

Assessment of ecological role of bacterial endophytes in vegetable production for human nutrition

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DEDICATION

This thesis is dedicated to all scientists who are working tirelessly to create solutions to many challenges facing humanity.

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ABSTRACT

Bacterial endophytes are a unique group of microbes inhabiting plant tissues for at least a part of their life cycle without causing any pathogenic symptoms. These microbes play significant roles in ecosystem functions and plant growth promotion (PGP) through biogeochemical cycling, biodegradation, nutrient solubilisation and production of phytohormones and bioactive compounds. These ecological roles impact the productivity and nutritional quality of plants and consequently, human health may be affected when plant materials are consumed. Being highly diverse in association and distribution, endophytes exist in every plant that has been investigated, including vegetable crops. Despite vegetables being an important component of a healthy diet and having substantial human health benefits, their endophytes are underexplored. Thus, using high-throughput sequencing, this study investigated the community composition and functional profile of bacterial endophytes inhabiting vegetables such as cabbage, lettuce, onion and spinach. The functional profile was investigated with PICRUST2 or Tax4Fun pipelines. Additionally, endophytes were recovered from vegetables using the culture-dependent method and identified by sequencing the partial 16S rRNA gene employing Sanger sequencing. The PGP potential was assessed using *in vitro* and greenhouse experiments. Results revealed that vegetables are colonized by an abundant and diverse group of bacterial endophytes, with Proteobacteria, followed by Actinobacteriodes being the most abundant phyla accounting for over 80% of the communities. *Pseudomonas*, *Pantoea*, *Bacillus*, *Serratia*, *Rahnella*¹, *Rhodococcus*, *Xanthomonas* and *Streptomyces* were the most relatively abundant genera. The abundance, diversity and richness of endophytic bacteria were lower in the leaves compared to the roots. In multivariate space, the non-metric multidimensional scaling plot revealed a substantial overlap in the bacterial communities of cabbage, lettuce and spinach, while onion samples clustered separately. Shared and unique bacterial communities vary between plant species and organ types, with onions having the highest number of unique amplicon sequence variants compared to other vegetables, suggesting plant structures may influence the composition and community structure of endophytes. Co-occurrence analysis showed that over 60% of the core genera belonged to the generalist, with the dominant taxa being *Pseudomonas*, *Bacillus*, *Agromyces*, *Nocardioides* and other unclassified genera in the

phyla Proteobacteria, Firmicutes and Bacteroidetes. The bacterial endophytes had a high prevalence of important pathways, such as CO₂-fixation and nitrate reduction pathways contributing to ecological functions. Recovered endophytes differentially produced indole-3-acetic acid and ammonium in the range 0.14-44.48 µg/mL and 0.77-93.24 mg/L, respectively, suggesting their PGP potential. *Bacillus cereus*, *Pseudomonas azotoformans*, *Stenotrophomonas maltophilia* and *Serratia liquefaciens* showed a high level of siderophore production, which was not significantly ($P > 0.05$) different across the plant and organ types. Approximately 20% of bacterial endophytes produce hydrolytic enzymes such as amylase, cellulase, gelatinase and protease, which contribute to their ecological functions. The degree of antibiotic susceptibility and cytotoxicity effects on HuTu-80 (human epithelial cells) reveals that some of the bacterial endophytes are resistant to different classes of antibiotics and are cytotoxic to the epithelial cells, suggesting their potential impact on human health when vegetables are consumed raw. The PGP endophytes susceptible to antibiotics and having no cytotoxic effect showed a promising PGP effect in a pot experiment. Bacterial endophytes improved vegetable seed germination rate, mean germination time and plant vigour index compared to the control. The inoculum of *Bacillus cereus*, *Pseudomonas azotoformans*, *Serratia liquefaciens* and *Enterobacter bugandensis* with efficient PGP potential used in the pot experiment significantly ($P < 0.05$) increased yield parameters including plant height, root length, wet and dry weight, and chlorophyll content over the control, suggesting they are good resources in bioformulations employed in the creation of biostimulants for improving plant productivity and nutritional value. Overall, endophytic bacterial community composition and diversity are influenced by plant and soil nutrient management types, suggesting a sustainable soil nutrient management that supports the assemblage and unique recruitment of beneficial endophytes by plants should be adopted. Such an approach will guarantee the microbiological quality of vegetables and consequently, human health when consumed. In conclusion, this study established that bacterial endophytes significantly improved plant growth and provided insights into how beneficial plant growth-promoting endophytes (PGPEs) can be harnessed in sustainable agricultural production. Although the *in vitro* and greenhouse experiments provided rapid screening of efficient PGPEs, further study in the field is necessary for a comprehensive knowledge of the benefits of endophytes in agroecosystems.

Keywords: Bacterial endophyte, microbial diversity, Sanger sequencing, plant health, high throughput sequencing, plant growth promotion, ecosystems, plant growth promotion.

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LIST OF ABBREVIATIONS

Measuring Units

cm: centimetre

°C: degree Celsius

E: East

g: gram(s)

g cm^{-3} : gram per centimetre cube

g kg^{-1} : gram per kilogram

h: hour(s)

Kg: kilogram

L: litre

m: metre

mg: milligram

μg : microgram

$\mu\text{g ml}^{-1}$: microgram per millilitre

μl : microliter

μM : micromole

mM: millimolar

min: minute(s)

M: molar

ng: nanogram

nm: nanometre

N: normality/Normal

%: percentage

pmol: picomole

S: South

S (in 16S rRNA): Sverberg unit

v/v: volume to volume

General abbreviations

(Pty) Ltd: property limited

ANOVA: analyses of variance

ASV: Amplicon sequence variant

C: Carbon

CEC: Cation exchange capacity

D: Dominance

DNA: deoxyribonucleic acid

H': Shannon-Weiner index of diversity

HCl: hydrochloric acid

HSD: Honest Significant difference

J': Evenness/Pieolu's evenness

KEGG: Kyoto Encyclopaedia of Genes and Genomes

KO: KEGG Orthology

KOH: Potassium hydroxide

LEfSe: Least discriminant analysis effect size

LSD: Least significant difference

LDA: Least discriminant analyses

OM: Organic matter

NGS: next-generation sequencing

NH₄: ammonium

NRF: National Research Foundation

OC: Organic carbon

OTU: Operational taxonomic unit

P: phosphorus

PCR: polymerase chain reaction

PERMANOVA: Permutational multivariate analysis of variation

PERMDISP: Permutational multivariate analyses of dispersion

RDA: redundancy analyses

RDP: Ribosomal database project

Ref.: Reference

rRNA: ribosomal ribonucleic acid

SR: Species richness/number of OTUs

SRA: Sequence read archives

SSU: small sub-unit

USA: United States

Preface

This thesis is compiled in article format following the guidelines for writing of the doctoral thesis from the 2023 edition of the Manual for Postgraduate Studies, North-West University. The American Psychological Association [APA] 6th edition referencing style was used for the bibliography. The articles in Chapters 2, 3, and 6 will be submitted to the journals listed below.

Chapter 2 article title: Ecological Role of endophytic microorganisms in sustainable crop production. Targeted Journal: Biotech3

Chapter 3 article title: High-throughput sequencing analysis of community diversity and functional structure of endophytic bacteria in edible vegetable species: potential Implication on Plant nutritional quality. Targeted Journal: Scientific report

Chapter 6 article title: Assessment of plant growth-promoting capability of bacterial endophytes. Targeted Journal: South African Journal of Botany

Declaration

I, Adekunle Raimi hereby declare that this thesis is my original work and that I have acknowledged all authors, contributors, and sources. I declare that the thesis has not been submitted to any other university for examination.



Adekunle Raimi

List of publications

1. Raimi, A., Adeleke, R., 2021. Bioprospecting of endophytic microorganisms for bioactive compounds of therapeutic importance. Archives of Microbiology (2021) 203:1917–1942, doi: 10.1007/s00203-021-02256-z

2. Raimi, A., Adeleke, R., 2023. 16S rRNA gene-based identification and plant growth-promoting potential of cultivable endophytic bacteria. *Agronomy Journal* 115, 1447–1462. doi: 10.1002/agj2.21241
3. Raimi, A.R., Ezeokoli, O.T., Adeleke, R.A., 2023. Soil nutrient management influences diversity, community association and functional structure of rhizosphere bacteriome under vegetable crop production. *Frontiers in Microbiology*. 14, 1–16. doi: 10.3389/fmicb.2023.1229873

CHAPTER 1 :

BACKGROUND AND PROBLEM STATEMENT

1.1 Introduction

Leafy vegetables such as beetroot, broccoli, cabbage, carrot, lettuce, onion and spinach are widely cultivated globally in home gardens, glasshouses and open field farming depending on the local agricultural practices and climatic conditions (Tshikororo et al., 2023). Spinach, *Spinacia oleracea*, is an annual flowering plant belonging to the family Amaranthaceae and order Caryophyllales. Lettuce (*Lactuca sativa* L.) is part of the daisy family Asteraceae (Křístková et al., 2008; Tshikororo et al., 2023), while onion, *Allium cepa* L., is a herbaceous biennial root crop in the family Amaryllidaceae (Farha et al., 2018). Another important biennial herbaceous edible taproot vegetable widely consumed is the carrot, along with cilantro and celery, belong to the Apiaceae family (Que et al., 2019). Some cruciferous vegetables used in the human diet include broccoli (*Brassica oleracea* L. var. *Italica*), kale (*B. oleracea* var. *viridis*), cabbage (*B. oleracea* var. *capitata*) and rape (*B. napus* var. *oleifera*) belonging to the family Brassicaceae (or Cruciferae) (Franzke et al., 2011; Minetor, 2005). Though mainly grown in the winter season, these crops are produced year-round as leaf vegetables for consumption and other health benefits to humans (Allemann & Young, 2005; Henning et al., 2017; Ülger et al., 2018). This study mainly focused on cabbage, lettuce, onion and spinach.

Vegetables are a key component of a healthy diet comprising essential nutrients such as folate, dietary fibres, potassium, magnesium, antioxidants, and vitamins A and C (Tshikororo et al., 2023; Ülger et al., 2018). These nutrients are important for maintaining and improving the overall health and well-being of humans. Some naturally occurring compounds in vegetables such as phytonutrients and vitamins have been demonstrated to have several health benefits, including reducing the risk of high blood pressure, cancer and heart attack (Henning et al., 2017; Oelofse and Averbek, 2012). The methods of preparing vegetable

diets affect available nutrients and their potential health benefits (Lee et al., 2018). It is highly recommended to eat raw or moderately cooked vegetables to retain their nutritional values and inherent beneficial microbiomes. Unprocessed vegetables in the form of salad impact human health through their microbiome, which influences human gut functioning (Nithya and Babu, 2017; Ülger et al., 2018). Thus, vegetables are a good source of important microbes with great potential in human health management and biotechnological advancement.

Due to the increasing awareness of the benefits of healthy diets, the global demand for vegetables has increased over the years, necessitating an increase in production (Mason-D'Croz et al., 2019). In 2021, the global gross production value of spinach doubled, while cabbage and onion increased by 9% and 3% compared to 2020 values, respectively (FAOSTAT, 2023). Coupled with the effect of a wide range of diseases and pests, vegetable crop health and productivity are greatly affected by the challenges of poor soil fertility and climate change (Phophi and Mafongoya, 2017). Several agricultural practices are widely employed to improve soil and crop productivity. Organic and conventional fertilisers have been the major soil nutrient management systems (Mukherjee et al., 2020) while various pesticides and organic substances are used to protect the plants against the attack of pests and diseases (Boyd and McNevin, 2015). However, these practices have various impacts on the soil and plant ecosystems (Thorat and More, 2022), emphasising the need to consciously choose economically viable practices that contribute to sustainable crop production.

1.2 Problem statement

Food security is a major issue among the developing nations of the world and the intensification of crop production globally has become increasingly important due to the rise in population and associated food demand (Frona et al., 2019). At present, agrochemicals such as chemical fertilizers and pesticides, are widely used for improving soil nutrient content, plant disease management and overall crop productivity (Pal and Gardener, 2006; Wang et al., 2018). However, these management practices cause numerous ecological

damages, including a decline in soil quality, contamination of water bodies, loss of soil biodiversity and pollution of the environment (Savci, 2012; Xun et al., 2016). Human health issues arising from biofumigation and bioconcentration have also become a critical concern. Chemical residue accumulates in organisms over time through contaminated food and surrounding water and air (Devi et al., 2022). In addition, due to the financial demands of procuring these agrochemicals, many developing nations lack access, limiting supplies to farmers (Raimi et al., 2017; Vanlauwe et al., 2014). Thus, only affordable sustainable agricultural practices with no fingerprint of ecological damage will be appropriate (Adeleke et al., 2019b; Raimi et al., 2021). The demand for sustainable agriculture has amplified the need to employ cost-effective, eco-friendly and sustainable technology such as bioinoculants and biopesticides to improve crop productivity (Singh et al., 2016).

To discover novel and competent microbial species for the production of bioinoculants and biological compounds to improve plant and human health, bioprospecting for beneficial microbes in diverse environments is crucial (Raimi and Adeleke, 2021). The soil, rhizosphere, water body, organic manure, compost and vermicompost are examples of ecosystems that have been well-investigated for ecologically important microbes. Various inoculants, including biofertilisers and biopesticides, have been developed over the years. Unfortunately, they are mostly non-native and, hence, are not specific to the local ecological terrain and climate, resulting in field inconsistency. Furthermore, the complex nature of soil microbial communities and the problems of integrating foreign species into an already established community are responsible for the low success of most exotic inoculants (Herrmann et al., 2015; Raimi et al., 2019). A major ecological risk of inoculum application is the disruption of soil microbial community structure and functions due to the non-native species, resulting in competitive interactions (Soumare et al., 2020).

The use of efficient bacterial endophytes, which have a beneficial association with the host plant, is a key strategy to combat these challenges (Raimi and Adeleke, 2023a). Bacterial endophytes have a wide range of plant growth-promoting benefits, including fixation of

nutrients (nitrogen and carbon dioxide), solubilisation and mineralisation of nutrients (phosphorus, potassium, and zinc) and production of phytohormones (1-aminocyclopropane-1-carboxylates, indole acetic acids and gibberellin) (Adeleke et al., 2019; Hardoim et al., 2015; Mashiane et al., 2018; Rana et al., 2020). Additionally, bacterial endophytes synthesise metabolites that have antagonistic properties against a wide range of phytopathogens, making them an essential agent in biopesticide production (Blumenstein et al., 2015; Prasad et al., 2019). This suggests the need to further intensify the investigation of endophytes for their biotechnological benefits.

1.3 Aim and objectives

The aim of the present study is to investigate the ecological roles of bacterial endophytes of ready-to-eat vegetables in crop production and their potential importance in human nutrition.

To achieve the aim, the specific objectives were:

1. To determine the structural and predicted functional differentiation of bacterial endophytes in selected vegetable crops and their potential ecological roles.
2. To evaluate the potential effects of soil nutrient management on rhizosphere bacteria and their subsequent recruitment as endophytic bacteria.
3. To isolate, characterise and assess the potential plant growth-promoting capabilities of bacterial endophytes associated with leafy vegetable crops.
4. To determine the plant growth-promoting capabilities of bacterial endophytes on selected vegetable crops in a greenhouse experiment.

The hypotheses of the study included the following:

- (i) The structural and functional diversity of the endophytic microbial community of vegetable crops vary with plant type and soil nutrient management.

- (ii) The endophytes of vegetables possess the ability to promote plant growth and contribute to ecological functions such as nitrogen fixation, phosphate solubilization and phytohormone production.

To test these hypotheses, ready-to-eat vegetables were sampled from four different farm sites, which have been in operation for at least 5 years (personal communication) and are either using organic fertilisers or conventional fertilisers. Bacterial endophytes were isolated and characterised following standardized methods. Specific information regarding the farm sites and experimental protocols used in the present study are described within the chapters.

1.4 Thesis outline

This thesis consists of 7 chapters, with the first chapter (the present chapter) being the “Introduction”, which provides the background, the problem statements, the aim, specific objectives and the research hypotheses for the present study. Chapter 2 is a general and systematic review of literature discussing the subject: “Ecological role of endophytic microorganisms in sustainable agriculture” and “bioprospecting of endophytic microorganisms for human health management”, respectively. Chapters 3, 4, 5, and 6 address the four objectives outlined above, structured as manuscripts for publications. Consequently, some information overlaps in these chapters were inevitable, especially as they relate to the site, sampling and methodology.

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CHAPTER 2 :

LITERATURE REVIEW

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2.1 Introduction

Microbial endophytes inhabit and associate with host plants, supporting diverse ecological processes and important ecosystem services (Prasanna et al., 2010). Endophytes are known to possess various bioactive compounds of therapeutic importance used in the production of drugs for human health management (Gouda et al., 2016). Thus, this study reviews the literature to gain insight into the numerous importance of microbial endophytes and their bioactive compounds in agroecosystems and biotechnological applications. The present chapter is divided into two sections. Section A is a general review, which describes the agroecological roles of endophytes, while section B is a systematic review, which assesses the potential applications of endophytes and their bioactive compounds in human health management. A summary of the findings and conclusion of the systematic review will be presented in section B to keep the literature review information brief and because of information overlaps with section A.

