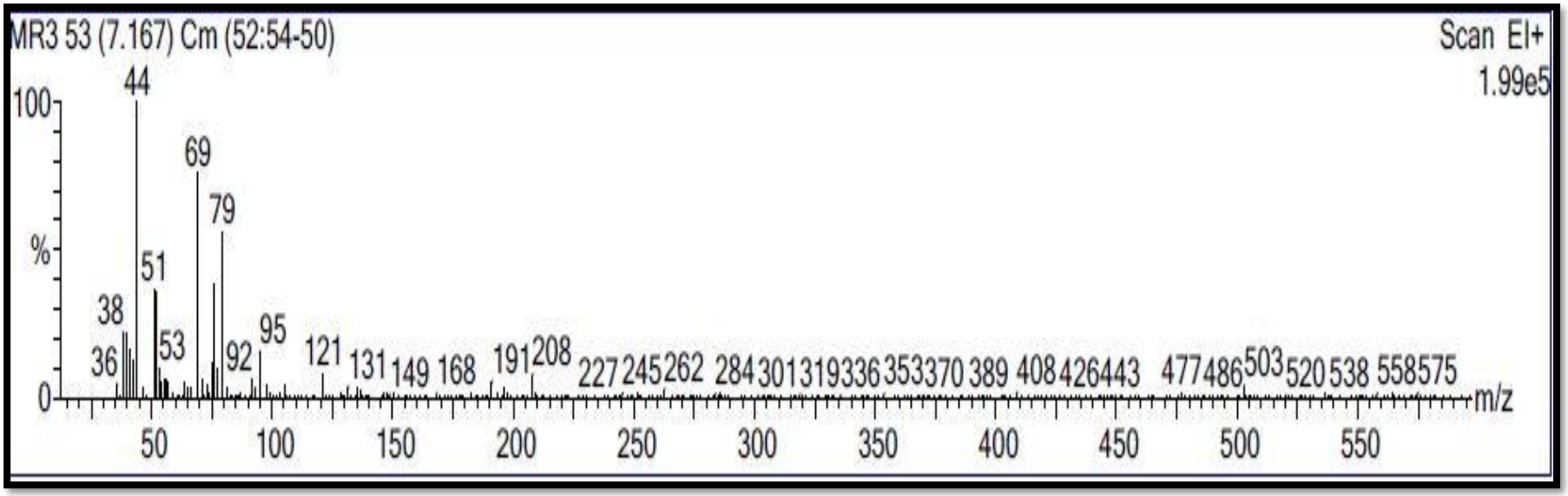


Fig. A11. The highest peak produced by *Bacillus sp*. at RT 7.167 min

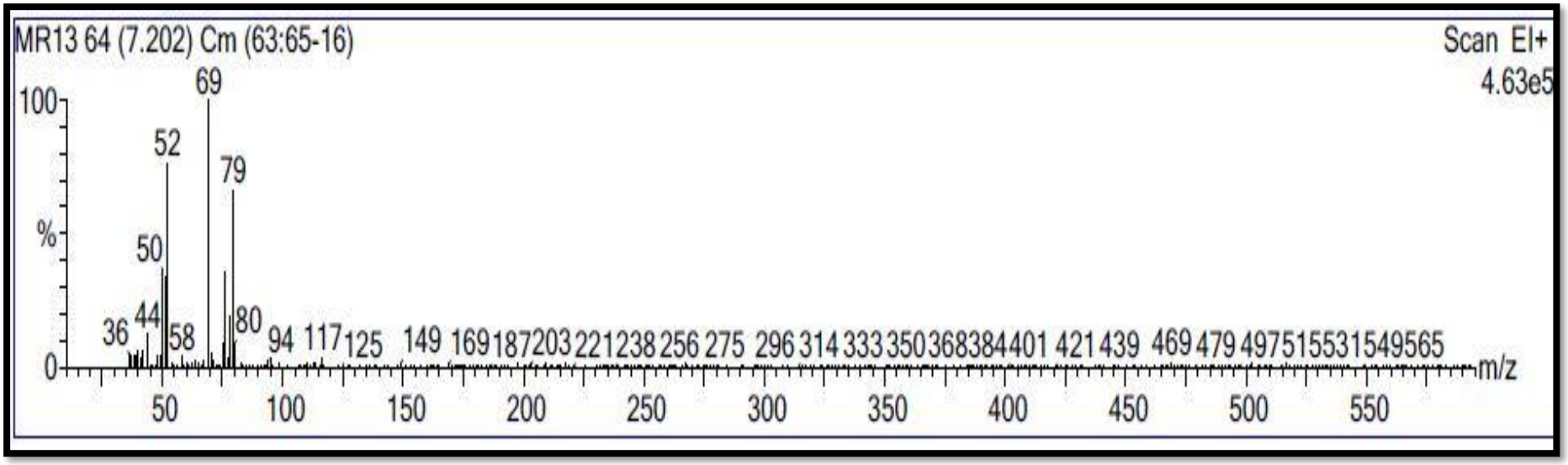


Fig. A12. The highest peak produced by *Streptomyces sp*. at RT 7.202 min

Table A5. Metabolites produced by *Bacillus* sp. and *Streptomyces* sp. based on the largest peak area

Bacteria	***RT***	***Area %***	***Compounds***	**Function**
Bacillus sp.	7.167	3.4%	Rhodopin	Naturally occurring carotenoid pigment which are known for their bright yellow to red colors. It serves as a light-harvesting pigment for photosynthesis.
			Cephaloridine	Semisynthetic antibiotic belonging to the cephalosporin class of beta-lactam antibiotics
Bacillus sp.	7.148	2.5%	(3-Ethynyl-3-methyloxiran-2 yl) methanol	Used as a reagent for the synthesis of various compounds, including pharmaceuticals, agrochemicals, and other organic molecules.
			2,4-Pentadienenitrile	Used in the production of pharmaceuticals, agrochemicals, and other fine chemicals.
Bacillus sp.	7.243	1.9%	Milbemycin B, 6,28-anhydro-15-chloro-25-isopropyl-13-dehydro-5-Odemethyl-4-methyl-	Milbemycin B is a macrocyclic lactone compound used as an antiparasitic agent.
			Lycoxanthin	Carotenoid pigment that is responsible for the red color of many fruits and vegetables, such as tomatoes, watermelons, and pink grapefruits. It is a highly effective antioxidant
			9-Octadecenoic acid (Z)-, octadecyl ester	Used in the cosmetics and personal care industry as a skin conditioning agent, emollient, and thickening agent.

Streptomyces sp.	7.202	14%	Boron, trihydro(pyridine)-, (T-4)-	Reduce a variety of functional groups, such as aldehydes, ketones, and carboxylic acids
			2-Pyridinecarboxylic acid	Important nutrient for some microorganism
Streptomyces sp.	7.373	5.9%	Rhodoxanthin	Natural carotenoid pigment with a deep red color
			Pregnan-20-one, 3,17,21tris[(trimethylsilyl)oxy]- ,O(phenylmethyl)oxime,(3á,5á)-	Synthetic derivative of the steroid hormone progesterone
			Echinenone	Natural carotenoid pigment with deep red colour and powerful antioxidant
Streptomyces sp.	7.015	1.8%	Semisynthetic cephalosporin antibiotic	Cephaloridine Used as a protecting group for amines in organic synthesis

Table A6. The functional metabolites of *Bacillus* (MR3)

Compound name	**Function**
Pyridinium, dinitromethylide-	Herbicides
1-Buten-3-yne	Potential treatment for cancer and viral infections./ Bioactive compound in medicine
Rhodopin	Natural pigment belonging to the class of carotenoids
Lycoxanthin	Carotenoid, a natural pigment that gives red, orange, or yellow color
Milbemycin B, 6,28-anhydro-15-chloro-25-isopropyl-13-dehydro-5-Odemethyl-4-methyl-	Drug used to treat parasitic infections
Carda-16,20(22)-dienolide,3-[(6-deoxy-3,4-Omethylenehexopyranos-2-ulos-1-yl)oxy]-5,11,14-trihydroxy-12-oxo-,(3á,5à,11à)-	Natural product with biological activities / steroidal compounds
1-(5-Bicyclo[2.2.1]heptyl)ethylamine	Flavouring substance
2,4-Pentadienenitrile	Herbicidal activity
Cephaloridine	Beta lactam antibiotic/Antibacterial activity
2-Pyridinecarboxylic acid	Herbicidal activity
Propanedinitrile	Pest control
Picolinamide	Nutrient supplement for livestock and poultry/ Plant growth regulator