2.2 Section A General Review: Ecological role of endophytic microorganisms in sustainable crop production

New studies and biotechnological developments have shown that microbial endophytes have important ecological roles, especially in improving agroecosystem management (Berg, 2009; Chhipa and Deshmukh, 2019) and human health (Alvin et al., 2014; Raimi and Adeleke, 2021). Endophytes contribute significantly to several ecological functions, including biogeochemical cycling, nutrient mineralisation and solubilisation, degradation and biocontrol of plant pathogens (Figure 2-1) (De Silva et al., 2018; Lugtenberg et al., 2016). They have a

wide application in biotechnology for the production of various biological compounds that are sustainable and ecologically friendly (Rana et al., 2020; Yan et al., 2018), suggesting the discovery of novel endophytes could expand their application in agrobiotechnology (Ludwig-Müller, 2015; Vigneshwari et al., 2019).

Endophytes inhabit and thrive in host plant tissues, exhibiting multidimensional interactions that may confer beneficial effects on the host (Khare et al., 2018). Due to co-evolutionary adaptation, endophytes have developed novel mechanisms such as antagonisms and resistance to toxic metabolites of the host. Thus, endophytes can promote efficient interventions without the risk of inhibition resulting from the toxicity of host metabolites (Venugopalan and Srivastava, 2015). This property contributes to their applicability in improving plant growth and productivity (Mashiane et al., 2018). Bacterial and fungal endophytes such as *Bacillus*, *Enterobacter*, *Pseudomonas*, *Rhizobia*, *Fusarium*, *Aspergillus* and *Alternaria* participate in plant growth promotion through biological nitrogen fixation (BNF), phosphate solubilization and the production of plant hormones such as cytokinins, gibberellins and indole acetic acid (IAA) (Figure 2-1) (Aydi et al., 2016; Khan et al., 2015, 2016). Endophyte also promotes stress tolerance and modulates the growth and development of the host plant (Redman et al., 2002; Ribeiro et al., 2018), and participate in the biological control of plant pathogens through various mechanisms such as antibiosis, competition, hypovirulence, predation and mycoparasitism (Mishra et al., 2014; Pieterse et al., 2014).

Considering the numerous ecological benefits of endophytes, especially in improving crop health and productivity (Marag and Suman, 2018), endophyte technology holds substantial potential to contribute to sustainable agricultural development through the reduction in the use of agrochemical inputs in crop production. Excessive use of chemical fertilizers and pesticides is associated with soil degradation, environmental pollution, loss of soil microbial diversity, as well as bioaccumulation in crops (Savci, 2012; Wood et al., 2015). Microbial endophytes provide a technology that is eco-friendly, safe and cost-effective with a wide

range of ecological benefits (Adeleke et al., 2019b; Berg, 2009). This review elucidates the evolution, biodiversity, and functional mechanisms of the plant-microbe interactions, focusing on the potential of endophytes that can be harnessed for the development of microbial inoculants useful in sustainable agriculture.

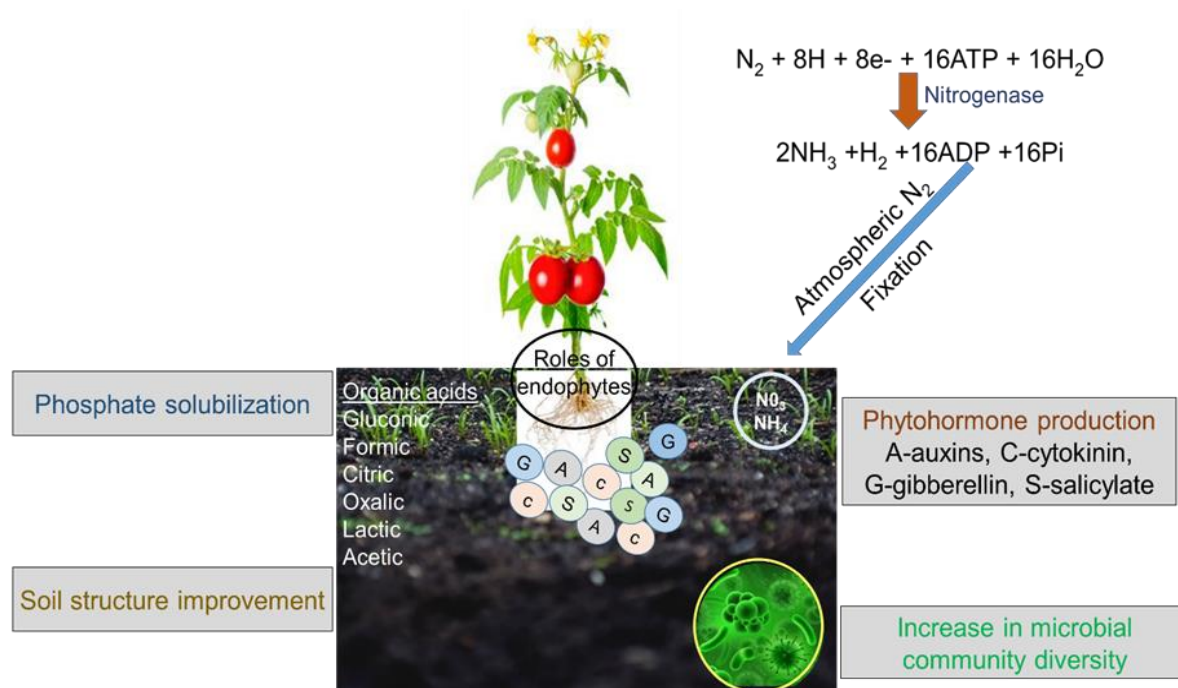


Figure 2-1: Plant growth promotion functions of endophytic bacteria and fungi. Endophytic microbes improve plant growth by participating in soil element cycling through atmospheric nitrogen fixation and solubilization of inorganic and organic phosphorus. Microbial endophytes also produce various phytohormones which enhance plant growth and development.

2.2.1 What are endophytes?

The definitions of endophytes vary widely, being influenced by many primary contexts, including life cycle (either transient or permanent), habitat within the host plant and the type of association (beneficial, latent or harmful) (Gouda et al., 2016; Hardoim et al., 2015). Generally, scholars who focus primarily on the organisms usually describe endophytes based on specific ecological or physiological traits, emphasizing features of host specificity and adaptation (Piontelli and Hormazabal, 2009). Researchers with a keen interest in descriptive ecology or biodiversity studies broadly include all microbes found within host

tissues as endophytes (Stone et al., 2000). A general definition describes endophytes as microbes that asymptotically reside within the internal tissues of host plants under the epidermal cell layer, where they colonise healthy living tissues via latent infections (Butin, 2000; Hardoim et al., 2015; Harrison and Griffin, 2020; Kloepper and Ryu, 2006). Endophyte infections are usually not conspicuous due to the infected tissues not showing symptoms (Stone et al., 2000) and can inhabit different microenvironments of the host, including rhizosphere, rhizoplane, root endosphere and phyllosphere (e.g., phylloplane, anthosphere, caulosphere, carposphere and spermosphere) (Figure 2-2), which provide unique niche conditions (Compant et al., 2010; Deng and Cao, 2016).

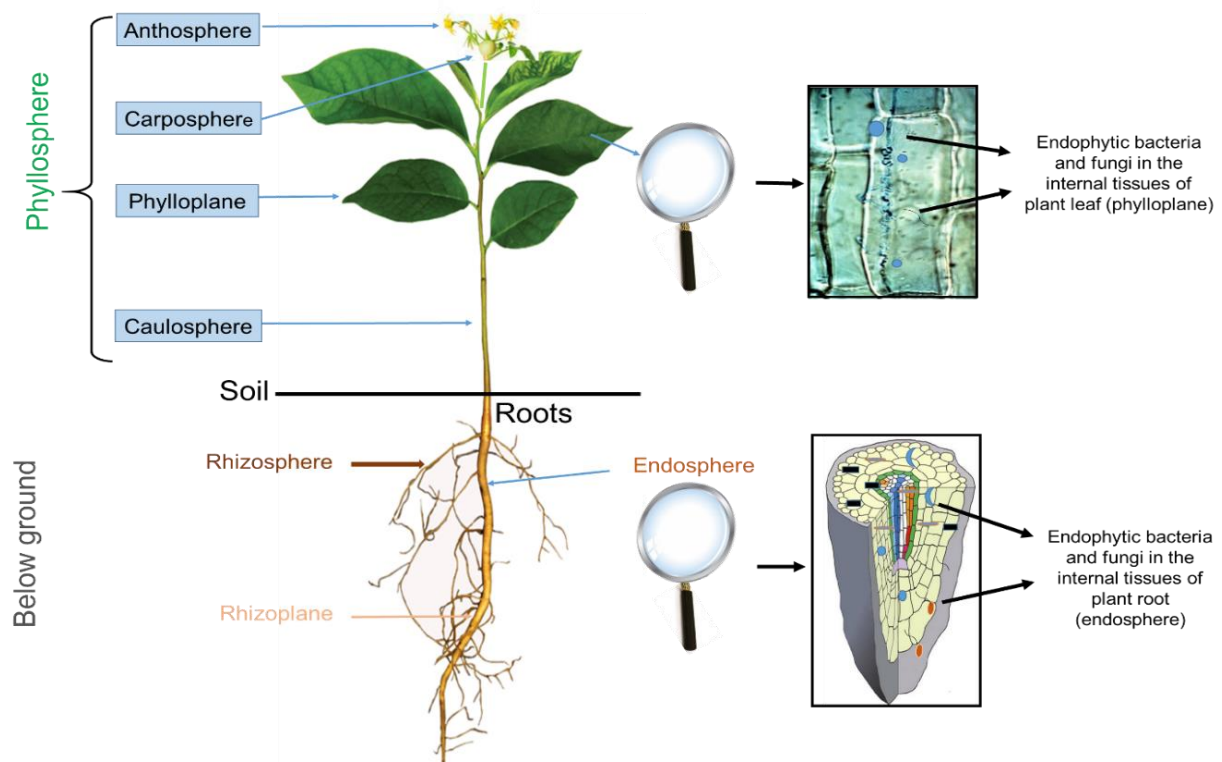


Figure 2-2: Endophytic bacteria and fungi colonizing different tissues of the host plant. The plant-associated micro-environments which the endophytes inhabit are the root endosphere (internal plant tissues below ground) and the phyllosphere (the above-ground plant parts). The above-ground portion consists of rhizoplane (surface of the plant root), and rhizosphere (thin zone of soil bordering the plant root) while the phyllosphere portion of plants is further divided into caulosphere (stem), phylloplane (leaf), Anthosphere (flower), and carposphere (fruit).

Another definition suggests that endophyte could be detrimental to the host plant (Sikora et al., 2007), causing infections that negatively impact host performance (Bacon et al., 2014; Sheibani-Tezerji et al., 2015). Most detailed definitions do not always consider the functional relationships between the endophytes and the host plants (Fesel and Zuccaro, 2016); rather, the habitat within the host is deemed as a key factor, causing the inconsistency in the term “endophyte”, which often varies depending on the specific study or situation of the researcher.

2.2.2 Ecology and evolution of endophytic microbial communities

Endophytes exist in various host plants ranging from algae (Rajivgandhi et al., 2016), medicinal plants (Gos et al., 2017), seagrasses (Supaphon et al., 2013), towering trees and vegetables (Kõiv et al., 2019). The majority of endophytes reported so far are fungi and bacteria, predominantly in the phyla Ascomycota, Basidiomycota (Casella et al., 2013; Padhi and Tayung, 2013; Vigneshwari et al., 2019), and Proteobacteria, Actinobacteria and Firmicutes (Kõiv et al., 2019; Ortiz-Galeana et al., 2018). It is estimated that over a million species of endophytes have been identified (Qin et al., 1996; Sun and Guo, 2012), with their host habitats ranging from mangroves, arid deserts, croplands, grasslands and savannahs, Arctic tundra, temperate and tropical forests (Arnold, 2008). According to Arnold and Lutzoni (2007), endophyte diversity decreases from the tropics to the northern boreal forests, with plants in the higher latitude region having relatively fewer populations of endophytes compared to tropical plants, which have a larger number of species (Arnold et al., 2000; Rodriguez et al., 2009). Endophytes are known to colonise both vascular and non-vascular plants, including mosses, algae, conifers, ferns, grasses and flowering plants (Chakravarty and Gaur, 2018; Nishad et al., 2019).

Recent studies have provided more insight into plant microbiome and the evolution theory of hologenome, which emphasised that plant genotype is the consequence of the interactions between a plant and its complex microbiome, functioning individually or in consortia (Compant et al., 2016) and that selective forces that influence the plant genome also impact

every associated species, including endophytic microbial communities (Saunders et al., 2010). The continuous biological cooperation and evolution between the endophyte and host have significantly influenced plant microbiome assembly (Akçay and Roughgarden, 2006). This association has emerged over time due to functional and survival exigencies such as environmental stress and nutritional limitations between the partners (Kondorosi et al., 2013). Ecological habitat and genetic component of the host are part of the pressure-choice factors for recruitment of endophytes (Kondorosi et al., 2013). Soil nutrient level, humidity, moisture, and temperature are factors influencing the type and quantity of plant exudates, metabolites and biological compounds, which in turn affect the recruitment and structure of the host plant endophytic community (Jia et al., 2016). This natural selection process has resulted in the assembly of diverse plant microbiome, which has various biotechnological importance.

The association between endophytes and host plants is recognised as symbiosis (Wani et al., 2015), (Harrison and Griffin, 2020; Jia et al., 2016) primarily mutualistic, a relationship where parties have a net benefit, including adaptation to a specific ecological niche or stressor (Yuan et al., 2010). The leguminous plant and rhizobia association is one of the earliest known endophyte-plant interactions. Rhizobia, such as *Azorhizobium*, *Bradyrhizobium*, *Rhizobium* and *Sinorhizobium* induce differentiation in root nodule structure, which enhances the nutrient absorption ability of their host plant (Akçay and Roughgarden, 2006; Kondorosi et al., 2013).

Endophytes colonise different niches in the host tissues, including roots, stems, leaves, flowers, fruits and seeds (Fig. 2-2) (Marag and Suman, 2018; Varma et al., 2017). The communities may be denser in the roots (Katoch et al., 2017; Marag and Suman, 2018), or in the leaves compared to other tissues (Dhayanithy et al., 2019; Hazalin et al., 2009), suggesting factors ranging from endophyte colonisation preference and plant ecosystems, nutrient composition and type of association greatly impact the composition and structure of endophyte communities in the host plant (Gaiero et al., 2013). Fungal endophytes have

been reported to colonise the leaves, stems and roots of lucerne (*Medicago sativa*) due to an array of secondary metabolites across the host tissues (Porrás-Alfaro and Bayman, 2011). In addition, farming practices, such as soil tillage, irrigation, fertilisers and pesticide application, influence the endophytic microbial community structure associated with cultivated plants. Therefore, the same plant species under different farming practices may differ in their endophyte community (Leff and Fierer, 2013; Marag and Suman, 2018; Varma et al., 2017). Every plant studied to date is a host to endophyte species, with hundreds to thousands of them colonising plants spanning different geographical locations (Gouda et al., 2016; Verma et al., 2017). This unique diversity and the fact that some endophytes may have transited from the rhizosphere to the plant is fascinating (Arnold, 2008), suggesting further research into endophytic technology to unravel more insights into plant-endophyte interaction dynamics and ecology is necessary.

2.2.3 Classification of endophytes

Based on genetics, biology and mechanism of transmission, endophytes are categorised into systemic and non-systemic endophytes (Faeth and Fagan, 2002). Systemic or true endophytes live within plant tissues throughout their entire life cycle without causing diseases to the host plant (Truyens et al., 2015; Wani et al., 2015), while non-systemic or transient endophytes inhabit the host tissues for at least a period in their life cycle and exhibit no apparent symptoms of disease (Frank et al., 2017). Under stressful or resource-limited conditions, non-systemic endophytes may become pathogenic, causing infections and damage to their host (Wani et al., 2015). Systemic endophytes are cocladogenetic, implying phylogenetically identical endophytes will always be present in a given host, even under varying environmental conditions (Wani et al., 2015). Non-systemic endophyte cohabitation is short-lived and seasonal, with diversity and functional properties varying with changes in the host's physiological structure (Botella and Diez, 2011). Based on colonisation strategies, endophytes may be obligate, facultative or opportunistic. Systemic endophytes are obligate (Hardoim et al., 2015) whereas non-systemic endophytes are opportunistic, only

infesting hosts under stochastic events through stem wounds and root hairs. Opportunistic endophytes are less competitive due to the lack of cellular machinery crucial for colonisation (Yuan et al., 2010). The vast majority of endophytic microbes belong to the facultative group, which are free-living and colonise plant tissues under suitable ecological conditions (Gaiero et al., 2013).

The fungal endophytes have been classified broadly into two major groups based on ecological groupings or in terms of their phylogeny, evolution or functional traits (Lugtenberg et al., 2016). The first group, clavicipitaceous (e.g., *Balansia* spp. and *Epichloë* spp.), are ecologically and economically essential with high prevalence in above-ground tissues of grass species. They are transmitted vertically through seed infection and confer drought resistance ability to the host, increase host biomass and enhance the production of bioactive compounds that are toxic to herbivores (Pressete et al., 2019). The second group, non-clavicipitaceous endophytes, are most common in asymptomatic tissues of non-vascular plants, including ferns, conifers and angiosperms (Rodriguez et al., 2009; Wani et al., 2015). Clay and Schardl (2002) identified clavicipitaceous as Type I (symptomatic and pathogenic), Type II (mixed interaction) and Type III (asymptomatic) endophytes. Rodriguez et al. (2009) further grouped the non-clavicipitaceous endophytes into three distinct categories based on criteria, such as transmission mechanisms, colonisation patterns, diversity richness, ecosystem functions, colonised tissues and host range. Class 2 comprises members of the subkingdom Dikarya, mainly Ascomycota and, to a lesser extent, Basidiomycota taxa. This group mostly colonises the leaves, roots and stems and possesses the ability to infect and confer stress tolerance on host plants. Class 3 are propagated via horizontal transmission and are mostly associated with vascular and non-vascular plants as well as woody and tropical trees, forming highly localised infections in the above-ground tissues of their host (Lugtenberg et al., 2016). Class 4, known as dark-septate endophytes due to their melanized dark septate hyphae, are facultative biotrophic fungi, mostly found in the Antarctic, arctic, alpine, and tropical zones, colonising the host plant roots (Jumpponen and Trappe, 1998).

Lugtenberg et al. (2016) suggested an additional class of endophytes that can infect and colonise both plants and insects. A possible class of endophytes with a unique ability to colonise both plants and animals is also worth investigating.

2.2.4 Propagation of endophytes in the host

Endophytes can be transmitted vertically or horizontally, or both ways as found in *Epichloë* (Tidke et al., 2018). Systemic endophytes are transmitted vertically from parents to offspring through seeds and vegetative propagules (Truyens et al., 2015), while non-systemic endophytes are transmitted horizontally to the host via spores dispersed by insects, wind, rain, and soil (Frank et al., 2017). Endophytes may naturally grow their hyphae into the embryo of a developing seed, infecting and colonising the growing seedlings (Gagic et al., 2018). For some groups of endophytes, colonisation possibly occurs through infection features such as appressoria or directly via the hyphal penetration of the host plant tissue (Genre and Lanfranco, 2016; Palanichamy et al., 2019; Rodriguez et al., 2009). Vertical transmission can present the possibility of manipulating endophytes in the laboratory through artificial inoculation of plants to develop a desired plant-endophyte partnership that has biotechnological applications (Gagic et al., 2018; Johnson et al., 2013).

2.2.5 Endophytes and host plant association

The exact mechanism and driving forces that activate the association between endophyte and host plant are still ambiguous (Hassani et al., 2018; Suryanarayanan et al., 2009); however, several hypotheses have been proposed. One such assumption is the signalling mechanism involving root exudates such as amino acids, carbohydrates and organic acids (Kandel et al., 2017). These microbial quorum-sensing compounds promote early communication between the endophyte and the host, subsequently influencing the colonisation process (Abdalla et al., 2017; Smith et al., 2015). The theory of “balanced antagonism” suggests that endophyte-host association exhibits high phenotypic plasticity from mutualism to antagonism, being influenced by ecological conditions and biological

interactions between the parties (Kusari et al., 2012; Wani et al., 2015). Depending on nutrient availability, ecological conditions and metabolic status of the host plant, the endophyte-plant interaction could result in beneficial or damaging effects (Eaton et al., 2011; Lahrmann et al., 2013). During mutualistic association, the host plant supports the endophytes by providing nutrients, spatial structure and protection against desiccation while also participating in the transmission of endophytes to the next generation (Aly et al., 2011; Lugtenberg et al., 2016). On the other hand, the host plant provides protection and nutrients to support the endophytes, while the endophytes influence the host plant's physiological and biological properties. Most of the recruited endophytes confer different beneficial values to the host plants, including improved growth and development, biocontrol of pathogens, enhanced stress tolerance (Rodriguez et al., 2009) and production of novel bioactive compounds, with applications in agrobiotechnology (Yan et al., 2018).

The biotrophic lifestyle of endophytes during colonisation is an essential feature. Multifarious cross-communication between different endophytes (e.g. archaea, bacteria, fungi and viruses), and their combined association with the host under diverse ecological selection pressures, impact the outcome of the cross-kingdom symbiosis (Kusari and Spiteller, 2011). A study has attributed the thermotolerance of *Dichanthelium lanuginosum* to the double-stranded virus that was harboured by the plant's fungal endophyte, *Curvularia protuberata* (Rodriguez et al., 2009). Thus, to explore the complex association of endophytes and plants, research should emphasise multitrophic association models analysing the communication impacts among interacting partners in a systems biology approach (Wani et al., 2015).

2.2.6 The ecological functions of endophytes

The association between endophytes and host plants plays a significant ecological role by contributing to ecosystem functions, including soil nutrient cycling and replenishment, and modelling of plant microbial communities and health (Adeleke et al., 2019; Haidar et al., 2018; White et al., 2019). Endophytes participate in biodegradation of plant materials, which is a key process in nutrient cycling (Osés et al., 2006; Qin et al., 1996). Being closely

associated with host tissues, endophytes have immediate access to nutrients and initiate decomposition during plant senescence, thus influencing the rate of the nutrient cycling process (Aly et al., 2011). Ecological roles of endophytes such as plant growth promotion, stress tolerance and biocontrol of phytopathogens have been reported (Figure 2-3) (Rodriguez et al., 2009; Varma et al., 2017; Wani et al., 2015). These benefits have made endophytes an essential factor in sustainable agriculture for improving crop productivity and health (Amatuzzi et al., 2018; Aydi et al., 2016; Kaddes et al., 2019)

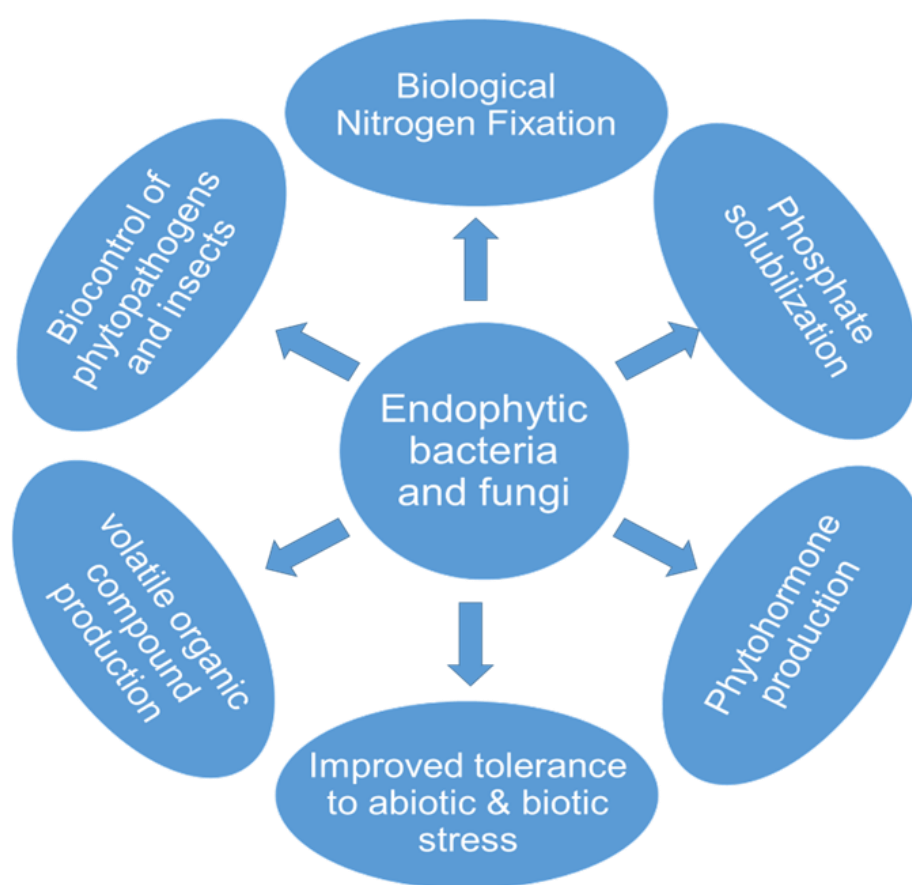


Figure 2-3: Bacterial and fungal endophytes mechanisms of plant growth promotion in sustainable agriculture. These mechanisms of action present endophytes as excellent resources in the production of inoculum for improving crop health and productivity.

2.2.7 Functions of microbial endophytes in sustainable agriculture

Various studies have revealed the potential of endophytic microbes in sustainable agriculture; presenting endophytes as useful resources in the production of bioinoculants for

improving crop productivity (Compant et al., 2010; Gaiero et al., 2013; Lugtenberg et al., 2016). Bacterial endophytes, including *Bacillus cereus*, *Pseudomonas* sp., *Paenibacillus macerans*, *Enterobacter* sp., and *Serratia grimesi* and fungal endophytes such as *Fusarium*, *Cladosporium*, *Penicillium*, *Pestalotiopsis*, and *Trichoderma* have been reported with plant growth-promoting traits such as nutrient fixation, solubilisation and mobilisation, and phytohormone production (De Silva et al., 2018; Hassan, 2017; Ribeiro et al., 2018; Wijesooriya and Deshappriya, 2016). The application of endophyte inoculants in sustainable agriculture is aimed at producing high quantity and quality agricultural produce without any ecological damage. This aim is achieved through several benefits expressed during the interaction of the endophyte inoculants and host plants (Gray and Smith, 2005), and major ones are elucidated.

2.2.7.1 Global element cycling by microbial endophytes.

The biogeochemical cycling of soil nutrients such as nitrogen, carbon, sulphur and phosphorous is a major ecological role of endophytes (Gray and Smith, 2005). Recent developments in genomics and biotechnology have allowed for significant advances in the knowledge of endophytes and the mechanisms they employ in nutrient cycling. Sessitsch et al., (2004) using metagenomics analysis, revealed that endophytic microbiomes of rice participate in the nitrification and ammonia oxidation process, a vital part of the BNF process. Similarly, folia endophytes have been proven to promote slow litter decomposition. This activity impacts the natural cycling rate of chemical elements (Wolfe et al., 2018), especially the nitrogen and carbon cycles, which contribute to the replenishment of ammonia, nitrate, carbon dioxide and other organic compounds in the ecosystem (Adeleke et al., 2019; Saikkonen et al., 2015). Nitrogen is an essential macronutrient needed for plant growth and development and forms a vital component of proteins, enzymes, nucleic acids, and chlorophylls in plants. Similarly, diazotrophic endophytes have been reported to enhance the acquisition of nitrogen by the host plant through BNF (Gupta et al., 2012).

Different amounts of nitrogen have been reportedly fixed in the soil through the endophyte-driven BNF process (Table 2-1) (Boyer et al. 2006; Ngetich et al. 2012; Raimi et al. 2017).

The BNF involves the trapping of atmospheric nitrogen directly into plant-usable forms of N, such as ammonia and nitrate by N-fixing bacteria using a specialised enzyme structure called nitrogenase (*nif*) (de Bruijn, 2015; Puri et al., 2018). For example, an obligate N-fixing endophyte, *Azoarcus* sp. has been found to express *nif* genes within colonised rice roots (Hurek and Reinhold-Hurek, 2003). Similar to the mechanism described for *Rhizobium*, the genomes of some endophytes comprise *nif*HDK operon that encodes dinitrogenase protein and dinitrogenase reductase protein (Carvalho et al., 2014). Dinitrogenase proteins including *nif*DK and MoFe protein contain molybdenum-iron cofactors where nitrogen reduction takes place whereas the dinitrogenase reductase protein comprised Fe protein and *nif*H involved in electron transfer from an electron donor to the nitrogenase protein (Carvalho et al., 2014; Monteiro et al., 2012). This biological process forms a significant source of nitrogen input in the agroecosystem and presents a promising alternative for inorganic nitrogen fertiliser application (Adeleke et al., 2019; Raimi et al., 2017; Vessey, 2003).

Diazotrophic endophytes are well-known for their associations with the roots of diverse host plants, including the economically important legume crops, which are usually nodulated (Carvalho et al., 2014). The formation of root nodules has also been reported in some endophytes and non-legume plants such as *Gunnera* sp., *Parasponia* sp., and actinorhizal plants (Santi et al., 2013). Thus, non-legumes, especially monocots, can obtain a considerable portion of their nitrogen need by forming an association with N-fixing endophytes (Table 2-1). Sugarcane (*Saccharum officinarum*) plants inoculated with diazotrophic endophytes are capable of deriving all their N needs from the fixed N (Triplett, 1996).

Table 2-1: Nitrogen-fixation in bacterial endophytes associated with different economic crops

Host Plant	Endophytes	N ₂	References
Yam (<i>Dioscorea esculenta</i> L.)	<i>Enterobacter cloacae</i> , <i>Xanthomonas translucens</i> ,	~65%	Ouyabe et al. (2019)
Pea (<i>Pisum sativum</i>)	<i>Rhizobium leguminosarum</i> bv. <i>viciae</i> strains	~ 63%	Yang et al. (2017)
Pine (<i>Pinus</i>) seedlings	<i>Pseudomonas migulae</i> AR1, <i>P. graminis</i> AN1, <i>P. lini</i> SN1	<53%	Padda et al. (2019)
Lodgepole pine (<i>Pinus contorta</i>) seedlings	<i>Paenibacillus polymyxa</i> P2b-2R	~ 48%	Padda et al. (2017)
Maize (<i>Zea mays</i>)	<i>Herbaspirillum seropedicae</i> , <i>Burkholderia unamae</i> <i>Azospirillum brasilense</i> ,	~ 29-82%	Van Deynze et al. (2018)
Black pepper (<i>Piper nigrum</i> L.)	<i>Bacillus amyloliquefaciens</i> , <i>B. cerus</i> , <i>B. subtilis</i> and <i>Lysinibacillus fusiformis</i>	~ 15%	Nguyen et al. (2019)
Rice (<i>Oryza sativa</i> L.)	<i>Azospirillum amazonense</i> Y2 (wild type strain)	~ 27.7%	Rodrigues et al. (2008)
	<i>Burkholderia</i>	~ 31%	Baldani et al. (2000)
	<i>Herbaspirillum</i>	~ 47%	Mirza et al. (2000)
	<i>Klebsiella pneumoniae</i> 324	~ 42%	Iniguez et al. (2005)
	<i>Rhizobium leguminosarum</i> bv. <i>trifolii</i>	~ 28%	Biswas et al. (2000); Yanni et al. (1997)
	<i>Burkholderia vietnamiensis</i>	~ 42%	Govindarajan et al. (2008)
	<i>Azospirillum</i> sp.	~ 27%	de Salamone et al. (2010)
Sugarcane (<i>Saccharum officinarum</i>)	<i>Achromobacter</i> , <i>Acinetobacter</i> , <i>Agrobacterium</i> , <i>Enterobacter</i> , <i>Pantoea</i> , <i>Pseudomonas</i> , <i>Rhanella</i> , <i>Shinella</i> , <i>Stenotrophomonas</i> , and <i>Xanthomonas</i>	~ 50%	Taulé et al. (2012)
	<i>Burkholderia</i> sp., <i>Herbaspirillum rubrisubalbicans</i> , <i>H. seropedicae</i> , <i>Gluconacetobacter diazotrophicus</i> , <i>Beijerinckia</i> , <i>Azospirillum</i> , <i>Klebsiella</i> , <i>Enterobacter</i> , <i>Erwinia</i> , <i>Herbaspirillum</i> and <i>Gluconacetobacter</i>	~ 74%	Urquiaga et al. (2012)
	<i>Microbacterium</i> sp., <i>Bacillus</i> ,	~18%	Abeysingha and Weeraratne (2010)
		~ 6%	Lin et al. (2012)
Elephant grass (<i>Pennisetum purpureum</i> Schum.)	<i>Herbaspirillum seropedicae</i> and <i>Herbaspirillum frisingense</i> , <i>Gluconacetobacter diazotrophicus</i> ,	~5.5%	de Morais et al. (2012)

This strategy for improving plant and soil nitrogen is achievable in sustainable agriculture if endophytic microbes capable of BNF are used for inoculating target crops. Some of the competent endophytic diazotrophic bacteria belong to the genera *Azoarcus*, *Azorhizobium*, *Gluconacetobacter*, *Herbaspirillum*, and *Rhizobium* (Haidar et al., 2018; Santi et al., 2013; Vessey, 2003). These endophytes occur in leaf mesophyll cells but a more considerable

amount of their communities are found colonising the cortex and stele of the root compared with the above-ground tissues (Carvalho et al., 2014; Kandel et al., 2017). Based on the increasing knowledge of the endosymbiotic process, a new perspective on harnessing N-fixing ability in non-leguminous plants should be considered.