Pyridine	Pesticides
Pyridinium, 1-(2-hydrazino-2-oxoethyl)-, chloride	Antioxidant
Pyridine, 2-(4-nitrobenzylidenamino)-	Bio-oil compound
Nickel, (ü-2-2-diallyl ether)(pyridine)	Trace element for plant growth
Boron, trihydro(pyridine)-, (T-4)-	Reducing agent
2-Pyridinecarboxaldehyde	Antibacterial activity
3-Methoxyamphetamine	Stimulant drug
Norpseudoephedrine	Appetite suppressant for the treatment of obesity
Cathinone	Central nervous system stimulant and a psychotropic drug
Norpseudoephedrine	Psychoactive drug
Cystine	Antioxidant activity
Tetraacetyl-d-xylonic nitrile	Anti-viral effects
N-(3,5-Dinitropyridin-2-yl)-L-aspartic acid	Biosynthesis of protein
Norephedrine	Nasal decongestant and an appetite suppressant
Allantoic acid	Carbon & nitrogen source for microbes
5H-Pyridazino[3,4-b][1,4]benzoxazine, 3-chloro-5-methyl-	Essential to bacterial cell viability

(R)-(-)-2-Amino-1-propanol	Used as a chiral intermediate for the synthesis of pharmaceutical products and agrochemicals.
Propanenitrile, 3-(methylamino)-	Intermediate antibiotic
2-Butyne-1,4-diol, diformate	Textile additives, corrosion inhibitors, plasticizers, synthetic resins and polyurethanes
1-Pentanol, 4-amino-	As a solvent in the manufacture of pharmaceuticals
Deoxyspergualin	Derivative of the antitumor antibiotic spergualin
Chlorozotocin	Cytostatic agent
dl-Phenylephrine	Medication compound

Table A7. The functional metabolites of *Streptomycetes* (MR13)

Compound name	**Function**
Aminocyanoacetic acid	Antimicrobial properties
Tetraacetyl-d-xylonic nitrile	Antiviral effects
Desmethyldoxepin	Antidepressant drug
Phosphoric triamide	Urease inhibitor
Cystine	Important in plant immunity
Pregn-5-en-20-one, 3,17-bis[(trimethylsilyl)oxy]-, O- (phenylmethyl)oxime, (3á)-	Unknown
Astaxanthin	Antioxidant
Milbemycin B	Bio-pesticide
4H-1-Benzopyran-4-one	Anti-inflammatory
Nickel	Plant micronutrient
Zirconium	Stimulate or inhibit enzymes in plant cell
[(1H)-Pyrrole-3-propanoic acid	Plant growth regulators
3,8,12-Triacetylingol 7-(4-methoxyphenyl)acetate	Unknown
trans-4-Nitro-4'-(octadecyloxy)chalcone	Pesticides and weed insecticide

Molybdenum	Nitrogen metabolism and synthesis of phytohormones, abscisic acid and indole-3-butryic acid
Triterpenoids(10-Acetoxy-2-hydroxy-1,2,6a,6b,9,9,12a-heptamethyl-1,3,4,5,6,6a,6b, 7,8,8a,9,10,11,12,12a,12b,13,14b-octadecahydro-2H-picene-4acarboxylic acid)	Plant adaptation
9-Octadecenoic acid (Z)-, 3-[(1-oxohexadecyl)oxy]-2-[(1- oxooctadecyl)oxy]propyl ester	Antifungal activity
(3,4-Dimethyl-5-oxo-2,5-dihydro-1H-pyrrol-2-yl)-[4,4-dimethyl-5-(2,3,3-trimethyl-5-methylthio-3,4-dihydro-2H-pyrrol-2-ylmethylene)pyrrolidin2-ylene]-thioaceticacid,S-(tert.-butyl) ester	Antifungal compound
2-(16-Acetoxy-11-hydroxy-4,8,10,14-tetramethyl-3-oxohexadecahydrocyclopenta[a]phenanthren-17-ylidene)-6-methyl-hept-5-enoicacid, methyl ester	Plant growth and development
Pregnane-11,20-dione, 3,17,21-tris[(trimethylsilyl)oxy]-,20-[O-(phenylmethyl)oxime], (3à,5á)-	Natural antioxidant
2,16,27,28Tetraazaheptacyclo[15.7.1.1(3,25).1(5,8).1(10,13).1(15,19).0(18,21)]nonacosa-1,3,5,7,9,11,13(28),14,17,19(29)-decaene-20, 22-dione, 12-ethyl-21-methoxy-6,11,26,29-tetramethyl-7-ethenyl	Unknown
Anodendroside E 2, monoacetate	Antimicrobial
7,8-Epoxylanostan-11-ol, 3-acetoxy	Antimicrobial

1,2-Bis(2,6-dinitro-4-trifluoromethylphenylamino)benzene	Herbicide
Tungsten(0), tetracarbonyl-(phenylmethoxycarbene)(ü-2-Ecycloctene)	Plant growth inducer
Rubixanthin acetate	Carotenoid
4'-Apo-á,.psi.-carotenoic acid, methyl ester	Plant growth regulator
Copper,[2,8,12,18-tetraethyl-3,7,13,17-tetramethyl-21H,23Hporphinato(2-)-N2,N22,N23,N24]-, (SP-4-1)-	
2,2-Bis[4-[(4,6-dichloro-1,3,5-triazin-2-yl)oxy]phenyl]-1,1,1,3,3,3-hexafluoropropane	Antibacterial and antifungal activity
5H-Cyclopropa[3,4]benz[1,2-e]azulen-5-one,4,9,9a-tris(acetyloxy)-3-[(acetyloxy)methyl]-1,1a,1b,4,4a,7a,7b,8,9,9a-decahydro-4a,7bdihydroxy-1,1,6,8-tetramethyl	Unknown
[8,8'-Bi-2H-naphtho[1,8-bc]furan]-2,2'-dione,3,3',4,4'-tetramethoxy-7,7'-dimethyl-5,5'-bis(1-methylethyl)-	Unknown
1H-Cyclopropa[3,4]benz[1,2-e]azulene-5,7b,9,9a-tetrol,1a,1b,4,4a,5,7a,8,9-octahydro-3-(hydroxymethyl)-1,1,6,8-tetramethyl-,5,9,9atriacetate,[1aR(1aà,1bá,4aá,5á,7aà,7bà,8à,9á,9aà)]	Anti-allergic, anti-inflammatory antibacterial
2 Milbemycin B, 6,28-anhydro-15-chloro-25-isopropyl-13-dehydro-5-Odemethyl-4-methyl	Macrolides
Rhodopin	Carotenoid
Rhodoxanthin	Xanthophyll pigment
Nickel	Micronutrient
Cephaloridine	Antibiotics

N-Hydroxymethyl fluoroacetamide	Antibacterial and antifungal activity
Ethanone, 1-(4-pyridinyl)-, oxime	Unknown
N-Vinylpyridinium bromide	Antibacterial
2-Pyridinecarboxylic acid	Antimicrobial
Picolinamide	Antibiotics
Cephaloridine	Antibiotics
Echinenone	Carotenoid pigments
Zeaxanthin	Carotenoid pigment
Carotene-3,3',8,19-tetrol, 7,8-dihydro-	Carotenoid pigment
D:A-Friedooleanan-7-one, 3-hydroxy-	Anti-tumour
Methyl 5,11,14-eicosatrienoate	Anti-inflammatory
Lycoxanthin	Carotenoid pigment
Astaxanthin	Keto-carotenoid
Zearalenone	potent estrogenic metabolite
4-Acetoxy-3-bromo-2,3'-dimethyl-5,5',8,8'-tetramethoxy-1,2'- binaphthalene-1',4'-dione	Unknown
Glycine	Increase nitrogen status and concentration of mineral elements in plant tissues

2-(5-(5-[Cyano-(9,9-dimethyl-1,4-dioxa-7-aza-spiro[4.4]non-7-en-8-yl)-methylene]-3,3-dimethylpyrrolidin-2-ylidenemethyl)-3,3-dimethylë1-pyrrolin-5-ylidenemethyl-4,4,5-trimethyl-ë1-pyrroline-5-carbonitrile]	Flavonoid
Cycloheximide tritms	Fungicide
L-Valine	Anti-algal
Calconcarboxylic acid	Azo dye
Benzothiophene-2-carboxamide	Plant growth regulator
Piperazine	Fungicide

Table A8. Unidentified compounds of *Bacillus* sp. (MR3)

Active compounds	Function
(-)-Norephedrine	Unknown
Ethanone, 1-(4-pyridinyl)-, oxime	Unknown
Bicyclo[3.3.1]non-6-en-3-ol	Unknown
2,4-Pentadienenitrile	Unknown
5-(2-Aminopropyl)-2-methylphenol	Unknown
Pyrido[1,2-a]benzimidazole-4-carbonitrile, 3-methyl-1-dimethylamino	Unknown
Benzeneethanamine, 2-fluoro-á,3-dihydroxy-N-methyl	Unknown
Benzeneethanamine, 4-fluoro-á,3-dihydroxy-N-methyl-	Unknown
Benzeneethanamine, 2,5-difluoro-á,3,4-trihydroxy-N-methyl-	Unknown
Benzeneethanamine, N-methyl-	Unknown
Dimethyl-(6-methyl-2-thioxo-[1,3,2]oxathiaphosphinan-2-yl)-amine	Unknown
1-Propanol, 2-amino-, (S)-	Unknown
Pyrido[3,4-d]imidazole, 1,6-dicarboxylic acid	Unknown
Benzhydrazide, 3,5-dichloro-2,6-dimethoxy	Unknown
1-(4-Acetamidoanilino)-3,7-dimethylbenzo[4,5]imidazo[1,2-a]pyridine-4-carbonitrile	Unknown
2-Butanol, 2-nitroso-, acetate (ester)	Unknown
3-Butyn-1-ol	Unknown
4-Cyclopropylcarbonyloxytetradecane	Unknown
2-Propenoic acid, 1-methylundecyl ester	Unknown
8,9,9,10,10,11-Hexafluoro-4,4-dimethyl-3,5-dioxatetracyclo[5.4.1.0(2,6).0(8,11)]dodecane	Unknown
2-(4,5-Dihydro-3-methyl-5-oxo-1-phenyl-4-pyrazolyl)-5-nitrobenzoicacid	Unknown
3-Cyclopropylcarbonyloxytetradecane	Unknown
2-Trifluoroacetoxydodecane	Unknown
5-(Prop-2-enoyloxy)pentadecane	Unknown
Tetraethyl 4,4'-(1,3-phenylene)bis(1,4-dihydro-2,6-dimethyl-3,5-pyridinedicarboxylate	Unknown

Piperidine, 1-acetyl-4-[4,5-dihydroxyphenyl]-	Unknown
N,N'-Pentamethylenebis[s-3-aminopropyl thiosulfuric acid]	Unknown
5-[(2,4-Dinitrophenyl)-hydrazonomethyl]-dihydrofuran-2-one	Unknown
Benzeneethanamine, 2-fluoro-á,3-dihydroxy-N-methyl-	Unknown
Benzhydrazide, 3,5-dichloro-2,6-dimethoxy-	Unknown
(2-Aziridinylethyl)amine	Unknown
5-(2-Aminopropyl)-2-methylphenol	Unknown
Benzeneethanamine, N-methyl-	Unknown
1-Methyl-2-phenoxyethylamine	Unknown
Benzeneethanamine, 2-fluoro-á,3-dihydroxy-N-methyl	Unknown
3-Hydroxy-N-methylphenethylamine	Unknown
Benzenemethanol, à-(1-aminoethyl)	Unknown
2-Butanamine, 3-methyl-	Unknown
Pyridine-3-carboxamide, 1,2-dihydro-4,6-dimethyl-2-thioxo-	Unknown
Stearic acid, 2-(9-octadecenyloxy)ethyl ester	Unknown
9-Octadecene, 1,1'-[1,2-ethanediylbis(oxy)]bis-, (Z,Z)	Unknown
9-Cycloheptadecen-1-one, (Z)-	Unknown
6H,8H-Benzo[10,11]chryseno[1,12-cd]pyran-6,8-dione	Unknown
5-Isoxazolepropanamine, N-methyl-3-(4-nitrophenyl)-	Unknown
2-[3-[à-Furylmethylamino]propylamino]ethylthiophosphate	Unknown
Pyridine-3-carboxamide, 1,2-dihydro-4,6-dimethyl-2-thioxo	Unknown
1,3-Adamantanediacetamide	Unknown
Glycyl-D-asparagine	Unknown
Butanoic acid, 4,4'-dithiobis[2-amino	Unknown
N-[3,5-Dinitropyridin-2-yl]proline	Unknown
Dimethyl-(6-methyl-2-thioxo-[1,3,2]oxathiaphosphinan-2-yl)-amine	Unknown
Pyridine-3-carboxamide, 1,2-dihydro-4,6-dimethyl-2-thioxo-	Unknown
5-Bromo-6-(3-hydroxy-propylamino)-1H-pyrimidine-2,4-dione	Unknown
Pyrazole[4,5-b]imidazole, 1-formyl-3-ethyl-6-á-d-ribofuranosyl	Unknown

Butanoic acid, 4-(1,3-dithian-2-yl)-4-hydroxyimino-, benzyl ester	Unknown
N-Chloroacetyl-3,6,9,12-tetraoxapentadec-14-yn-1-amine	Unknown
Acetamide, N-methyl-N-[4-[4-fluoro-1-hexahydropyridyl]-2-butynyl]-	Unknown
26-Deoxy-26-ethylaminodihydroneotigogenin	Unknown
1,6-Diaminohexane-N,N,N',N'-tetraacetic acid	Unknown
Benzenemethanol, 2-(2-aminopropoxy)-3-methyl-	Unknown
5H-Thiazolo[3,2-a]pyrimidine-7-carboxylic acid, 5-oxo	Unknown
Heptano[a]purin-2,6-dione, 1,3-dimethyl-	Unknown
2-Naphthalenol, 1-[(2-pyridinylimino)methyl]-	Unknown
4-[5-Dimethylamino-2-hydroxy-2-pentyl]-2-phenylquinonine	Unknown
Pregnan-18-oic acid, 20-hydroxy-, ç-lactone, (5à)-	Unknown
Propanenitrile, 3-(5-diethylamino-1-methyl-3-pentynyloxy)-	Unknown
2-Butanamine, 3-methyl-	Unknown
2-Heptanol, 6-amino-2-methyl	Unknown
Thiophene-3-ol, tetrahydro-, 1,1-dioxide	Unknown
4-Fluorohistamine	Unknown
Ethyne, fluoro	Unknown
Propanamide, N-methyl-2-amino-	Unknown
Benzeneethanamine, 2-fluoro-á,3-dihydroxy-N-methyl	Unknown
Acetamide, 2-cyano	Unknown
1,4-Naphthoquinone, 6-acetyl-2,5,7-trihydroxy-	Unknown
4-(Trimethylsilyloxy)-3-methoxycinnamaldehyde	Unknown
4-Acetoxy-2-butanone 2,4-dinitrophenylhydrazone	Unknown
Tryptamine, N,N'-diacetyl-6-fluoro-5-methoxy	Unknown
4-Chlorobenzenesulfonamide, N-tert.-butyldimethylsilyl	Unknown
2-Chloro-3-methoxydibenzo-p-dioxin	Unknown
Thiazolidin-4-one, 3-ethyl-5-[3-(2,2,3,3-tetrafluoroethoxybenzylidene)-2-thioxo-	Unknown
7H-[1,2,4]Triazolo[3,4-b][1,3,4]thiadiazin-6-amine, 3-(2-pyrazinyl)-	Unknown
Pyrimidine, 5-bromo-4,6-dimethoxy	Unknown

N,N'-Bis(Carbobenzyloxy)-lysine methyl(ester)	Unknown
Benzhydrazide, 3,5-dichloro-2,6-dimethoxy	Unknown
Gentamicin a	Unknown
1-(3-Azido-4-methoxymethyl-6-methyl-thieno[2,3-b]pyridin-2-yl)-ethanone	Unknown
à-Aminoisobutyronitrile	Unknown
5-Bromo-6-(3-hydroxy-propylamino)-1H-pyrimidine-2,4-dione	Unknown
5-Ethyl-3-(3-pyridyliminomethyl)-2-thiophenethiol	Unknown
Benzothieno[2,3-d]tetrazolo[5,1-f]pyrimidine, 8,9,10,11-tetrahydro-5-methylthio-	Unknown
10-Hydroxy-2,4a,6a,6b,9,9,12a-heptamethyl-1,2,3,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,12b,13,14b-eicosahydropicene-2-carboxylic acid	Unknown
5,6-Azulenedimethanol, 1,2,3,3a,8,8a-hexahydro-2,2,8-trimethyl-,(3aà,8á,8aà)-	Unknown
Benzazepin-1-one, 1,2,3,4-tetrahydro-7,8,9-trimethoxy	Unknown
1,3,4-Metheno-1H-cyclobuta[cd]pentalene, 1,1a,2,2,3,4,5,5,5a,5bdecachlorooctahydro	Unknown
2,6-Diazatricyclo[3.3.1.1(3.7)]decane, 2,6-dimethyl-4,8-dichloro	Unknown
6,9-Dimethyl-3-nitro-6,7-dihydro-9H-5-oxa-9-azabenzocyclohepten-8-one	Unknown
N,N'-Bis(Carbobenzyloxy)-lysine methyl(ester)	Unknown
2-Penten-1-one, 2-methyl-1,3-diphenyl-	Unknown
Tricyclo[2.2.2.1(2,5)]nonane, 3-cyano-3-trimethylsilyloxy	Unknown
2-Thiazolamine, 4-(3,4-dimethoxyphenyl)-5-methyl	Unknown
Tricyclo[3.3.1.1(3,7)]decanone, 4-hydroxy-, (1à,3á,4à,5à,7á)-	Unknown
Pyrido[1,2-a]benzimidazole-4-carbonitrile, 3-methyl-1-dimethylamino	Unknown
3-Amino-7-(trifluoromethyl)[1,2,4]triazolo[4,3-a]pyrimidin-5-ol	Unknown
Pyrazole[4,5-b]imidazole, 1-formyl-3-ethyl-6-á-d-ribofuranosyl	Unknown
3',5'-Dichloromethotrexate	Unknown
Pyrimidin-4-ol, 2,6-diamino-5-(2-furfurylidenamino	Unknown
N-[4-Methylbenzenesulfonyl]-N'-[1-[6-chloro-1,4-dihydro-1-hydroxy-2-methyl-4-oxo-2-quinolinyl]ethylidene]hydrazine	Unknown
8-Azanonane, 4-cyano-4-(3,4-dimethoxyphenyl	Unknown
N,N-Diethyl-2-(4-chlorophenyl)-3-morpholino- thioacrylamide	Unknown