2.2.7.2 Endophytes participate in nutrient solubilization and mobilization

Endophytes can solubilise complex compounds of phosphorous (P), iron (Fe), potassium (K), and zinc (Zn) in the soil, thereby improving macro- and micronutrient uptake by host plants (Marag and Suman, 2018). Aside from leaching, P fertilisers applied in the agricultural field usually form insoluble complexes with soil elements such as aluminium, iron, copper and calcium, and become unavailable to plants (Figure 4) (Walia et al., 2017). Of the average soil P, which is about 0.05% (w/w), approximately 0.1% is accessible to plants, suggesting the solubilisation strategy is crucial for improving soil available P (Zhu et al., 2011). Interestingly, microbial technology has provided an effective strategy for the manageability of precipitated forms of nutrients in the soils (Figure 2-4) (Adeleke et al., 2019). Endophytes including *Enterobacter*, *Micrococcus*, *Bacillus*, *Pseudomonas*, and *Pantoea*, (Table 2-2) facilitate efficient nutrient solubilisation and mobilization (Sessitsch and Mitter, 2015; Verma et al., 2015). Currently, there is an increasing awareness about utilizing P-solubilising bacterial and fungal inoculants to enhance P-uptake in plants. The inoculants of endophytes solubilise compounds of P such as aluminium and iron phosphate under low pH soils and calcium phosphates in alkaline soils (Walia et al., 2017). For example, *Piriformospora indica* found in the roots of monocots and dicots have been reported to improve phosphate absorption in the host (Kumar et al., 2011).

The primary strategy used by solubilising bacterial endophytes involves chelation, acidolysis, organic acid production and exchange of cations (Figure 2-4) (Hassan, 2017; Marag and Suman, 2018). Several nutrient-solubilising endophytes hydrolyse insoluble inorganic P using organic acids such as citric, lactic, gluconic, acetic and oxalic acids. The phosphate-

solubilizing endophytes mineralize organic P through hydrolysis by secreting phosphatase enzymes, which can save up to 50 kg/ha of phosphate (P_2O_5) fertilizer (Raimi et al., 2017).

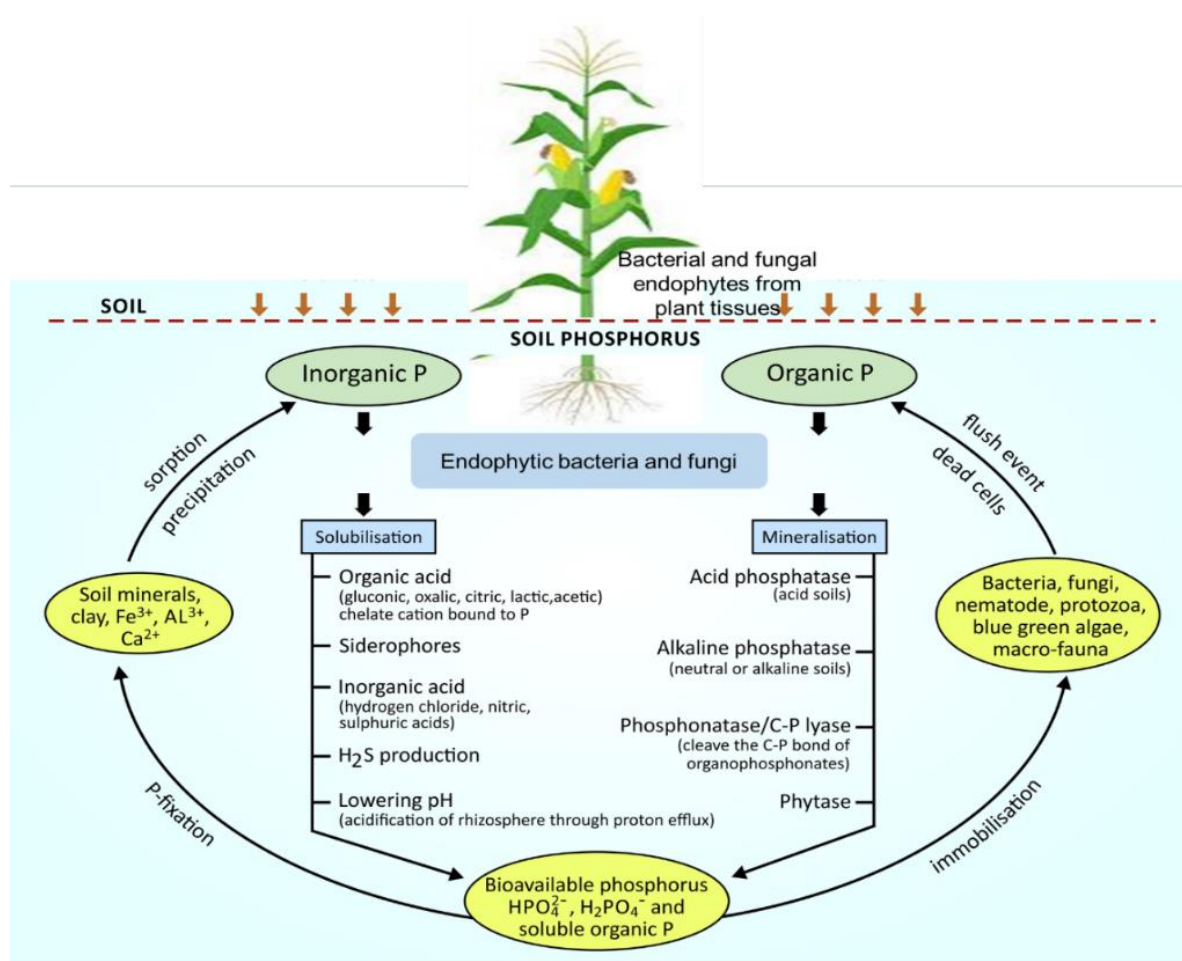


Figure 2-4: Mechanism of soil phosphorus solubilization by phosphate solubilizing endophytic bacteria and fungi. Endophytes solubilise inorganic phosphorus by producing organic acids (gluconic, citric, lactic and acetic acids) and inorganic acids (hydrogen chloride, nitric and sulphuric acids) or by lowering the soil pH through acidification of the rhizosphere. Endophytic microbes usually mineralise soil organic phosphorus through the production of enzymes such as acid and alkaline phosphatase (adapted from Raimi et al, 2017).

Endophytes, especially fungi, may stimulate the development of long root hairs through hyphal extension or hormonal stimulation in the host (Walia et al., 2017). The extended root hair improves the exudation of compounds, including phenolic acids around the rhizosphere, which enhance absorption or mobilization of P and Al tolerance through chelation (Rodriguez et al., 2009). Endophytes inhabiting the seedling roots of *Phoenix dactylifera* L. (date palm),

including *Acinetobacter pittii*, *Bacillus endophyticus*, *Achromobacter* sp. (which solubilise K), and the genera *Bacillus*, *Chryseobacterium*, *Enterobacter*, *Paenibacillus*, and *Rhodococcus* (which solubilise Zn) have been reported to enhance nutrient uptake in the host (Table 2-2) (Yaish et al., 2015).

For a better supply of soluble nutrients to the crops, nutrient-solubilising inoculants should have an intimate association with the host plant (Gaiero et al., 2013). Most times, endophyte-host association differs with plant species and tissue type (Li et al., 2020), suggesting solubilisation efficiency may be contingent on the types of relationship between the endophyte and the host plant. It is also crucial to note that the endophyte communities in the host tissues vary with plant growth stages (Marag and Suman, 2018; Mashiane et al., 2018). The nutrient compositions in the host tissues at different growth stages differ, thereby influencing the functional and species diversity of the endophytes at different maturation stages of the host plant (Kandel et al., 2017; Marag and Suman, 2018).

2.2.7.3 Phytohormones of endophytes contribute to plant growth promotion.

One of the essential mechanisms of endophytes in promoting plant growth is the production of phytohormones, which stimulate and regulate plant responses to ecological changes (Table 2-2) (Gilbert et al., 2018). Endophytes have been reported to synthesise a wide range of phytohormones especially the “classical five” which are auxins (indole acetic acid), ethylene, cytokinin, abscisic acids and gibberellins (Boivin et al., 2016; Lambrecht et al., 2000; Li et al., 2018). These hormones improve plant fitness and competitive abilities (Aly et al., 2011). Auxins and cytokinins have been linked to the regulation of root formation and architecture (Boivin et al., 2016; Li et al., 2018) while gibberellins participate in the early stage of plant development, promoting shoot and root growth as well as increasing root hair density (Khan et al., 2012; Waqas et al., 2012).

Table 2-2: Plant growth-promoting endophytic bacteria and fungi

Endophytes	Host plants	functions	Activity	References
<i>Pseudomonas</i> , <i>Sphingobacterium</i> , <i>Enterobacter</i> , <i>Lysinibacillus</i> ,	Wheat <i>Triticum aestivum</i> L.	P-solubilization Indole acetic acids	3.61-6.44 mg of P	Emami et al. (2020)
<i>Pseudomonas fluorescens</i> smppsap5	Brown sarson (<i>Brassica rapa</i>)	P-solubilization	313.58 mg/L P	Padder et al. (2016)
<i>Pantoea dispersa</i>	Cassava (<i>Manihot esculenta</i> Crantz)	P-solubilization	41.4-55.8 mg/L P	Chen et al. (2014)
<i>Burkholderia cenocepacia</i> CR318	Maize (<i>Zea mays</i> L.)	P-solubilization K-solubilization Indole acetic acids	~ 30 µg/mL IAA	You et al. (2020)
<i>Bacillus cereus</i>	Felty germander (<i>Teucrium polium</i>)	P-solubilization	3.3-7.7mm zone P	Hassan (2017)
<i>Bacillus subtilis</i>		Indole acetic acids	18-30 µg/mL IAA	
<i>Bacillus aryabhatai</i>	Maize (<i>Zea mays</i> L.)	P- solubilization	1.8-24.8 µg/mg P	Marag and Suman (2018)
<i>Pantoea cyripedii</i>		K-solubilization	9.4-101 µg/mg IAA	
<i>Bacillus licheniformis</i>		Indole acetic acids		
<i>Klebsiella</i> sp.				
<i>Pantoea dispersa</i>				
<i>Klebsiella variicola</i>				
<i>Pantoea</i> sp.				
<i>Agrobacterium larrymoore</i>				
<i>Penicillium chrysogenum</i>	Felty germander (<i>Teucrium polium</i>)	P-solubilization	6-9.3 mm clear zone	Hassan (2017)
<i>P. crustosum</i>		Indole acetic acid	27-58 µg/mL IAA	

According to Khan et al. (2015), endophytic *Fusarium tricinctum* and *Alternaria alternata* isolated from *Solanum nigrum*, in comparison with the uninoculated rice plants, significantly enhanced growth attributes of Dongjin rice plants, including chlorophyll content, root and shoot length, and biomass yield due to auxin production. The abundant naturally occurring auxins function in cell elongation and division, vascular differentiation, root formation, apical dominance, and tropism (Lambrecht et al., 2000; Petrasek et al., 2019). Amongst the variously studied auxins, indole-3-acetic acid is the most reported plant growth regulator due to its physiological, biochemical and genetic structures (Gilbert et al., 2018). Other plant hormones produced by endophytes include jasmonates, brassinosteroids, salicylic acids and strigolactones (Ek-Ramos et al., 2019). Under drought conditions, *Piriformospora indica* associated with maize plant caused increased growth in the host through improved oxidative potentials of the roots and gene stimulation for hormone functioning, including the genes responsible for abscisic acid, cytokinins, auxin, and salicylic acid (Zhang et al., 2018).

2.2.7.4 Diverse enzymes of endophytes improve crop health

Endophytic microbes have been widely reported in the production of commercially essential enzymes, including protease, amylase, esterase, cellulase, and pectinase with various applications in agrobiotechnology (Nair and Padmavathy, 2014). The extracellular enzyme hemicellulase found in endophytic *Acremonium zeae* inhabiting *Zea mays* has a hydrolytic activity, with application in bioconversion of lignocellulose into fermentable sugars. Similarly, *Colletotrichum siamense* synthesises extracellular protease while *Fusarium nematophilum* produces amylase, cellulase, glutenase, and protease (Katoch et al., 2017). These enzymes contribute to the biocontrol and adaptation abilities of the fungal endophytes under ecological stress conditions (Katoch et al., 2017). In addition, the extracts of *Burkholderia gladioli* and *Trichoderma* sp. have a high level of hydrolytic enzymes, including chitinase and amylase, which suppress the growth of pathogenic fungi (De Silva et al., 2018). Chitinase degrades chitin into chitooligosaccharide, a compound with significant applications in the pharmaceutical and biotechnological industry (Wang et al., 2007). The potent antagonistic

activity of endophytic *Trichoderma virens* associated with *Saccharum officinarum* L. (sugarcane) against the pineapple disease pathogen (*Ceratocystis paradoxa*) is due to the production of chitinases such as endochitinases, chitobiosidases and N-acetyl- β -D-glucosaminidases (Romão-Dumaresq et al., 2012). Thus, endophytes producing valuable biologically active enzymes are useful for crop health management strategies (Ek-Ramos et al., 2019).

Furthermore, endophytes produce ethylene, an essential hormone involved in root initiation, extension, and development (Iqbal et al., 2017; Monteiro et al., 2012). The rate of ethylene production in plants increases during seed germination and seedling growth. However, when ethylene is produced in excess during plants' response to stress-induced stimuli, it is damaging to the plant, causing inhibition of cell division, root and shoot growth and DNA synthesis (Santoyo et al., 2016; Yaish et al., 2015). 1-aminocyclopropane-1-carboxylate (ACC) deaminase can alleviate the damaging effects of ethylene (Penrose et al., 2001). Some endophytes possess ACC deaminase, an enzyme that regulates the production of ACC, ethylene precursor, from the ethylene biosynthesis pathway to produce α -ketobutyrate and ammonia (Marag and Suman, 2018). ACC deaminase acts as a sink for ACC by decreasing the amount of accumulated ACC through the absorption and metabolism of ACC (Yaish et al., 2015). Moreover, the association of endophytic microbes could reprogram some plant communication pathways effectively and thus influence host plant enzyme production and recruited microbial community (Etesami et al., 2014; Khamwan et al., 2018). In sugarcane, for example, genes encoding an ethylene receptor and two putative ERF (ethylene-responsive factor) transcription factors were distinctively modulated in response to pathogenic or beneficial microbe interactions, respectively, suggesting endophytes could activate some levels of defence response to appropriate interactions, or in specialised signalling spate (Trujillo et al., 2008; Vardharajula et al., 2017).

2.2.7.5 The endophyte-plant association induces stress tolerance in the host plant

The endophyte association promotes stress tolerance of the host plants to various biotic and abiotic stresses (Yan et al., 2018). They act as a biological trigger to activate the host defence mechanisms, thereby enhancing the plant tolerance ability to abiotic stress such as drought through osmoprotectant and cell wall elasticity enhancement, antioxidant system up-regulation, and IAA and gibberellin production (Hardoim et al., 2015). Endophyte-mediated stress tolerance against drought, salinity, and high temperature have been reported in different host plants (Nanda et al., 2019). According to Redman et al. (2002), the mutualistic symbiosis of the thermotolerance host and endophytic *Curvularia* sp. was responsible for the heat tolerance ability of the host plant, *Dichanthelium lanuginosum* found in geothermal soil. Similarly, the high level of metal sequestration and chelation by endophytic *Bacillus* sp., *Aspergillus sydowii* and *Penicillium chrysogenum* is a strategy used by endophytes for improving the tolerance of associated host plants towards heavy metals and pathogens (Chowdappa et al., n.d.; Deng and Cao, 2016). This iron-chelating ability of endophytes is attributed to siderophore production, which is a major source of iron for the host plant growth and development (Haidar et al., 2018). Siderophore also acts to reduce the infectivity potential of plant pathogens by tightly binding with free iron in the soil, thereby reducing the available iron required for pathogen metabolism and growth, leading to the death of pathogens (Marag and Suman, 2018; Raimi et al., 2020). Moreover, it has been shown that the abscisic acid and gibberellins produced by endophytic *Azospirillum lipoferum* contribute to alleviating drought stress effects in maize crops (Cohen et al., 2009).

Plant's unique ecological niche drives various biological activities that may influence genetic modifications or physiological structures in their endophytic microbes, thereby, contributing to the building of resistance against external stress signals in the host plants (Harrison and Griffin, 2020; Porras-Alfaro et al., 2008; Yuan et al., 2010). Considering the theory of hologenome, the total endophytes associated with plants play vital roles in the adaptation of the host to biotic and abiotic stress and thus influence the evolved or evolving holobiont

(Hassani et al., 2018; Zilber-Rosenberg and Rosenberg, 2008). For example, the growth and drought tolerance of hybrid poplar were enhanced by endophyte consortia inoculation (Khan et al., 2016). However, it is still unclear if genomic signatures are available for microbial community-linked fitness phenotypes in the genome and microbiome of host plants (Hassani et al., 2018). Most probably not every microbe that makes up the plant holobiont influences the evolution trajectories of the parties and the holobiont fitness may not always be positively impacted by the selective pressure (Smith et al., 2015; Vandenkoornhuyse et al., 2015).