1,3,5-Triazin-2-amine, 4-chloro-6-(2-difluoromethylthiophenylamino	Unknown
2-(Benzylsulfanyl-fluoro-methylene)-malonic acid dimethyl ester	Unknown
2-Hydroxy-2-methyl-succinic acid, bis-(2-oxo-2-phenyl-ethyl ester	Unknown
Borneol, methyl(tetramethylene)silyl ether	Unknown
2,5-Ethano-2H-azocino[4,3-b]indole, 4-ethylidene-1,3,4,5,6,7-hexahydro-6-methylene-, [r-(E)]-	Unknown
Isoborneol, methyl(tetramethylene)silyl ether	Unknown
4-Acetyl-2-(4-chlorophenyl)-6,7,8,8a-tetrahydro-2H-pyrrolo[1,2-d][1,2,4]triazin-1-one	Unknown
7H-1,2,4-Triazolo[4,3-b]-1,2,4-triazol-7-amine, 3-(5-methyl-1,3,4-oxadiazol-2-ylmethylthio)-	Unknown
Propionic acid, 3-bis(diethylphosphonatomethyl)amino-	Unknown
Chlorozotocin	Unknown
1-[3-(4-Bromophenyl)-2-thioureido]-1-deoxy-b-d-glucopyranose 2,3,4,6-tetraacetate	Unknown
Curan-17-oic acid, 19-(acetyloxy)-2,16-didehydro-20-hydroxy-,methyl ester, (19S)-	Unknown
Hydrazinecarbothioamide, 2-(2-thienylmethylene	Unknown
2,4a-Epidioxy-4a,5,6,7,8,8a-hexahydro-2-[2-benzoyloxypropyl]-5,5,8a-trimethylbenzopyrane	Unknown
2,4(1H)-Cyclo-3,4-secoakuammilanium,3,17-dihydroxy-16-(methoxycarbonyl)-4-methyl-(3á,16R)-	Unknown
1-(3-Azido-4-methoxymethyl-6-methyl-thieno[2,3-b]pyridin-2-yl)-ethanone	Unknown
Pregan-20-one, 2-hydroxy-5,6-epoxy-15-methyl	Unknown
5,6-Dichloro-2-[methylthio]benzimidazole	Unknown
2-Hexanamine, 4-methyl-	Unknown
3-Pyridinecarboxaldehyde, O-acetyloxime, (E)-	Unknown
Thiophene-3-ol, tetrahydro-, 1,1-dioxide	Unknown
1,2,5-Oxadiazol-3-carboxamide, 4,4'-azobis-, 2,2'-dioxide	Unknown
1,1'-Biphenyl, 2-fluoro-2'-hydroxy-6,3'-dimethoxy	Unknown
(2-Aziridinylethyl)amine	Unknown
1-Propanol, 2-amino-, (ñ)-	Unknown
Ethyne, fluoro-	Unknown

8-Azanonane, 4-cyano-4-(3,4-dimethoxyphenyl	Unknown
l-Alanine ethylamide	Unknown
2-Butanamine, 3-methyl	Unknown
N-dl-Alanylglycine	Unknown
Benzenemethanol, à-(1-aminoethyl	Unknown
Ethyne, fluoro-	Unknown
á-Alanine, N-methyl-, ethyl ester	Unknown
Butanoic acid, 3-amino-2-methyl	Unknown
3-Chloro-1-methoxydibenzo-p-dioxin	Unknown
4H-Thiopyrano[4',3':4,5]furo[2,3-d]pyrimidin-3(6H)-amine, 5,8-dihydro-4-imino-6,6-dimethyl-	Unknown
2-Chloro-3-methoxydibenzo-p-dioxin	Unknown
2H-Thieno[3,4-g]indazole, 8-propylthio	Unknown
à-l-Mannose semicarbazone pentaacetate	Unknown
2-(Acridin-9-ylamino)-3-methyl-butyric acid	Unknown
Acetamide, N-[2-(acetyloxy)-2-[4-(acetyloxy)-3-methoxyphenyl]ethyl	Unknown
Thieno[2,3-c]furan-3-carbonitrile, 2-amino-4,6-dihydro-4,4,6,6-tetramethyl-	Unknown
Murrayatin	Unknown
trans-Bicyclo[4.3.0]nonane, 1-methyl-9-(2-octyl)-5-(2-trimethylsilyloxy)-4-(1-methyl-2,4 -di(trimethylsilyloxy)cyclohexyl	Unknown
Pentanamide, N-1H-purin-6-yl-	Unknown
5-Bromo-4-nitroimidazole-2-[2-thioacetic acid	Unknown
Purine-2,6-dione, 8-(3-ethoxypropylamino)-1,3-dimethyl-3,9-dihydro-	Unknown
3-Carbomethoxy-4-[p-chlorophenyl]-2-[[N'-p-nitrophenyl]ureido]-5,5-dimethyl-4,5-dihydrofuran	Unknown
Carbamic acid, N-[10,11-dihydro-5-(2-methylamino-1-oxoethyl)-3-5H-dibenzo[b,f]azepinyl]-, ethyl ester	Unknown
Thiophene-3-carboxylic acid, 2-amino-4-(4-chlorophenyl)-, ethyl ester	Unknown
1,4-Thiazine, perhydro-1-benzoylimino-4-(3-chloro-5-trifluoromethyl-2-pyridyl)-	Unknown

Tetraethyl 4,4'-(1,3-phenylene)bis(1,4-dihydro-2,6-dimethyl-3,5-pyridinedicarboxylate)	Unknown
3-Ethoxy-1,1,1,5,5,5-hexamethyl-3-(trimethylsiloxy)trisiloxane	Unknown
Pyridine-3-carbonitrile, 2-amino-4-(4-methoxyphenyl)-5-methyl-6-propyl-	Unknown
Ethenylamine, N-benzoyl-2-[3,5-di-t-butyl-4-hydroxyphenyl]-	Unknown
3-(ç-Methylaminopropyl)-5-(4-bromophenyl)-2-methyl-2H-pyrazole	Unknown
3-Carbomethoxy-4-[p-chlorophenyl]-2-[[N'-p-nitrophenyl]ureido]-5,5-dimethyl-4,5-dihydrofuran	Unknown
Pyrido[1,2-a]benzimidazole-4-carbonitrile, 3-methyl-1-dimethylamino	Unknown
Octadecane, 1,1'-[1,3-propanediylbis(oxy)]bis	Unknown
Quinoline-3-carboxylic acid, 1,4-dihydro-7-chloro-1-ethyl-6-nitro-4-oxo-, ethyl ester	Unknown
1,6-Dihydropyridine-3-carboxylic acid, 5-cyano-2-ethyl-6-oxo-4-phenyl-, methyl ester	Unknown
4-Acetyloxyimino-6,6-dimethyl-3-methylsulfanyl-4,5,6,7-tetrahydrobenzo[c]thiophene-1-carboxylic acid methyl ester	Unknown

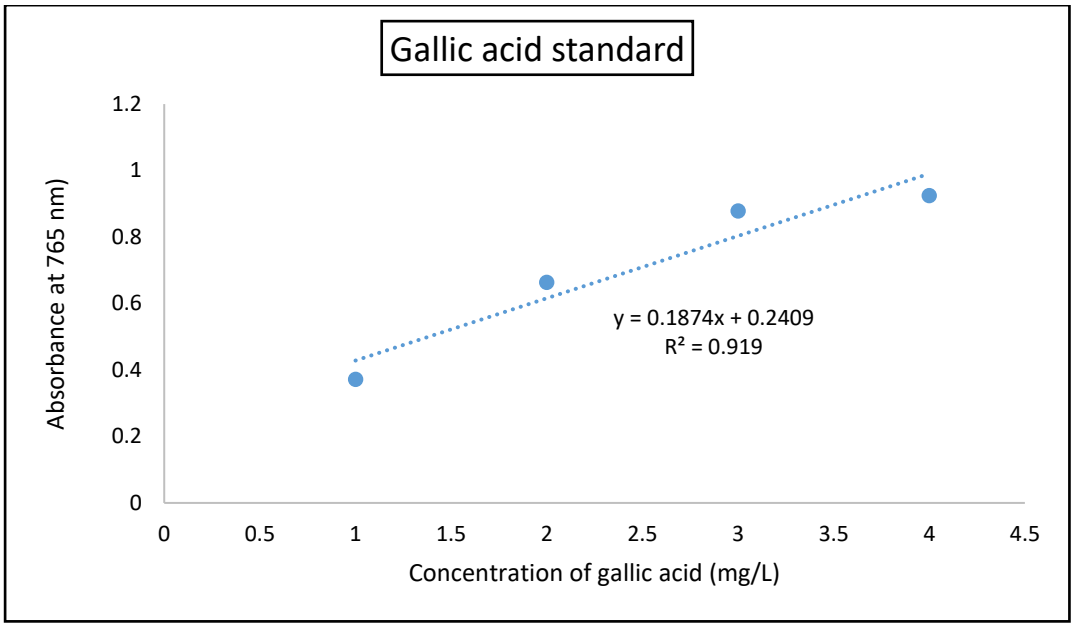


Fig. A9 Gallic acid standard graph

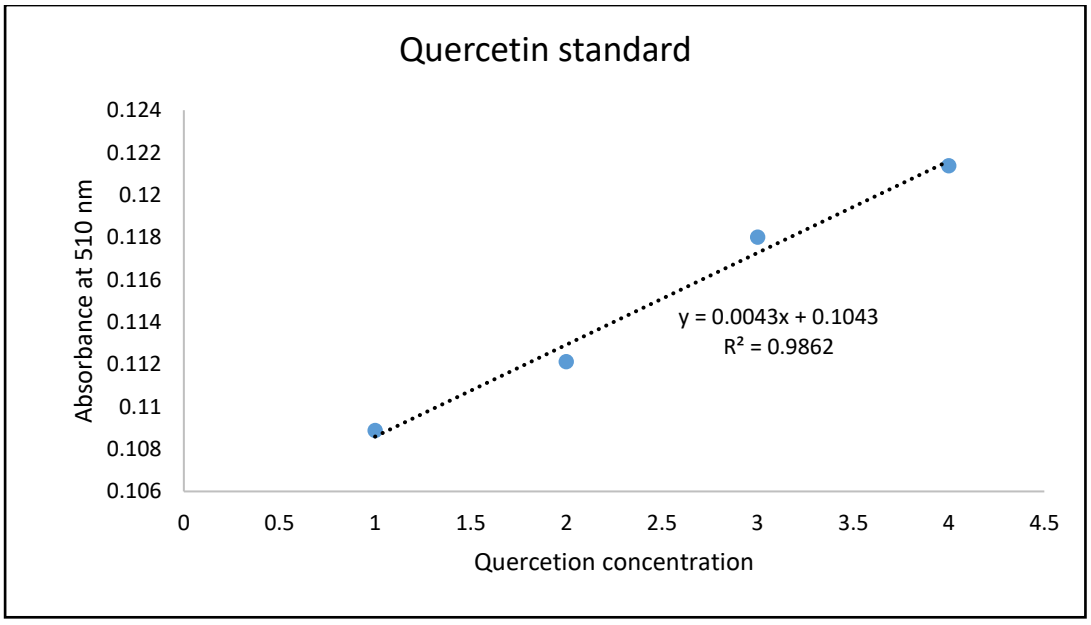


Fig. A10 Quercetin standard graph

Table A11. Physico-chemical properties of moringa leaf nano-emulsion

Oil: Moringa leave extract	Aqueous phase	Oil phase: Aqueous phase	Size (d. nm)	Poly-dispersity index	Zeta potential (mV)	Conductivity (mS/cm)
1:10 (Y)	1% surfactant in dH_2O	5:95	134±3.63	0.53±0.01	27.5±0.44	0.05±0.00
1:10 (Y)	1% surfactant in dH_2O	10:90	121±1.66	0.48±0.02	26.6±0.54	0.09±0.00
1:10 (Y)	1% surfactant in dH_2O	20:80	185±13.2	0.53±0.04	24.6.8±0.53	0.18±0.00
1:10 (Y)	1% surfactant in dH_2O	50:50	182±1.21	0.47±0.01	27.7.0±1.28	0.39±0.00
1:10 (Y)	5% surfactant in dH_2O	5:95	215±1.87	0.41±0.01	24.9.5±1.23	0.05±0.00
1:10 (Y)	5% surfactant in dH_2O	10:90	188±2.94	0.46±0.01	24.1.6±0.74	0.09±0.00
1:10 (Y)	5% surfactant in dH_2O	20:80	182±0.86	0.42±0.01	24.1.8±0.33	0.17±0.00
1:10 (Y)	5% surfactant in dH_2O	50:50	140±1.25	0.63±0.01	25.1.0±0.81	0.45±0.00
1:10 (Y)	10 % surfactant in dH_2O	5:95	212±3.83	0.39±0.01	24.8.5±0.28	0.04±0.00
1:10 (Y)	10 % surfactant in dH_2O	10:90	219±2.21	0.38±0.03	19.9.6±0.33	0.10±0.00
1:10 (Y)	10 % surfactant in dH_2O	20:80	191±4.00	0.39±0.02	21.2±0.26	0.15±0.00
1:10 (Y)	10 % surfactant in dH_2O	50:50	176±7.28	0.42±0.08	19.2.0±0.40	0.44±0.00
1:10 (Y)	1% surfactant in dH_2O + 0.3% modified starch in dH_2O	5:95	123±0.04	0.38±0.01	16.6.5±0.36	0.06±0.00

1:10 (Y)	1% surfactant in dH_2O + 0.3% modified starch in dH_2O	10:90	136±2.77	0.47±0.01	8.36.6±1.11	0.10±0.00
1:10 (Y)	1% surfactant in dH_2O + 0.3% modified starch in dH_2O	20:80	120±0.16	0.57±0.03	16.5.8±0.54	0.19±0.00
1:10 (Y)	1% surfactant in dH_2O + 0.3% modified starch in dH_2O	50:50	146±0.59	0.23±0.00	22.8.0±0.40	0.40±0.00
1:10 (Y)	5% surfactant in dH_2O + 0.3% modified starch in dH_2O	5:95	172±1.84	0.43±0.01	16.5±0.33	0.06±0.00
1:10 (Y)	5% surfactant in dH_2O + 0.3% modified starch in dH_2O	10:90	162±4.81	0.40±0.02	16.5.6±0.08	0.1±0.00
1:10 (Y)	5% surfactant in dH_2O + 0.3% modified starch in dH_2O	20:80	151±0.96	0.39±0.00	16.8.8±0.26	0.18±0.00
1:10 (Y)	5% surfactant in dH_2O + 0.3% modified starch in dH_2O	50:50	148±4.39	0.39±0.07	11.3.0±0.32	0.46±0.00
1:10 (Y)	10% surfactant in dH_2O + 0.3% modified starch in dH_2O	5:95	179±1.69	0.29±0.00	11.8.5±0.28	0.06±0.00
1:10 (Y)	10% surfactant in dH_2O + 0.3% modified starch in dH_2O	10:90	191±1.09	0.38±0.02	9.6.6±0.44	0.10±0.00
1:10 (Y)	10% surfactant in dH_2O + 0.3% modified starch in dH_2O	20:80	175±2.09	0.37±0.00	11.1.8±0.65	0.17±0.00
1:10 (Y)	10% surfactant in dH_2O + 0.3% modified starch in dH_2O	50:50	176±4.22	0.45±0.01	12.4.0±0.44	0.47±0.00
1:5 (Z)	1% surfactant in dH_2O	5:95	113±0.85	0.31±0.01	25.1.5±0.41	0.04±9.42
1:5 (Z)	1% surfactant in dH_2O	10:90	134±1.97	0.35±0.00	23.9.6±0.20	0.08±0.00
1:5 (Z)	1% surfactant in dH_2O	20:80	177±1.79	0.38±0.01	19.6.8±0.20	0.16±0.00
1:5 (Z)	1% surfactant in dH_2O	50:50	202±6.24	0.43±0.01	13.6.0±0.41	0.41±0.00
1:5 (Z)	5% surfactant in dH_2O	5:95	206±1.20	0.48±0.00	22.5.5±0.30	0.04±0.00
1:5 (Z)	5% surfactant in dH_2O	10:90	190±4.81	0.64±0.09	20.1.6±0.18	0.07±0.00
1:5 (Z)	5% surfactant in dH_2O	20:80	250±3.64	0.44±0.09	14.6.8±0.57	0.14±0.00
1:5 (Z)	5% surfactant in dH_2O	50:50	283±3.34	0.48±0.00	8.89.0±0.22	0.41±0.00