2.2.7.6 Volatile organic compounds of endophytes and their biocontrol potential

Endophytes produce a plethora of volatile organic compounds (VOCs), which play essential roles in microbe-microbe and microbe-plant interactions. Many known benefits of endophytes are principally attributed to their VOCs (Ryu et al., 2003; Schulz-Bohm et al., 2017). VOCs are hydrophobic, characteristically small, and carbon-based compounds ($< C_{15}$) with high vapour pressure (≥ 0.01 kPa at $20^{\circ}C$), low molecular mass (< 300 Da), low boiling point, and a lipophilic moiety (Kaddes et al., 2019; Schulz-Bohm et al., 2017). As a result of these properties, VOCs readily diffuse and evaporate through the atmosphere, rhizosphere, soil environment and plant tissues (Effmert et al., 2012; Schulz-Bohm et al., 2017). Volatile organic compounds are secondary metabolites but are categorised differently because of their unique properties and special techniques for their detection. Various VOCs are derivatives of alkenes, alcohols, aldehydes, benzenoids, esters, sulphides, and phenols (Chowdhury et al., n.d.). VOC mixtures can be separated and identified in the laboratory by using a combination of chemical profiles built on gas or liquid chromatography-mass spectrometry (GC-MS, LC-MS), or nuclear magnetic resonance (NMR) (Arora et al., 2016; Lai et al., 2013; Wu et al., 2015).

The biosynthesis of a particular VOC could be triggered or repressed during interspecific microbial interaction in the ecosystem (Garnica-Vergara et al., 2016; Kaddes et al., 2019). VOCs provide organisms with rapid and accurate signals to identify neighbouring species,

both mutualistic and antagonistic, and to launch necessary responses for the association (Nisa et al., 2015; Roy and Banerjee, 2019). This knowledge could be harnessed for improving crop growth and productivity and for effectively promoting crop resistance and defence against pathogens causing both pre- and postharvest diseases (Roy and Banerjee, 2019). Several VOCs of agroecological importance have been identified from diverse endophytic microbes. According to Ryu et al. (2003), 2,3-butanediol and acetoin produced by species of *Bacillus* caused a substantial improvement in the growth of *Arabidopsis thaliana*. In addition, VOCs regulate the homeostasis of auxins and cell expansion and contribute to host plant photosynthetic efficiency with an increase in chlorophyll content (Zhang et al., 2007). VOCs could also enhance plant tolerance to abiotic stress such as metals, salt and drought. The *Bacillus* strain GB03 improves iron uptake in *Arabidopsis* by promoting mRNA levels for the Fe-deficiency-induced transcription factor 1 and two other target genes, the ferric reductase *FRO2* and the iron transporter gene *IRT1*, which participate in mediating VOC-induced iron uptake (Farag et al., 2013).

Endophytes, including *Muscodor*, *Noduliosporium*, and *Trichoderma*, modulate plant defence systems against fungal pathogens through the inhibitory activities of their VOCs (Mitchell et al., 2010). The endophytic *Muscodor crispans* from *Ananas ananassoides* (wild pineapple) have high biological activity against diverse pathogens including *Alternaria helianthin*, *Botrytis cinerea*, *Fusarium culmorum* and *Verticillium dahlia* (Mitchell et al., 2010). Some of the VOCs from *M. crispans* are listed by the United States Food and Drug Administration as harmless compounds to humans (Lugtenberg et al., 2016). The VOCs have properties and structures analogous to natural compounds, which make them beneficial in the biotechnology and agricultural industries (Mari et al., 2014). Endophytes, including *Fusarium oxysporum*, *Oxyporus latemarginatus* and *Trichoderma arundinaceum* produce a wide range of VOCs with inhibitory activities against a broad range of phytopathogens, such as *Alternaria alternata*, *Aspergillus niger*, *Botrytis cinerea*, *Colletotrichum gloeosporioides*, and *Penicillium digitatum* (Lee et al., 2009; Malmierca et al., 2012; Zhang et al., 2014). *Oxyporus*

latemarginatus EF069 synthesises 5-pentyl-2-furaldehyde, widely used in the preservation and reduction of damages during post-harvest due to its ability to inhibit the growth of *Botrytis cinerea* and *Rhizoctonia solani*, causing apple decay and moth orchids root rot, respectively (Lee et al., 2009). Postharvest losses of crops such as grains, fruit and vegetables are critical challenges affecting food productivity (Kumar and Kalita, 2017; Singh and Sharma, 2018), suggesting the VOCs of endophytes could present active agents for alleviating these agronomic challenges.

Furthermore, the trichodiene produced by *T. arundinaceum* was responsible for the bioactivity of the endophyte against the pathogen, *B. cinerea* (Malmierca et al., 2012). In a study by Ajith and Lakshmidēvi, *Trichoderma* species, including *T. harzianum*, *T. saturnisporum*, and *T. reesei*, inhibited pathogenic *Colletotrichum capsici* within the range of 30 to 67%. The biocontrol activity was attributed to VOCs such as terpenes, principally limonene, β -phellandrene, and 1,8-cineole synthesized by the endophyte (Ajith and Lakshmidēvi, 2010). Thus, endophytes have profound applications in the production of inoculum for the control of soil-borne pathogens (Kaddes et al., 2019). Strobel introduced the mycofumigation concept, a novel biocontrol for postharvest diseases of fruit and vegetables (Strobel, 2006). Notably, the efficiency of inoculum used for biocontrol is dependent on different factors, including the type of endophyte and VOC, quantity of inoculum, and disease type. Thus, the exploration and discovery of new endophytes and their beneficial VOCs are vital for developing a novel and effective biocontrol strategy.

2.2.7.7 Endophytic bacteria and fungi with insecticidal properties

A vast number of insects destroy various crops of economic importance and are constantly developing resistance against chemical insecticides (Qin et al., 1996), suggesting a new strategy using entomopathogenic microbes as biocontrol may be an option (El-Sayed et al., 2020). Similar to biofertilisers and biofungicides, the awareness and market share of biopesticides are on the rise (Berg, 2009; Thatoi et al., 2013). A large number of endophytic microbes are known to produce secondary metabolites such as *N*-formyl loline, naphthalene,

and nodulisporic acid with insecticidal activity (Figure 2-5) (Qin et al., 1996; Ryan et al., 2008). Nodulisporic acid is a potent indole diterpene isolated from *Nodulisporium* sp., an endophyte of *Bontia daphnoides* with bioactivity against blowfly larvae and grasshoppers due to the activation of glutamate-gated chloride (GluCl) channels of the insects (Smith et al., 2000).

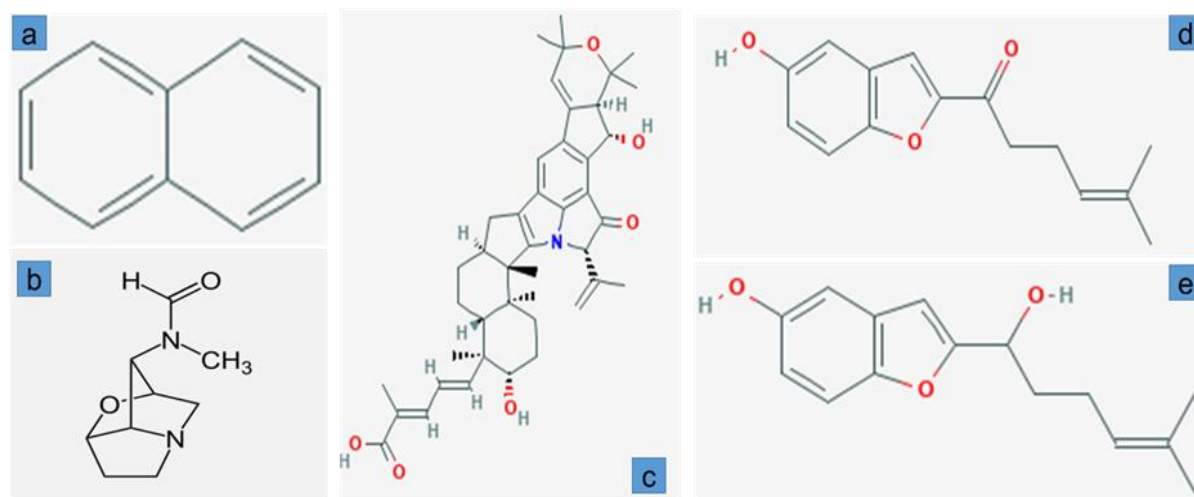


Figure 2-5: Bioactive Secondary metabolites of endophytes with potent biological activity against pests. The compounds (a) Naphthalene (b) *N*-formyllooline (c) Nodulisporic acid (d) 5-hydroxy-2-(1-oxo-5'-methyl-4'-hexenyl)benzofuran (e) 5-hydroxy-2-(1-hydroxy-5-methyl-4-hexenyl)benzofuran have high insecticidal activities (Pubchem.ncbi.nlm.nih.gov/).

Other insecticidal agents known to target GluCl_s include milbemycins, which is a bacterial product. The discovery of endophytes with novel and natural compounds that target GluCl_s underscores the confidence that a more effective strategy for accomplishing insecticidal activity is possible (Smith et al., 2000). Naphthalene, which is produced by the novel endophytic *Muscodor vitigenus*, is an active component of mothballs that repels insects such as the wheat stem sawfly *Cephus cinctus* (Daisy et al., 2002). The endophytic fungus, *Epichloë*, produces alkaloid ergovaline and lolitrem B (Philippe, 2016). Lolitrem B, though able to inhibit the growth of Argentine stem weevil larvae, is a major tremorgenic mycotoxin that causes ryegrass staggers syndrome in grazing livestock such as horses, cattle and sheep (Philippe, 2016). Similarly, ergovaline stimulates vasoconstriction of blood vessels, causing severe health issues in grazing animals when endophyte-infested tall fescue

pastures with fescue toxicosis are grazed (Klotz et al., 2016). Though ergovaline and lolitrem B have potent activity against insects, the associated adverse effects limit their use in bioinsecticide production (Lugtenberg et al., 2016).

The defence mechanism of some plants towards pest and pathogens are enhanced by the ability of their associated endophytes to produce potent insecticidal compounds (Zhao et al., 2020). In a study by El-Sayed and others, endophytic *Sarocladium strictum* and *Aspergillus nidulans* isolated from *Cynancum acutum* and *Lantana camara*, respectively, showed potent insecticidal activity against a second larval instar of *Spodoptera littoralis*. *S. littoralis* is a polyphagous insect pest that causes significant destruction of economic crops (El-Sayed et al., 2020). Similarly, *Streptomyces* sp. BS-1, an endophyte of *Stemona sessilifolia*, produces ten new insecticidal endostemonines A-J, naturally occurring pyrrole-2-carboxylic ester derivatives comprising different fatty acid chains (Zhao et al., 2020). These insecticidal compounds exhibit intense lethal activity (LC₅₀ value range 3.55-32 mg/L after 72 h) against *Aphis gossypii* and moderate toxic activity against *Tetranychus urticae* (Zhao et al., 2020). Four new penicixanthenes, which are xanthene derivatives, were discovered from *Penicillium* sp. JY246, an endophyte of *Ceriops tagal* (Bai et al., 2019). Two of the compounds, penicixanthene B and C, inhibit the activity of newly hatched larvae of *Helicoverpa armigera* Hubner with an IC₅₀ value of 100 and 200 µg/mL, respectively. The compound penicixanthene A, C and D, exhibited potent toxicity against newly-hatched larvae of *Culex quinquefasciatus* with an LC₅₀ range of 11.6 to 38.5 µg/mL (Bai et al., 2019). Amongst the fungi recovered from strawberry leaves, the genera *Aspergillus*, *Cladosporium*, *Paecilomyces*, and *Diaporthe* were tested against third-instar larvae of *Duponchelia fovealis*, and *Paecilomyces* reportedly induced the highest death rate in the pathogen (Amatuzzi et al., 2018). The resistance of the elm plant against the beetle, *Physocnemem brevilinenu*, the vector of the Dutch elm disease agent, *Ceratocystis ulmi*, is attributed to its endophyte, *Phomopsis oblonga* that produces insecticidal compounds such as ergot alkaloids and mycotoxins (Dutta et al., 2014). Compounds such as 5-hydroxy-2-(1-oxo-5'-methyl-4'-

hexenyl)benzofuran and 5-hydroxy-2-(1-hydroxy-5'-methyl-4'-hexenyl)benzofuran (Figure 2-5) have been found in endophytic fungi of wintergreen, *Gaultheria procumbens*. These toxins disintegrate spruce budworms and improve wintergreen yield (Findlay et al. 1997).

Another good example is *Leifsonia xyli* subsp. *cynodonits* that has been genetically modified with the insecticidal gene of *Bacillus thuringensis*. The transformed *Leifsonia* produces delta-endotoxin, a natural agent with bioactivity against insects such as *Lepidoptera* and *Coleoptera* (Li et al., 2007; Rutherford et al., 2002). The endophyte-host association enhances the secretion of the toxin inside host plant tissues, thus protecting the host against the target insects (Li et al., 2007; Wani et al., 2015). Studies have also shown that grasses with a high content of associated endophytes develop resistance to attack by certain insects (Dutta et al., 2014). These beneficial endophytes and their bioactive compounds of entomopathogenic importance can be engineered as a crucial strategy for improving the host plant's natural defence against ravaging insects and for developing new biopesticides. While diverse endophytes with an array of bioactive compounds exist, there are still numerous unexploited endophytes with substantial potential in the production of several novel and natural products of economic interest (Ryan et al., 2008).

2.2.7.8 Microbial endophytes as biocontrol agents

Biocontrol strategies involve the use of beneficial organisms including endophytes to promote positive plant response to alleviate the effects of pest and pathogen attacks (Tranier et al., 2014). Agricultural productivity is adversely affected by diseases and pathogens, causing approximately 10-16% reduction in global food production (De Silva et al., 2018). The antagonistic ability of endophytes against pathogens, due to their bioactive secondary metabolites, has proven to be an excellent strategy for improving plant protection and disease management (Siew et al., 2019). Some of the biocontrol applications have been discussed in the previous sections. Endophytes, including *Bacillus*, *Pseudomonas*, *Enterobacter*, *Pantoea*, *Paenibacillus*, *Streptomyces*, *Colletotrichum*, *Trichoderma* and

nonpathogenic *Fusarium* genera are used as biocontrol agents against pathogens of economically important crops such as cucumber, maize, potato and wheat (Table 2-3) (Díaz Herrera et al., 2016; Marag and Suman, 2018; Vurukonda et al., 2018). A significant advantage of biological control agents of endophyte origin is that they can be applied directly as an inoculant to the seeds or seedlings, thereby optimising their functionality in the target environment and reducing waste that would have occurred during the treatment of a large quantity of soil or already established plants in the field (O'Callaghan 2016).

According to Abdallah et al. (2016), endophytic *Bacillus cereus* and *Alcaligen faecalis* inhabiting *Nicotiana glauca* (tobacco) plant can inhibit *Fusarium oxysporum* f. sp. *Lycopersici* that causes Fusarium wilt of tomato. In the same vein, endophytic *Paecilomyces* was found to induce inhibition against *Duponchelia fovealis*, a new pest of strawberries (Amatuzzi et al., 2018). Fungal endophytes such as *Metarhizium anisopliae* and *Beauveria bassiana* have entomopathogenic ability, protecting their host plants against insect pests (Bamisile et al., 2018). The endophytic *Trichoderma harzianum* exhibits biocontrol ability against pathogens *Pythium aphanidermatum*, *Rhizoctonia solani* and *Gaeumannomyces graminis* var. *tritici* (Vinayarani and Prakash, 2018) while *Fusarium oxysporum* isolated from the roots of red pepper had high biocontrol efficacy against tomato late blight caused by *Phytophthora infestans* (Kim et al., 2007). Endophytes can also protect host plants against the attack of nematodes (De Silva et al., 2018). A study reported that *Microbacterium esteraromaticum* and *Kocuria varians* from *Tagetes* spp. can suppress root-lesion nematode (*Pratylenchus penetrans*) through attenuation of nematode proliferation. Thus, endophytes could be beneficial residual microflora for cultivating other crops in rotation with the host plants (Sturz and Kimpinski, 2004).

The primary mechanism used by microbial agents for the biocontrol of phytopathogens is mainly antagonism, which may be via antibiosis, competition, mycoparasitism, hypovirulence and predation (Haggag and Mohamed, 2007; Mishra et al., 2014). These mechanisms have been reviewed in different studies (De Silva et al., 2018; Ghorbanpour et al., 2018; Köhl et

al., 2019; Pal and Gardener, 2006). In brief, antibiosis is the ability of endophytes to produce toxic antimicrobials, which inhibit the growth of pathogenic microbes (Kloepper and Ryu, 2006). Antibiotics, such as pyrrolnitrin and iturin A synthesized by endophytic *Serratia plymuthica* and *Bacillus subtilis*, respectively, have been reported to inhibit fungal pathogens (Cho et al., 2002).

Table 2-3: Endophytic fungi and biocontrol of phytopathogen

Beneficial endophyte	Host plant	Phytopathogen
<i>Trichoderma asperellum</i>	Lettuce (<i>Lactuca sativa</i>) leaves	<i>Corynespora cassicola</i> <i>Curvularia aerea</i> , <i>Fusarium solani</i>
<i>Aspergillus flavipes</i>	Candyleaf (<i>Stevia rebaudiana</i>)	<i>Sclerotinia sclerotiorum</i>
<i>Penicillium</i> spp.	Banana (<i>Musa</i> spp.)	<i>Fusarium oxysprum</i> , <i>Fusarium</i> sp.
<i>Rhizopycnis vagum</i>	Giger (<i>Zingiber officinale</i> Rosc.)	<i>Rhizoctonia solani</i> <i>Colletotrichum acutatum</i> <i>Fusarium oxysporum</i>
<i>Muscodor yucatanensis</i>	Lemon mint (<i>Monarda citriodora</i>)	<i>Sclerotinia</i> sp. <i>Aspergillus flavus</i> , <i>A. fumigatus</i>
<i>Trichoderma gamsii</i>	Ginseng (<i>Panax ginseng</i>)	<i>Rhizoctonia solani</i> , <i>Pythium</i> spp. <i>Botrytis cinerea</i> <i>Cylindrocarpon destructans</i>
<i>Penicillium simplicissimum</i>	Cotton (<i>Gossypium hirsutum</i>)	<i>Verticillium dahliae</i>
<i>Alternaria</i> sp.	Olive (<i>Olea europaea</i> L.)	<i>Colletotrichum gloeosporiodes</i>
<i>Epicoccum nigrum</i>	Sugarcane (<i>Saccharum officinarum</i>)	<i>Fusarium verticillioides</i> <i>Ceratocystis paradoxa</i>
<i>Penicillium commune</i>	Lemon mint (<i>Monarda citriodora</i>)	<i>Sclerotinia</i> sp.

Adapted from Segaran and Sathiavelu (2019)

A broad range of antibiotics exists, including oomycin A, tropolone, amphisin and phenazine produced by *Pseudomonas* as well as kanosamine, xanthobacin and zwittermicin A,

synthesized by *Bacillus* sp., *Stenotrophomonas* and *Streptomyces* (Rana et al., 2020). Through competition, endophytes can deprive pathogens of space, water and nutrients, by colonizing shared habitats (plant tissues) and sequestering nutrients, thereby limiting the growth and infectivity potential of the pathogens (Ghorbanpour et al., 2018). Endophytes with a highly competitive ability include *Pseudomonas* and *Trichoderma* species. *Pseudomonas* suppresses the growth of *Fusarium oxysporum* f. sp. *dianthi* by producing siderophore, a peptide with a high affinity for iron, which increases competition for nutrients, thereby controlling infection (Guzmán-Guzmán et al., 2017; Lecomte et al., 2016). The environment, adaptation to a unique niche, and endophyte species affect the competition efficiency. However, to ultimately outcompete the pathogens, biocontrol agents must possess effective and diverse colonisation strategies with high growth rates.

Furthermore, the biocontrol mechanisms could occur via mycoparasitism, a situation where a living fungus (host) is parasitized by and acts as a source of nutrients for another fungus and this may be mycoparasite or predator (Hassani et al., 2018; Karlsson et al., 2017). Mycoparasites live on their host (another fungus) and physically penetrate the host hyphae through the development of special organs such as haustoria and secretion of enzymes and secondary metabolites that degrade the host's cells for easy uptake of nutrients (Zhang et al., 2014). Cell wall-degrading enzymes are critical factors in mycoparasitism. *Trichoderma* produces extracellular enzymes, including protease, endochitinase, and β -1,3-glucanases, which degrade fungal cell walls, e.g. of *F. oxysporum* hyphae (Lecomte et al., 2016; Vos et al., 2015). In predation, an organism hunts and kills another, mainly for consumption, usually under inadequate nutrient conditions. Similarly, bacterial mycophagy involves the ability of some bacteria to grow at the expense of pathogenic fungal hyphae actively. Predation is pathogen non-specific and mostly provides less predictable levels of disease control (Pal and Gardener, 2006).

The pre-inoculation of plants with avirulent or hypovirulent strains of phytopathogen could trigger resistance against diverse virulent microbes causing diseases through plant-induced

systemic resistance (Ghorbanpour et al., 2018). The hypovirulent strains may also cause upregulation of various pathogenesis-related and defence-related proteins such as chitinases, and phytoalexins (Bailey et al., 2012). It may transmit its hypovirulence-associated mycovirus into the invading pathogen, thereby reducing the virulence of the pathogen (Kyrychenko et al., 2018). The hypovirulent strain of *Cryphonectria parasitica* has been utilized to reduce the pathogenicity of the virulent strain, which causes chestnut blight in European *Castanea sativa* plants (Ghorbanpour et al., 2018). Also, the nonpathogenic strain *F. oxysporum* Fo47 has been used to trigger defence responses in tomato plants against the pathogenic *F.oxysporum f. sp. lycopersici* (Lecomte et al., 2016). Considering the enormous importance of endophytes in biological control, a continued investigation pipeline towards the commercialization of endophytic microbes will contribute immensely to sustainable agriculture.

2.2.8 Screening and efficiency of beneficial endophytes

There are different strategies used in identifying potential beneficial endophytic microbes, and *in vitro* and *in vivo* experiments have been the widely used methods (De Silva et al., 2018). The biocontrol efficiency of endophytes is usually determined by assessing the prevalence and severity of pathogenic diseases in the host plant according to defined disease indices in the greenhouse (Lecomte et al., 2016; Sturz and Kimpinski, 2004). The greenhouse experiments also seek to determine the physiological status of inoculated plants by measuring changes in activities, including transpiration, antioxidant production, enzymatic activities, and the production of plant growth-promoting and defence compounds. Other plant growth parameters such as plant height, dry and fresh weight, and flowering date are also determined (Lecomte et al., 2016). The potential of endophytes as suitable inoculant agents has been ascertained under *in vitro* and *in vivo* experiments with promising results. Moreover, field trial experiments have been widely used to further determine the adaptability and efficiency of beneficial endophytes under agri-environmental conditions, which vary amongst different ecosystems (Vurukonda et al., 2018).

The effects exhibited by endophytes on host plants are major factors to be considered when screening for beneficial endophytes. Such effects differ from one endophyte to the other. A study revealed endophytic *Alcaligenes faecalis* and *Bacillus cereus* to have antagonistic effects against the Fusarium wilt of tomatoes but with different levels of effectiveness (Aydi et al., 2016). The possible factors causing the differences in the antagonistic activities of biocontrol agents (BCA) have been reported. According to Busby et al. (2016), the variations in the biology of pathogens influence the antagonistic strategies of endophytes. Also, the synergistic production of metabolites by microbial consortia (Hassani et al., 2018) or the synthesis of different types of metabolites by individual microbes with diverse potent activities (Vurukonda et al., 2018) influence the level of antagonistic activities against many pathogens. It could also be that some microbial BCAs interact with plants and enhance the inducement of resistance or priming of plant defence mechanisms against pathogens without any direct interaction with the targeted pathogens (Köhl et al., 2019).

Successful retrieval and selection of endophytes with novel secondary metabolites and incomparable bioactivity require identifying a unique host plant. Such a plant must possess novel strategies for survival in a unique ecosystem with unusual biology (Lugtenberg et al. 2016). Also, the ethnobotanical history of the plant relating to special applications in agriculture amongst the indigenous people must be well-established and realistic. More so, such plants must be endemic, easy to access, and cultivable with an exceptionally extended lifespan and should have the ability to grow in environments that support high biodiversity (Strobel, 2003). For instance, several plant species which harbour a large number of endophytes, producing a broad spectrum of unique secondary metabolites, have been supported by the ecosystem of the Amazonian rainforest for a very long time (Harrison and Griffin, 2020; Liotti et al., 2018).

2.2.9 Endophytic bacterial communities are potential opportunistic pathogens

Despite having beneficial impacts on plants and humans, some endophytes have been reported as opportunistic pathogens (Berg et al., 2014; Nithya and Babu, 2017). Such

pathogens, including endophytic *Salmonella* and *Shigella*, cause different disease outbreaks and constitute a health risk for consumers of plant products, mostly ready-to-eat vegetables and fruits (Akhtyamova, 2013; Kõiv et al., 2019). Although opportunistic pathogens may not cause infections in a healthy immunocompetent host, they take advantage of the host's compromised immune system to cause infections (Berg et al., 2014). According to Parke and Gurian-Sherman (2001), some active biocontrol agents, including endophytic *Burkholderia cepacia*, *Pantoea agglomerans*, and *Stenotrophomonas maltophilia* have been confirmed as opportunistic human pathogens. Similarly, pathogens, including endophytic *Bordetella bronchiseptica*, *Enterobacter aerogenes*, *Listeria monocytogenes*, *Salmonella enterica* and *Staphylococcus aureus*, have been reported in vegetables such as carrots and onions (Nithya and Babu, 2017). Therefore, to reduce the incidence of crop and human pathogens, the microbial endophytes which constitute a high health risk to plants and humans should not be used in inoculum formulation for agricultural purposes.

2.2.10 Endophytic bacterial inoculant formulation

The formulation process is a critical step in the production of bio-inoculants, as it significantly influences the stability, functionality, and efficacy of the final product (Mari et al., 2014). Most biological agents are made into dehydrated forms and maintained in a dry state to ease application using standard distribution and storage channels (Mari et al., 2014). However, product dehydration often leads to death or loss of viability in some microorganisms during the process of drying and storage. Similarly, other undesirable effects, including protein denaturation and cell mutation, may occur (Morgan et al., 2006). Therefore, to reduce or prevent adverse effects, appropriate media used as cryoprotectants must be well evaluated during freeze-drying treatment to ascertain that the functional viability of the inoculant strains is preserved. In the case of *Rahnella aquatilis*, which effectively inhibited *Botrytis cinerea* of Red Delicious apples, the best formulation was achieved when the cells were freeze-dried in a mixture of skimmed 10% non-fat milk. Interestingly, the freeze-dried cells maintained 98%

viability after four years with a slight decrease in antagonistic ability when compared to fresh cells (Navarta et al., 2011).

On the other hand, other studies have reported liquid formulations to present better quality products and easier application compared to dry formulations (including granules and powdered products) (Herrmann and Lesueur, 2013; Pindi and Satyanarayana, 2012). Due to the moisture supplied and readily available nutrients in the liquid products, microbial strains are easily maintained, and their functional traits are preserved throughout the storage period and during field application (Raimi et al., 2020; Schulz and Thelen, 2008). Nevertheless, both liquid and solid formulations have been widely used in the production of inoculants supplied to different agromarkets. One of the primary reasons for developing an effective formulation process is to produce bioinoculant products with a shelf-life of at least six months or more under the right storage conditions (Malusà et al., 2012). Storage of inoculants under different ecological conditions has been reported to affect product viability and shelf life, suggesting an effective formulation is necessary to produce inoculants that can perform effectively under diverse environmental and storage conditions (Abd El-Fattah et al., 2013; Kaljeet et al., 2011).

Many beneficial endophytes have been widely used in the commercial production of inoculants for improving plant productivity (Johnson et al., 2013). For example, *Epichloa* endophytes with pastoral agricultural benefits have been commercialized in perennial ryegrass and tall fescue production in Australia, New Zealand, the United States and South America, where forage grasses are principally for feeding ruminants (Young et al., 2013). These countries have identified novel *Epichloa* endophytes for the development and breeding of special forage grasses with safe properties for feeding livestock. Due to their ability to improve host plant survival and adaptability, especially in unique ecosystems and under biotic and abiotic stresses, *Epichloa* endophytes have been adopted by farmers, and new strains contribute an estimated US\$ 200 million per annum to the New Zealand economy (Johnson et al., 2013).

2.2.11 Conservation and natural traits of endophytes

The effect of anthropogenic alterations of the ecosystem on the abundance and diversity of endophyte communities is still not fully established (Petrini, 1991). However, the loss of endophytic microbes and their intrinsic functional traits in host plants are well demonstrated in different studies (Mousa et al., 2015; White et al., 2019). Wild plant species have higher numbers of unique endophytes with elevated potent biological activity than domesticated relatives (Leff et al., 2017). A study examined *Rhizobium* species from nitrogen-fertilized and unfertilized clover plantations. The findings showed that the *Rhizobium* from the 22-year fertilizer-fed area was less mutualistic and the plant had reduced biomass of 17-30% and less chlorophyll content compared to the control (Weese et al., 2015). Under continued stress conditions, cultivated rice plants were found to maintain a 100% colonisation level, while under a reduced stress situation the endophyte colonisation decreased to 65% (Redman et al., 2011). These results show that endophytes may lose traits or features necessary for optimal partnership under changed conditions. According to Lugtenberg et al. (2016), growing plants with reduced or non-selective pressure may cause a reduction in the ability of endophytes to improve nutrient acquisition and biocontrol of phytopathogens. Also, the application of systemic fungicides or heat treatment of seeds may destroy endophytes alongside the targeted pathogens (Fetcher, 2005; Williams, 1984). The field treatment of seedlings and mature plants with fungicides also eliminates some of the associated endophyte communities. More so, synthetic chemicals used in the coating of seeds or as carrier bases or liquid suspensions for inoculants may be detrimental to endophytes and their intrinsic properties, especially during prolonged storage of commercial inoculants or seed products (Santos et al., 2019). Thus, agricultural practices which have detrimental effects on associated endophytes and their intrinsic properties should be avoided.

However, the documentation and preservation of the wild plant microbiome during crop breeding and management can limit the loss of endophytes and their beneficial properties. Vital information such as the geographical location where the plant was first domesticated

and its native habitat should be documented; peradventure, there may be a need to revisit and obtain initial wild samples of the organism (Lugtenberg et al., 2016). Besides, efforts should be made to conserve not only the host plants but also their associated endophytes. Agronomists and managers of plant germplasm resources need to consider endophytes as a crucial part of the host plant. Moreover, planting seeds that have undergone a series of cultivation or manipulation to increase the number of seeds should be discouraged because the endophytes and their functional properties may have been lost under different selective pressures (Leinonen et al., 2019; Rodriguez et al., 2009). Over the years, plant and endophytes' co-evolution has developed unique genetic traits essential for establishing an intimate and beneficial association (Mishra et al., 2018; Nanda et al., 2019). Hence, missing beneficial endophytes could be reintroduced into a host via artificial inoculation to improve host plant productivity. Unequivocally, the selection, identification and characterization of endophytes and their genetic and beneficial properties are necessary before they are totally lost.

2.2.12 The commercialization of endophytic microbial agents

The commercialization of bioinoculants is fundamentally essential for extensive coverage and regular supply to end-users- i.e. the farmers and for product standardization and quality control (Herrmann et al., 2015; Leggett and Gleddie, 1995). To achieve sustainable and cost-effective production of inoculants of endophytes, multi-step processes that also promote the production of quality products must be instituted. The processes include (1) the recovery of beneficial and novel endophytic microbes from the natural environment such as soil, rhizosphere and plant tissues (Parnell et al., 2016), (2) the assessment of the potential functional characteristics of the selected microbes under *in vitro*, and *in vivo* (glasshouse and field) experiments to obtain the most potent strain that is suitable for product formulation, (3) mass multiplication or production of selected strain(s), (4) delivery compatibility and (5) registration of inoculant products (Kloepper and Ryu, 2006; Parnell et al., 2016). The type of inoculant products depends on the beneficial endophytes, plant

species to be protected, target pathogen, and agroecosystem conditions (Nishad et al., 2019; Pal and Gardener, 2006). Endophytes such as *Paenibacillus* sp. and *Pantoea* sp. from wheat seeds (Díaz Herrera et al., 2016), *Phialocephala sphareoides* inhabiting Norway spruce (Terhonen et al., 2016), and *Paecilomyces* sp. from strawberry (Amatuzzi et al., 2018) are some excellent examples of microbial resources for the production of inoculants. Essentially, bioprospecting and the use of endophytes for enhancing plant development and adaptation, have been recently reviewed (Hardoim et al., 2015; Hassan, 2017; Macías-Rubalcava and Sánchez-Fernández, 2017; Porras-Alfaro and Bayman, 2011) and suggest an increasing awareness amongst researchers of the yet unexplored novel endophytes for sustainable crop production

The numerous potential benefits of endophytes (discussed in this review) and the fact that the benefits can be harnessed and replicated in agroecosystems have become the impetus for the commercialization of endophytes. Though there are some commercial products in the agro-markets, more efforts should be made to commercialize potent endophytic microbial agents into useable inoculant products. Lugtenberg et al. (2016) reviewed some commercial products such as *BioEnsure®-Corn*, which can increase crop yield to about 25-80% under heavy drought stress and by 7% under low drought stress. Moreover, the plant utilized less than 50% of water under reasonable conditions and moderate drought stress. Another product, *BioEnsure®-Rice*, is marketed to cause yield increase under drought and salt stress and decrease water usage in plants by up to 25-40%. These products are made as liquid formulations used for seed treatment (<http://www.adaptivesymbioticttechnologies.com/press-publications.html>).