1:5 (Z)	10% surfactant in dH_2O	5:95	247±1.86	0.46±0.00	19.4.5±0.64	0.04±0.00
1:5 (Z)	10% surfactant in dH_2O	10:90	203±2.11	0.43±0.01	22.2.6±0.44	0.08±0.00
1:5 (Z)	10% surfactant in dH_2O	20:80	271±2.08	0.47±0.01	19.2.8±0.14	0.129±0.00
1:5 (Z)	10% surfactant in dH_2O	50:50	255±9.20	0.47±0.02	11.7.0±0.29	0.42±0.00
1:5 (Z)	1% surfactant in dH_2O + 0.3% modified starch in dH_2O	5:95	133±4.07	0.37±0.00	18.2.5±0.16	0.04±8.16
1:5 (Z)	1% surfactant in dH_2O + 0.3% modified starch in dH_2O	10:90	132±1.10	0.34±0.00	16.8.6±0.16	0.08±0.00
1:5 (Z)	1% surfactant in dH_2O + 0.3% modified starch in dH_2O	20:80	198±1.04	0.40±0.00	12.5.8±0.40	0.16±0.00
1:5 (Z)	1% surfactant in dH_2O + 0.3% modified starch in dH_2O	50:50	266±20.5	0.60±0.03	11.3.0±0.12	0.40±0.00
1:5 (Z)	5% surfactant in dH_2O + 0.3% modified starch in dH_2O	5:95	190±2.28	0.49±0.04	21.5±0.37	0.06±0.00
1:5 (Z)	5% surfactant in dH_2O + 0.3% modified starch in dH_2O	10:90	159±6.72	0.42±0.09	14.4.6±0.09	0.09±0.42
1:5 (Z)	5% surfactant in dH_2O + 0.3% modified starch in dH_2O	20:80	191±1.92	0.45±0.02	8.07.8±0.42	0.17±0.00
1:5 (Z)	5% surfactant in dH_2O + 0.3% modified starch in dH_2O	50:50	228±4.61	0.46±0.00	8.6.0±0.12	0.41±0.00
1:5 (Z)	10% surfactant in dH_2O + 0.3% modified starch in dH_2O	5:95	193±1.34	0.41±0.01	2.06.5±0.03	0.01±0.00
1:5 (Z)	10% surfactant in dH_2O + 0.3% modified starch in dH_2O	10:90	206±1.59	0.40±0.02	12.7.6±0.23	0.09±0.00
1:5 (Z)	10% surfactant in dH_2O + 0.3% modified starch in dH_2O	20:80	191±0.90	0.39±0.02	11.1.8±0.14	0.17±0.00
1:5 (Z)	10% surfactant in dH_2O + 0.3% modified starch in dH_2O	50:50	210±1.91	0.40±0.00	8.52.0±0.12	0.41±0.00

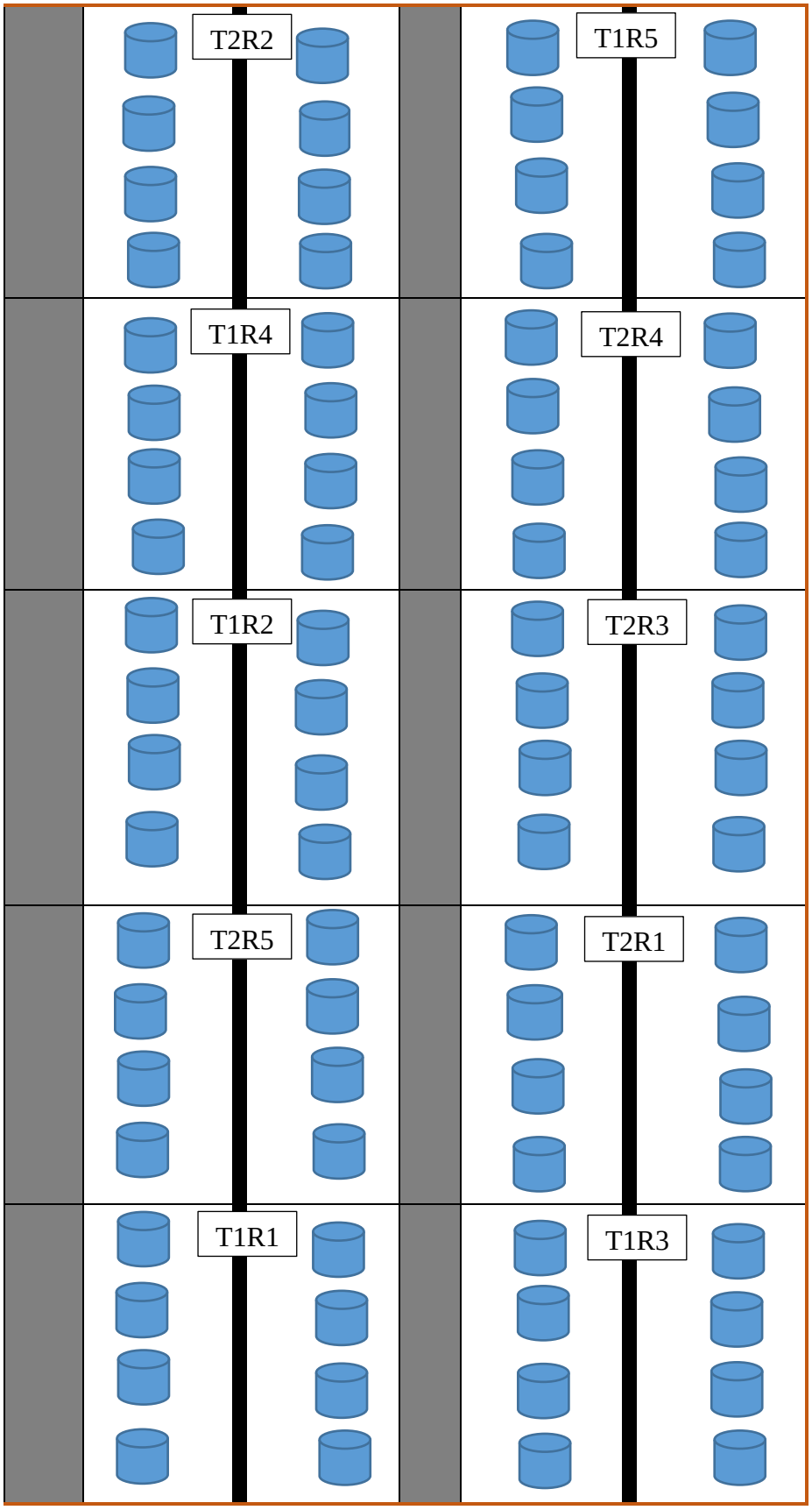


Fig. A12. Experimental layout for chilli cultivation using fertigation system. Shade-house fertigation experimental design for combined application of nano-encapsulated endophytic bacteria and moringa leaf extract

Table A13. Fertigation schedule according to the Department of Agriculture, Malaysia

Week	**Volume**	**EC value (µS/cm) and pH (5.7-6.5)**
21 days after sowing	600 ml	1.2 (Tray dipping)
25 days after sowing	600 mL	1.5 (Tray dipping)
30 days after sowing	600 ml	1.8 (Tray dipping)
Week 1 after transplanting	900 ml	2.0 (Fertigation)
Week 2 after transplanting	1000 ml	2.2 (Fertigation)
Week 3 after transplanting	1000 ml	2.3 (Fertigation)
Week 4 after transplanting	1200 ml	2.4 (Fertigation)
Week 5 after transplanting	1200 ml	2.5 (Fertigation)
Week 6 after transplanting	1400 ml	2.5 (Fertigation)
Week 7 after transplanting	1400 ml	2.6(Fertigation)
Week 8 after transplanting	1600 ml	2.6 (Fertigation)
Week 9 after transplanting	2000 ml	2.8 (Fertigation)
Week 10 after transplanting	2200 ml	2.8 (Fertigation)
Week 11 after transplanting	2000 ml	2.8 (Fertigation)
Week 12 after transplanting	2200 ml	2.8 (Fertigation)