2.3 Section B: Systematic Review on Bioprospecting of endophytic microorganisms for bioactive compounds of therapeutic importance – Summary, Results and Conclusion

In recent years, biotechnologists have adopted bioprospecting of microorganisms that synthesise bioactive substances of medicinal importance as a method for unravelling novel and natural compounds (Manganyi et al., 2018; Martinez-Klimova et al., 2017). While plant materials have conventionally remained the main source of natural bioactive compounds for drug development (Kouipou Toghueo and Boyom, 2019) the rise in demand and shortage in the supply of plant materials, coupled with the growing challenges of ethnopharmacological plants becoming endangered species, have caused an increase in the use of synthetic materials to produce chemical compounds (Rajamanikyam et al., 2017). However, synthetic compounds used in drug development and human disease management often have fewer therapeutic benefits and can cause adverse effects, such as anaemia and anorexia in cancer patients (CancerQuest, 2017; Karimi et al., 2015). Hence, alternative sources and new technologies for producing natural and safe bioproducts are urgently needed (Ek-Ramos et al., 2019). Bioprocessing for microbial endophytes that synthesise bioactive compounds of therapeutic importance is one such approach (Gupta et al., 2020; Jamwal and Gandhi, 2019).

Endophytes are microbes that live within healthy plant tissues, intra and intercellular space, without inflicting harmful effects or diseases on the host (Gouda et al., 2016). Over time, endophytes and hosts have developed a mutually beneficial relationship, which is beneficial to both parties and also promotes ecological sustainability (Adeleke et al., 2019a; Palanichamy et al., 2018). Both parties have been reported to produce analogous metabolites and cooperate to enhance their physiological stability and functionality. Understanding the mechanisms involved in endophyte-plant interactions can be exploited for ecosystem management and efficient production of diverse beneficial compounds (Venugopalan and Srivastava, 2015). For instance, manipulating growth conditions or

efficient strains to encourage effective colonisation by endophytes can enhance host plant productivity (Faeth and Fagan, 2002), and the introduction of a particular endophyte into a host plant may stimulate a genetic variation that improves secondary metabolite production in both the host and its endophyte (Firáková et al., 2007). Such a strategy may provide insights into how desired bioactive compounds of therapeutic importance can be synthesised directly using bio-engineering (Jia et al., 2016; Kumaran et al., 2009).

Literature has estimated that over a million species of microbial endophytes exist in various plant species, which have potential applications in drug development and human health management (Blackwell, 2011; Yu et al., 2010). Notwithstanding this, only a few of these have been studied, suggesting there are immense opportunities for many undiscovered endophytes and a popular subject for exploration (Harrison and Griffin, 2020; Tidke et al., 2018). Many bioactive compounds of endophytes, such as alkaloids, flavonoids, phenolics, polyketides, and terpenoids (Figure 2-6), have therapeutic benefits for human diseases like cancer, tumours, and bacterial and fungal infections (Gupta et al., 2020; Seo et al., 2010; Xie et al., 2017). Therefore, exploring novel endophytes with bioactive compounds of medicinal importance could significantly promote human health management.

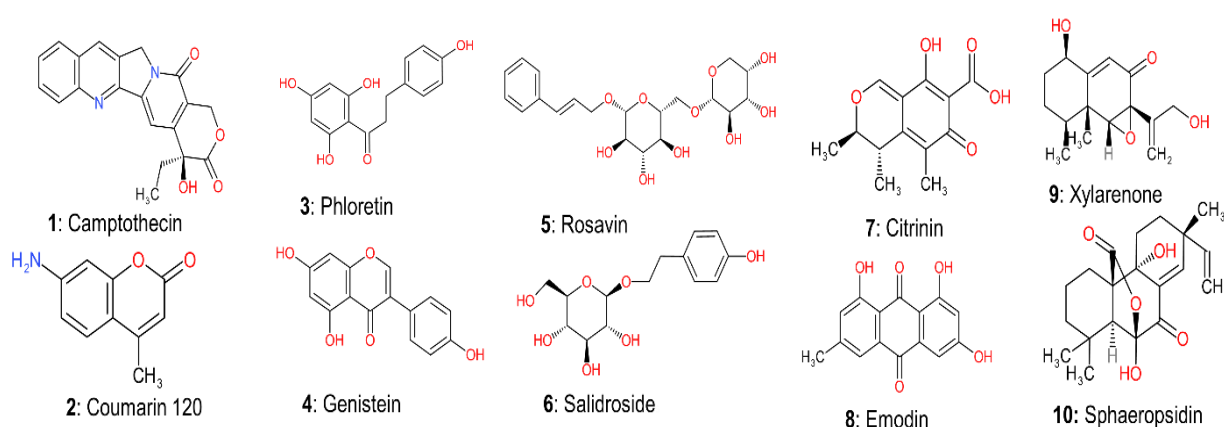


Figure 2-6: Structure of bioactive compounds of bacterial and fungal endophytes with therapeutic benefits. Alkaloids (1, 2), flavonoids (3,4), phenolics (5, 6), polyketides (7,8), and terpenoids (9,10) have various therapeutic potentials such as antitumour, antioxidant, anticancer, antifungal and antibacterial properties making them a useful resource in drug development.

Endophytes offer various benefits in sustainable agriculture, already reviewed in the above chapter. However, research on endophytes with bioactive compounds for therapeutic applications is limited. This information is not always well-collated in the literature, making it difficult to comprehend the current status. Therefore, a review of the available literature is necessary to advance our knowledge and provide valuable information for both applied and basic research towards improving human health and our ecosystem.

In this review, we assessed the potential therapeutic applications of endophytes reported in the literature. Specifically, studies with hypotheses and conclusions about the applications of endophytes and their bioactive compounds were reviewed. An increased interest in endophyte research has been observed in the last 20 years, so published articles from the years 2000 to 2019 were included in this systematic review (PRISMA guidelines used is explained in the published article).

2.3.1 Potential benefits of microbial endophytes in human health management

Over the years, the exponential rise in human health challenges and their subsequent management has been an enormous task amongst health practitioners and stakeholders, including biotechnologists and pharmacologists. The challenge has also been exacerbated by the adverse effects of the various chemical compounds used in the synthesis of drugs for the therapeutic management of human health (CancerQuest, 2017). Thus, considering the well-known benefits, especially in the synthesis of novel bioactive secondary metabolites, which have applications in biotechnological and pharmaceutical industries, endophytes present a good source of biological resources for the production of drugs for human health management (Gouda et al., 2016).

Microbial endophytes have diverse biological activities such as antibacterial and antifungal properties against clinically important pathogens including, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Shigella flexneri*, *Mycobacterium tuberculosis*, *Saccharomyces cerevisiae* and *Candida albicans* (Casella et al., 2013; Jouda et al., 2016; Kyekyeku et al.,

2017; Praptiwi et al., 2018). The endophytic fungi *Pleosporales* sp. and *Trichoderma* IIM-B4 exhibit antifungal activity against *C. albicans* (Katoch et al. 2019; Li et al. 2019). Similarly, *Aspergillus clavatonanicus* strain MJ31, an endophytic fungus isolated from the roots of *Mirabilis jalapa* L., had high antimicrobial potential against *Staphylococcus aureus*, *Micrococcus luteus* and *Bacillus subtilis*. These endophytes and their bioactive compounds are widely used in the treatment of bacterial and fungal diseases (Mishra et al., 2017).

As a result of being a rich source of natural metabolites exhibiting a broad spectrum of biological activities, endophytes have found applications as anticancer, antidiabetic, antiviral, antimalaria, and antioxidant agents (Chakravarty and Gaur, 2018). A vast diversity of bioactive metabolites of endophytes with substantial pharmaceutical applications such as camptothecin, cytochalasins, taxol, griseofulvin, pericin, terpenoids, phenolic acid flavonoids have been reported and explored as a bioresource for drug discovery (Swamy et al., 2016). Endophyte exploration has helped in the production of safe, efficient and economical drugs for the treatment of different human ailments such as cancer. The alkaloid from endophytes, for example, has been used in the production of commercially effective drugs such as camptothecin and vincristine (Kuriakose et al., 2016; Ran et al., 2017). Many of the chemotherapy treatments for leukaemia, lymphoma and lung cancer use indole-derivative anticancer drugs. One such medication is vincristine, marketed under the trade name “Oncovin R”, which was initially obtained from the *Catharanthus roseus* plant. Of late, vincristine is produced by *Fusarium oxysporum*, an endophyte of *C. roseus* (Zhang et al., 2006). Similarly, a novel compound, sorokiniol, isolated from *Bipolaris sorokiniana* LK12, an endophyte of the alkaloid-rich medicinal plant *Rhazya stricta*, is an essential lead drug for the treatment of Alzheimer's disease (Ali et al., 2016).

Furthermore, novel compounds that may not be produced using chemical processes are synthesised by endophytes using stereo- and region-selective reactions (Pimentel et al., 2011). Metabolites of endophytes demonstrate an excellent source of natural drugs with characteristics that suggest them as viable candidates for production processes in different

industries. The WHO has described antimicrobial resistance to pathogens as a growing global public threat to human health (WHO, 2021). Of importance are the infections due to methicillin-resistant *Staphylococcus aureus*, penicillin-resistant *Streptococcus pneumoniae* and vancomycin-resistant *Enterococcus* pathogens. Considering the global spread of resistant pathogenic microbes and the unsustainable production of drugs using chemical synthesis, the search for unique endophytes as bioresources to overcome the challenges is crucial in biotechnological development (Joseph and Priya, 2011). Endophytic *Fusarium* sp. and *Aeromicrobium ponti* LGMB491 show high antibacterial activity against the methicillin-resistant *Staphylococcus aureus* (Gos et al., 2017; Ratnaweera et al., 2015). Antibiotics such as miconazole, ketoconazole, fluconazole, ampicillin and rifampicin have been detected in endophytes such as *Aspergillus clavatonanicus*. Miconazole is an imidazole antifungal agent commonly used to cure yeast and dermatophyte infections such as vaginal, skin and nail infections. Ketoconazole is used mainly for the treatment of skin dermatophytosis caused by different species of *Candida* (Mishra et al., 2017).

2.3.2 Source of endophytic microbes

Endophytes exist in all known plant species, including vegetable crops such as carrot (*Daucus carota* subsp. *sativus*), cabbage (*Brassica oleracea*), spinach (*Spinacia oleracea*), beetroot (*Beta vulgaris* subsp. *vulgaris*), cucumber (*Cucumis sativus* L.) and lettuce (*Lactuca sativa*) (Kim et al., 2007; Nithya and Babu, 2017). Their diversity is influenced by farming and processing techniques. Farm practices such as soil tillage, nutrient management systems and crop types differentiate organic farming from conventional farming practices. (Hardoim et al., 2015; Leff and Fierer, 2013; Marag and Suman, 2018), suggesting crop endophytic community diversity is different across agroecosystems.

Various disinfection measures are intended to eliminate the entire microbial community of vegetables. However, only the surface microbes may be removed, the endophytes within the internal tissues of plants are rarely excluded. Thus, some of the endophytic microbes surviving disinfection may pose health risks to consumers. According to Parke and Gurian-

sherman, (2001), several active biocontrol agents, including *Stenotrophomonas maltophilia*, *Pantoea agglomerans*, and *Burkholderia cepacia*, have also been confirmed as opportunistic human pathogens. Hence, one of the overall objectives of the present study is thought to investigate the potential opportunistic human pathogens existing as latent endophytes in different ready-to-eat vegetables.

2.3.3 Antibiotic resistance of potential endophytic microbes, a critical health risk

Different studies have reported the presence of antibiotic-resistant microbes in various environments, including water bodies, soil and plants (Chen et al., 2016; Manyi-Loh et al., 2018; Shen et al., 2021). Vegetable crops cultivated in agricultural soils under organic fertilisers possess different levels of antibiotic-resistant and pathogenic microbes (Al-Kharousi et al., 2018; Kljujev et al., 2018). From the one-health concept, the contamination of soil and plants may pose health challenges to consumers of plant materials, especially leafy vegetables, which may not be cooked before consumption. Thus, it is essential to investigate the antibiotic resistance of the endophytic community of vegetables to have insight into the associated potentially pathogenic microbes. The most common antibiotic-resistant bacteria of medical importance are Gram-negative bacteria and mostly foodborne pathogens, including *Campylobacter* sp., *Escherichia coli* O157: H7, and *Salmonella* sp. among others (Bintsis, 2017). Bacterial endophytes in the genera *Pseudomonas*, *Xanthomonas*, *Staphylococcus*, *Bacillus*, *Paenibacillus*, *Kocuria* and *Microbacteria* inhabiting ready-to-eat vegetables such as lettuce, carrot, cucumber, radish and tomato have been reported to have antibiotic-resistant genes. The occurrence of these endophytes has also been reported in wastewater and as pathogens in human (Scaccia et al., 2021). The indiscriminate application of agrochemical inputs and organic waste in agroecosystems has contributed to the growing risk of antibiotic resistance in soil microbes (Manyi-Loh et al., 2018; Zhu et al., 2017). Antimicrobial resistance has become a rising challenge that is undermining the efficacy of antibiotics used in disease treatment, and constituting a potential risk to humans, animals and the environment. Importantly, there is a need for consolidated

attention of scientists and stakeholders to address or alleviate the imminent risk (Silbergeld et al., 2008; WHO, 2021).

2.4 Conclusion

The diverse bacterial and fungal endophytes and the growing knowledge of their beneficial associations with a wide range of host plants hold promise for developing novel and valuable endophyte-host plant partners with applications in agroecosystem sustainability. Plant-endophyte associations possess enormous potential in improving crop health and productivity through biogeochemical cycling, soil nutrient replenishment, yield increase, enhanced stress tolerance, bioactive metabolite production and biocontrol of pathogens. It is crucial to understand the mechanisms of endophytic associations that elicit essential agronomic benefits and harness such for sustainable crop production. This review revealed that bacterial and fungal endophytes are viable agents in the formulation and production of bioinoculants for improving crop productivity. More so, using bio-engineering techniques, endophytes with desirable traits can be (re)introduced into plants, thus creating formidable and novel associations that are economically and commercially viable for improving crop productivity.

Based on the data from the systematic review, endophytes are promising and reliable sources of bioactive compounds that can be employed in human health management. Several studies established that endophytes possess at least one of the multiple therapeutic qualities, including anticancer, antibacterial, antifungal, antitumor antioxidant and antitumour activities. Literature showed that over 100 plant species, mainly medicinal plants, have been well-investigated from various ecosystems. In addition, this study revealed there is a knowledge gap in developing countries, particularly in Africa, which has limited available information. This presents a great opportunity for more research in this region to discover novel endophytic microbes that can synthesize new and potent bioactive compounds of medicinal importance. Some endophytes can produce bioactive compounds analogous to those of their host plants. This knowledge is essential in bioengineering for efficient

production of bioactive compounds with therapeutic benefits, especially from microbial endophytes of an endangered host plant species. There were a high number of *in vitro* experiments reported, and to fully understand the effectiveness of bioactive compounds, researchers must go beyond laboratory testing.

While fungal endophytes have been extensively studied, there is a need to intensify efforts on bacterial endophyte research for more discoveries of beneficial compounds. This observation informed the basis of investigating bacterial endophyte communities in the next chapter. In addition, researchers must explore both known and novel bioactive compounds from endophytes for biological product development, allowing for a conclusive exploration of already discovered compounds. Despite their importance, less than 5% of total plant diversity has been explored for their microbial endophytes and natural products and bioactivities. While several plants, including medicinal plants, shrubs, feed, fibre and grain crops have been well investigated, vegetable crops, which hold a gateway to natural and effective human health management are less investigated for the ecological role and biotechnological importance of their bacterial endophytes, suggesting the reason this research focused on vegetable crops. Addressing these areas of endophytic research may lead to the discovery of more novel and potent natural bioactive compounds of biotechnological importance. The economic importance of bacterial endophytes on human nutrition can also be inferred.

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CHAPTER 3

HIGH-THROUGHPUT SEQUENCING ANALYSIS OF COMMUNITY DIVERSITY AND FUNCTIONAL STRUCTURE OF ENDOPHYTIC BACTERIA IN EDIBLE VEGETABLE SPECIES: POTENTIAL IMPLICATION ON PLANT NUTRITIONAL QUALITY

3.1 Introduction

Leafy edible vegetables are essential for maintaining a healthy diet because they contain key nutrients such as dietary fibre, folate, potassium and vitamins (Nithya and Babu, 2017). Interestingly, vegetables also contain endophytic microbial communities within their tissues (Tadych and White, 2019), which is one of the most varied and complex components performing crucial roles in ecosystem functioning and host plant health (Hardoim et al., 2015; Khare et al., 2018). Endophytic bacteria have been implicated in different agroecological roles that contribute to sustainable agriculture (Adeleke et al., 2019; Santoyo et al., 2016). They also produce several biochemical compounds with human health importance (Raimi and Adeleke, 2021; Singh et al., 2017), suggesting insight into the community composition and functional diversity of bacterial endophytes may provide a better understanding of plant-microbe interactions and how it can further be utilised in biotechnological development. Despite being well cultivated and consumed, vegetables are less explored for bacterial endophyte communities and their potential importance related to plant nutritional values.