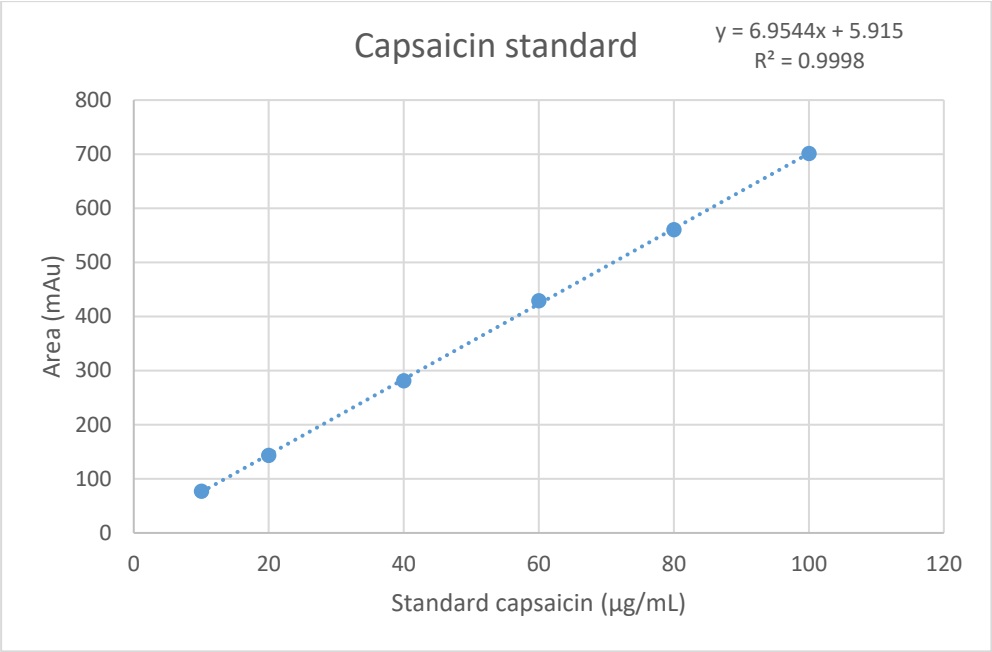


Fig. A14. Standard graph of capsaicin

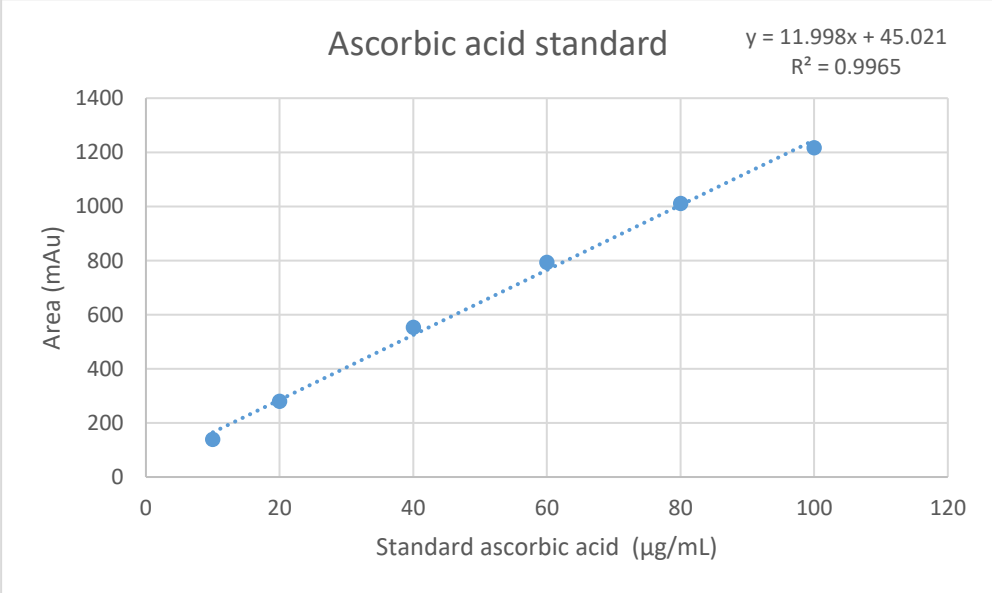


Fig. A15 Standard graph of ascorbic acid

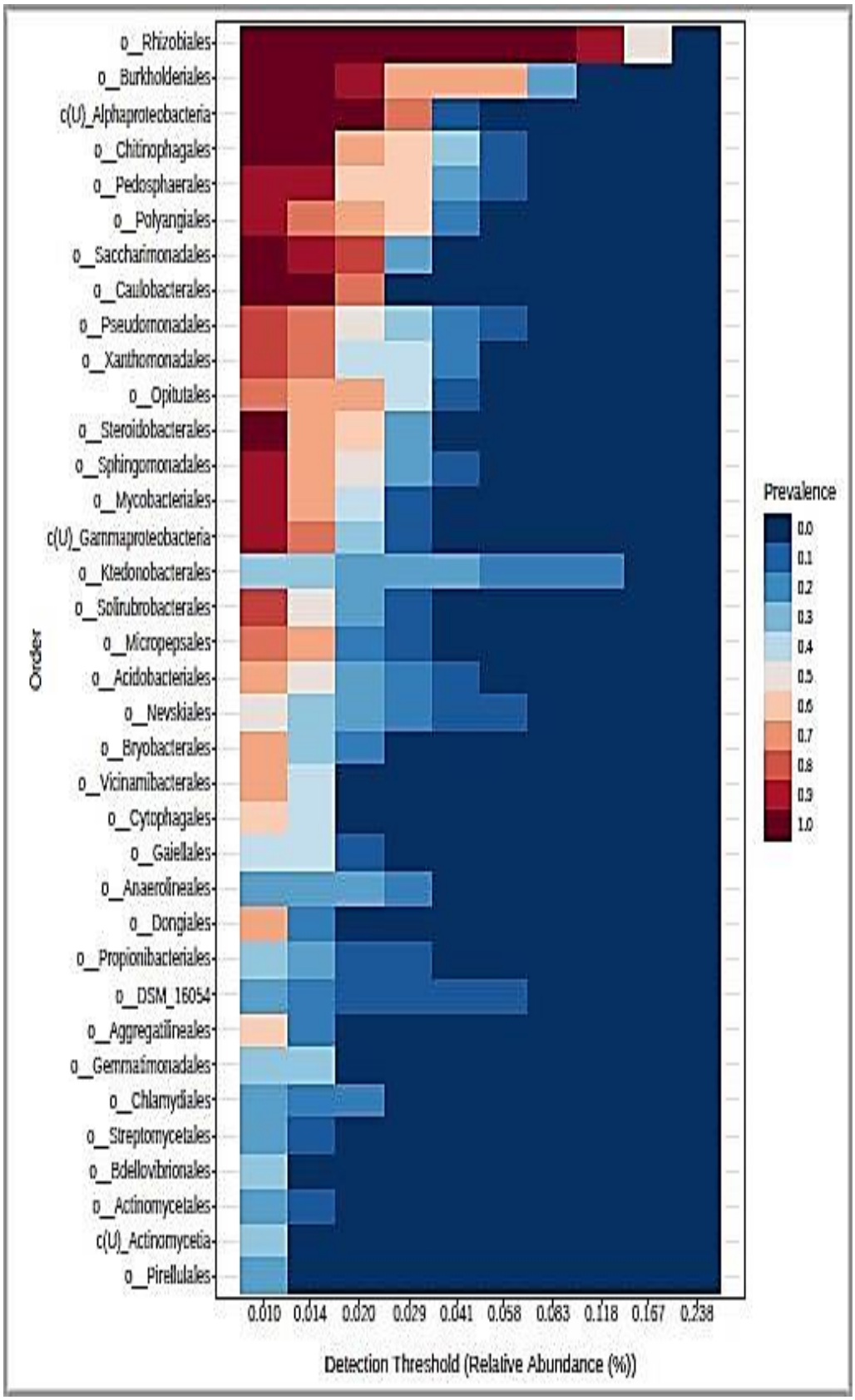


Fig. A16. Chilli root microbiome at order level

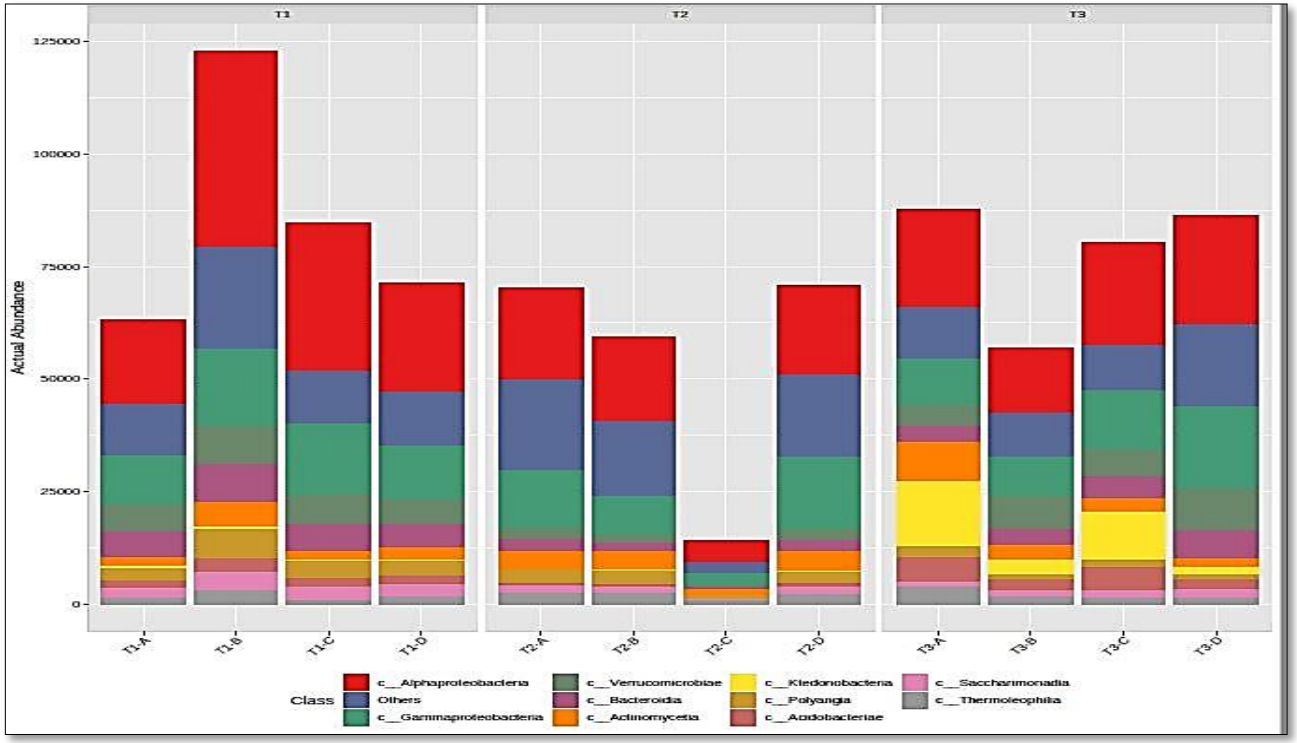


Fig. A17. Chilli root microbiome at class level

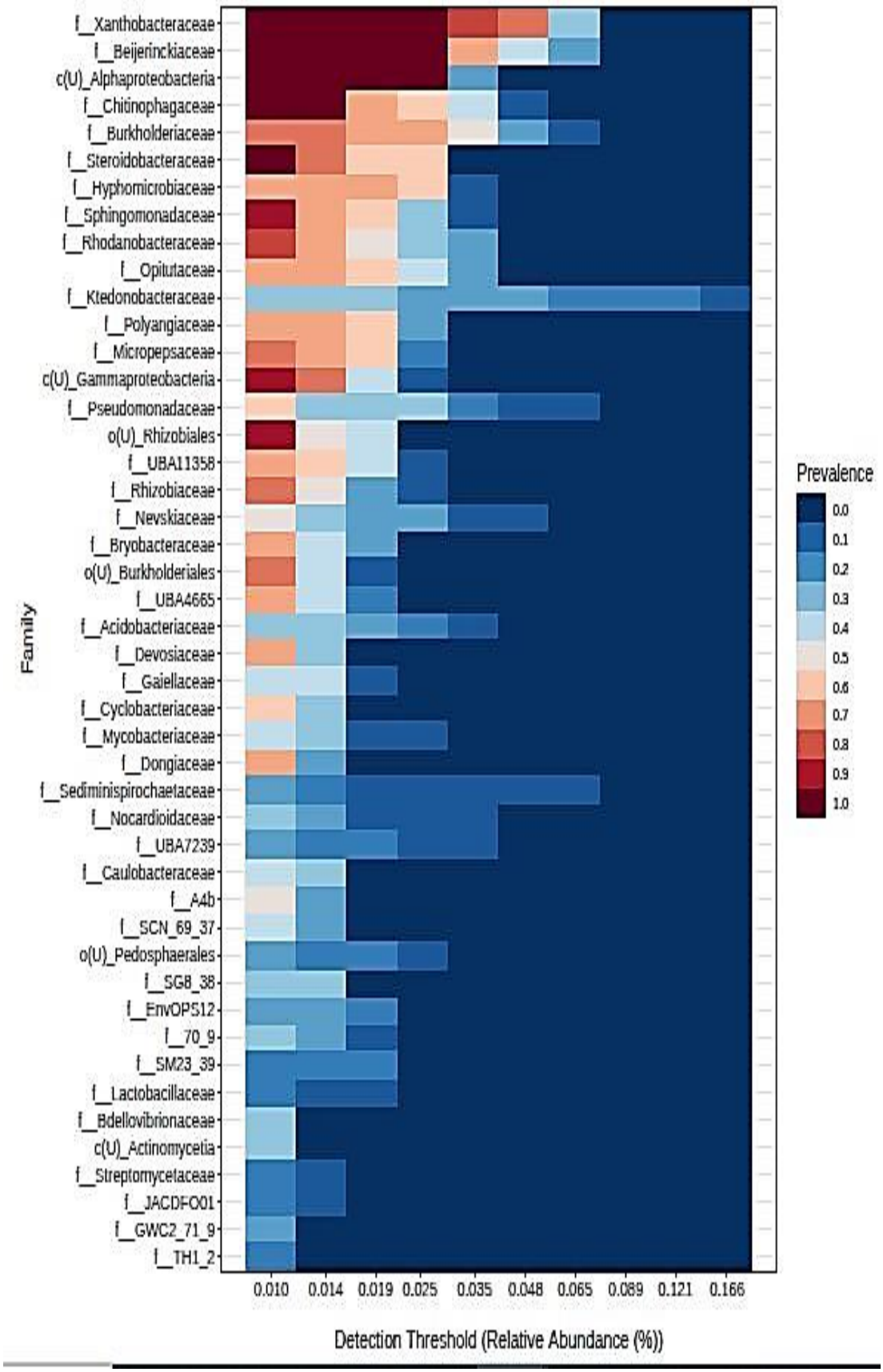


Fig. A18. Chilli root microbiome at family level

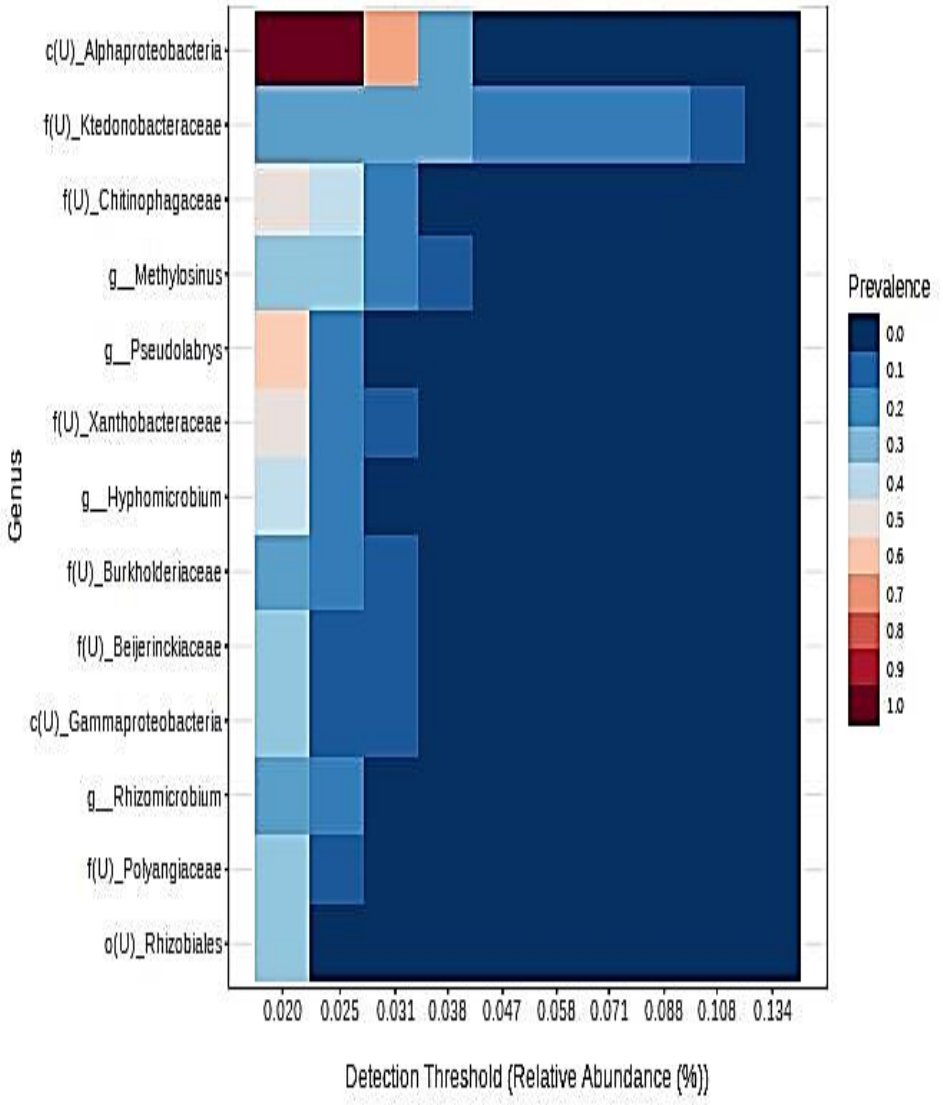


Fig. A19. Chilli root microbiome at genus level