The demand for vegetables has increased over the years, prompting the need to increase their production (Mason-D'Croz et al., 2019). The major strategies for enhancing soil nutrient richness to support increased vegetable production are organic and conventional fertiliser applications (Cheng et al., 2010; Ngetich et al., 2012). Organic fertilisers, including green waste, animal manure, and compost, are a sustainable option for improving plant health and quality (Lazcano and Domínguez, 2014). In contrast, conventional fertilisers, such as

chemical fertilisers, pesticides, and fungicides, though enhancing plant productivity, can have undesirable impacts on the environment, including soil structure degradation, microbial community loss and pollution of air and water bodies (Diacono and Montemurro, 2010). Both types of fertilisers impact the diversity and functional structure of soil and plant microbes, as well as their metabolism, enzyme activities and genetic repertoire (Pittarello et al., 2021; Zhang et al., 2022). These agroecological practices also drive the prevalence of antibiotic resistance in soil, microbes and plants (Chen et al., 2016; Flandroy et al., 2018). A study has reported the presence of antibiotic-resistant genes in the endophytes and phyllosphere of lettuce and endive planted in manure-amended soil (Wang et al., 2015). Under the One-Health concept, these practices may potentially affect human nutrition through the microbiological quality of plant food, especially vegetables that are eaten raw (Scaccia et al., 2021). Hence, it is crucial to comprehensively understand the impact of soil nutrient management, specifically organic and conventional fertilisers, on community composition and functions of bacterial endophytes as well as the influence on agroecosystems and plant health and nutritional quality.

Furthermore, endophytic bacteria play an important role in plant growth and development by improving nutrient uptake (Hassan, 2017), resistance to pests and pathogens (Hallmann and Berg, 2006; Wiewióra et al., 2015) and biotic and abiotic stress tolerance (Nanda et al., 2019). Studies have shown that bacterial endophytes such as *Acinetobacter*, *Serratia*, *Bacillus* and *Stenotrophomonas* could produce indole acetic acid (IAA) and solubilise phosphate with antagonistic potential against pathogens such as *Colletotrichum* and *Fusarium* (Wang et al., 2016; Xia et al., 2015). A better knowledge of the mechanisms that drive these benefits under different ecosystems is crucial to microbial technology that is aimed at improving crop yield and health (Berg et al., 2014b; Khare et al., 2018). Factors, including geographic locations, plant types (species, genotypes and organs) (Christian et al., 2016) and agronomic practices, such as fertiliser types, irrigation and soil tillage impact soil and plant ecosystems (Azarbad, 2022), which consequently shape the bacterial

endophyte diversity and functional structure (Xia et al., 2015). However, these ecological and biological factors have received less attention (Wagner et al., 2016; Xia et al., 2015), especially as it relates to vegetable production.

Microbial community structures are mostly characterised using compositional and diversity metrics (An et al., 2019; Hoch et al., 2019); however, recent knowledge of the dynamics of the interactions among different taxa in a community has been harnessed to elucidate more on microbial community structure and functions (Pan et al., 2021). According to Banerjee et al., (2018a), closely associated taxa that greatly interact to exert a considerable impact on microbial structure and functions, either individually or in a group regardless of their abundance are termed keystone taxa. Several keystone taxa exist in many ecosystems, including deserts (Zhou et al., 2022), dense forests (Yang et al., 2021) and grasslands (Banerjee et al., 2018b) performing diverse but unique ecological functions. Improved knowledge of microbial interactions could reveal a simplified strategy for assessing microbiomes to better identify key mechanisms driving plant-microbe interactions and keystone species that have the potential to impact plant growth and human nutrition.

Understanding the complex interactions between plants and microbes has been a challenge due to microbial cultivation limitations (Torsvik and Øvreås, 2002). However, microbiome research in biological sciences has been transformed by next-generation sequencing, which evaluates the relationship between microbes and host plants through environmental DNA analysis using specific marker genes (Shokralla et al., 2012). This study used high throughput sequencing analysis to compare the abundance and diversity of bacterial endophytes of vegetable crops across plant species, organs (leaf and root) and fertiliser types (organic and conventional). By using network analysis, keystone taxa with unique functions that potentially impact plant and human nutritional quality are identified. In addition, the study examines if there is any correlation between bacterial community diversity and functions across plant species and fertiliser types. The functional profile was evaluated using the phylogenetic investigation of communities by reconstruction of unobserved states 2

(PICRUSt2) pipeline (version 2.3.0) (Douglas et al., 2020). Moreover, this study hypothesised that bacterial metabolism pathways are impacted by the plant nutrient content, species, and fertiliser type. A distinctive endophytic community structure that varies across vegetable species, organs and fertiliser types is hypothesised, providing evidence that microbial communities and functions exhibit patterns relative to biological and ecological factors such as plant species and fertiliser type. Understanding such patterns in plant-microbe interactions can provide valuable information about agronomic strategies that best support sustainable agriculture and could improve plant health and human nutritional management.

3.2 Materials and Methods

3.2.1 Study site and vegetable sampling

Vegetable-production farms situated in the North West and Gauteng provinces of South Africa were used for this study. The North West farms, SF (26°47'43.5"S 27°02'18.3" E) and BF (26°42'55.0"S 27°04'59.6"E) are located in Potchefstroom, JB Marks municipality, while the Gauteng province farms, JF (26°11'40.1"S 28°03'58.3"E) and TF (26°03'16.0"S 27°40'13.3"E) are situated in Bertrams and Mogale city, respectively (Figure 3-1). The geographical location and climate parameters are presented in Supplementary Table 3-1. Soil classification for the North West and Gauteng province farms is clayey and sandy loam, respectively. To improve soil nutrient richness and plant productivity, the North West farms use plant waste, cow, goat, and poultry manure and are categorised as organic fertiliser (OF) farms, while the Gauteng farms use chemical fertilisers and pesticides, and are classified as conventional fertiliser (CF) farms in the present study.

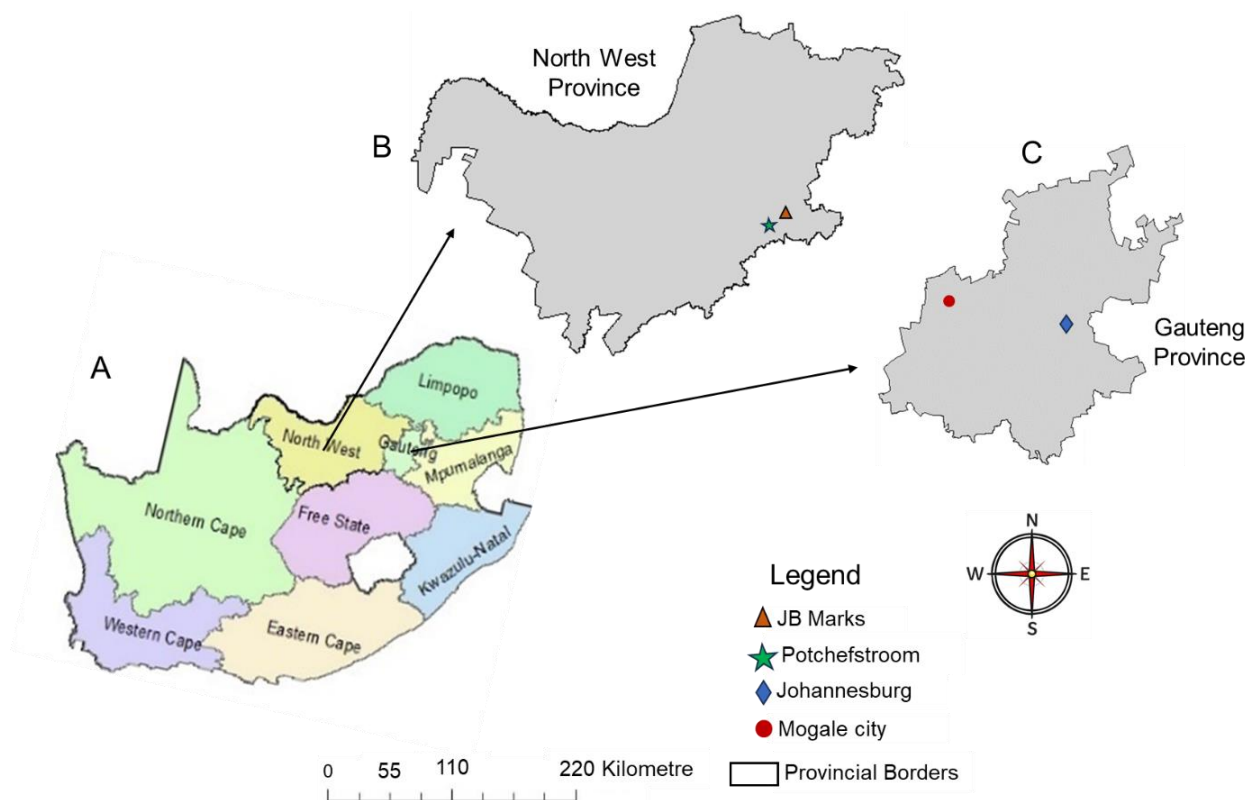


Figure 3-1: Location of sampling sites. (A) Map of South Africa (B) Map of North West provinces and (C) Map of Gauteng province.

Vegetable crops, cabbage (*Brassica oleracea*), lettuce (*Lactuca sativa*), Onion (*Allium cepa*) and spinach (*Spinacia oleracea*) were sampled from the farms. To ensure unbiased sampling, healthy plants of the same age and size were collected, based on visual inspection. Using a sterile garden fork, a whole vegetable crop was carefully uprooted without damaging the tissues and shaken vigorously to remove bulk soil from the roots. Rhizosphere soil was subsequently recovered from the roots (as described in Chapter 4). From each farm, five biological replicates of each vegetable were randomly sampled and immediately placed in sterile zip-lock bags and transported to the laboratory on ice to maintain microbiological integrity. The plant tissues were washed with distilled water and the phyllosphere (comprising the leaf and stem) and root were carefully lacerated using a sterile blade. To eliminate the interference of viable and nonviable epiphytic microbes and obtain only endophytic bacteria, plant tissues were sterilised following the method described by McKinnon (2016). The vegetable nutrient content was analysed at the institute for Industrial

Crops, Agricultural Research Council, following the methods described in the official methods of analysis of the Association of Official Analytical Chemist (Bodily 1956).

3.2.2 Bacterial 16S rRNA gene library preparation

Total genomic DNA was extracted from the plant organs (pulverised phyllosphere and root) using Qiagen DNeasy Plant kits (QIAGEN®, Hilden, Germany), following the manufacturer's instructions. The DNA was quantified with a Nanodrop™-1000 (Nanodrop Technologies Inc., Wilmington, USA), while the integrity and size were checked in a 1% agarose gel electrophoresis. The 16S rRNA gene was amplified in a polymerase chain reaction using Illumina barcoded primers, 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3') targeting V3-V4 region (Klindworth et al., 2013). The library preparation was performed as described by van Wyk et al., (2017). Paired-end (300 bp) sequencing of the libraries was performed on Illumina MiSeq sequencer using Nextera v3 kit (Illumina Inc., San Diego, USA) at the Sequencing facility of the Unit for Environmental Sciences and Management, North-West University, Potchefstroom, South Africa.

3.2.3 Bioinformatics analysis

After demultiplexing and trimming of barcodes using MiSeq Reporter software (Illumina Inc., San Diego, USA), the quality of the reads was checked with FastQC v. 0.11.9 (Babraham Bioinformatics, UK), and trimmed using Trimmomatic software v. 0.38 (Bolger et al., 2014). The DADA2 plugin in QIIME2 (Bolyen et al. 2019) was then utilised to correct for errors, remove chimaeras and cluster the sequences at 100% similarities to amplicon sequence variance (ASVs) as described in the supplementary information (Supplementary Text S1). Taxonomic classification, phylogenetic relationship, and alpha and beta diversity analyses were performed in QIIME2 and R software v. 4.1.3 (R Development Core Team, 2018). The ASV countable obtained were rarefied to an even depth of 7340 in R software. Alpha diversity, based on Chao1, Shannon, Simpson and Pielou's evenness were used for community diversity, richness, and uniformity analyses. Sequencing depth adequacy in each

sample was evaluated with the Good's coverage index and rarefaction curve analysis. Endophytic bacterial community abundance and composition were calculated and compared between the fixed factors (plant species, organ and fertiliser types) using beta diversity, based on weighted and unweighted unifracs distances in R studio. Using the ggVennDiagram package in R, a Venn diagram was made to show the presence and distribution of unique and shared species. Core species are the 'stable' part of the community exhibiting key functions that impact host physiological performance, and may be associated with the unique or shared species (Alibrandi et al., 2020).

3.2.4 Prediction of endophytic bacterial functional profile

The functional metabolic profile of the endophytic bacterial communities was computed with the Phylogenetic investigation of communities by reconstruction of unobserved states 2 (PICRUSt2) pipeline (version 2.3.0) (Douglas et al., 2020). The pipeline was executed in QIIME2 software using the ASV sequence and abundance table generated against the SILVA rRNA (138) database as an input file. The output files included gene family and pathway abundances (Douglas et al., 2020). The average Nearest Sequenced Taxon Index (NSTI) score was 0.134 ± 0.024 ($\pm$ SD) and 3% of ASVs with NSTI above the maximum cut-off score of 2 were expunged. The non-metric multidimensional scaling (NMDS) and the redundancy analysis (RDA) plot were used to investigate the effects of plant species, plant nutrient content and fertiliser type on the endophytic bacterial community functions. A subset of essential pathways involved in key ecosystem functions contributing to decomposition, metabolism, degradation and biosynthesis and relating to plant nutritional quality were analysed.

3.2.5 Microbial co-occurrence network analysis

Microbial network analysis was performed to examine the interactions among bacterial communities across the fixed factors, using the random matrix theory (RMT) in molecular ecological network analysis (MENA) pipeline (<http://ieg2.ou.edu/MENA/>) (Deng et al., 2012).

Detailed procedures for constructing and analysing networks are well elucidated in previous studies (Deng et al., 2012; Zhou et al., 2011); however, major steps are summarised in supplementary information (Supplementary Text S2). Unnormalized data was used for the analysis and ASVs present in at least 20% of the samples were considered to ensure statistical consistency and reliability. Microbial community interactions were clustered into modules based on their within (Z_i) and among (closeness) (P_i) module connectivity criteria, causing nodes to be grouped into four sub-categories: peripherals, connectors, module and network hubs, using the threshold values for Z_i and P_i as follows: peripherals ($Z_i < 2.5$; $P_i < 0.62$), connectors ($Z_i < 2.5$; $P_i > 0.62$), network hubs ($Z_i > 2.5$; $P_i > 0.62$) and module hubs ($Z_i > 2.5$; $P_i < 0.62$) (Guimera and Amaral, 2005). The constructed network was visualized using the interactive tool Gephi v 0.9.2. Details of the network analysis are available in supplementary information (Supplementary Text S3).

3.2.6 Statistical analyses

R software (version 4.1.3) (R Development Core Team, 2018) was employed for the statistical analyses, with a statistically significant level set at probability (P) < 0.05. The normality of data was verified using the Shapiro-Wilk test and non-normally distributed data were transformed using log, square root, or sine function. Normally and non-normally distributed data were analysed using parametric and non-parametric tests, respectively. The Mantel test was used to assess correlations between vegetable nutrient content, bacterial community composition and metabolic pathways. The relative proportion of each ASV count was log-transformed ($\log(x) + 1$, where $x > 0$) with the “decostand ()” function in the vegan package (version 2.55) of R software for multivariate analyses of the microbial community. Bacterial community structure in multivariate space was visualised with a non-metric multidimensional scaling (NMDS) and an unweighted pair-group method with arithmetic mean (UPGMA) using the vegan and dendextend (v. 1.12.0) packages in R software. Differences between groups were tested with weighted and unweighted unifracs distances dissimilarity using permutational multivariate analysis of variance (PERMANOVA) and

Permutational test for homogeneity of multivariate dispersions (PERMDISP). The Linear Discriminant Analysis (LDA) Effect size (LEfSe) was used to detect the most differentially abundant community and pathways (Mann-Whitney U test, $P < 0.05$, LDA score > 2) between sample groups. The differentially abundant bacterial communities that are statistically significant were visualized as bar plots using the micro-eco package (Liu et al., 2021). An indicator species analysis was performed for the predicted pathways to detect the most discriminatory pathways between vegetable species and across the fertiliser types. Furthermore, to determine the plant nutrient content that best explains the variations in the microbial community composition and pathways across the sample group, a redundancy analysis (RDA) plot was made using an automatic stepwise model selection ("ordistep ()") in the vegan package of R software. Variables with multicollinearity (variance inflation factor > 10) were excluded from the final plot. Log-transformed nutrient data and bacterial species composition relative counts ($>1\%$ genus taxa) were used for the RDA plot.

3.2.7 Data availability

The 16S rRNA gene sequence data is available in the sequence read archives (SRA) of the National Centre for Biotechnological Information as part of a BioProject under the SRA accession number PRJNA1037753 (<https://www.ncbi.nlm.nih.gov/bioproject/1037753>).

3.3 Results

3.3.1 Vegetable nutrient content between the plant species cultivated under organic and conventional fertilisers

The vegetable nutrient content varies between plant species and fertilizer types (Table 3-1). Compared to other vegetables, spinach had the highest amount of most nutrients, including N, Mg, Na, Cu, and Mn across all the farms and fertiliser types). Similarly, spinach had the highest value for Fe, and Zn in all the farms except in farms SF and TF where lettuce and

onion had the highest mean values, respectively. For Ca, cabbage was highest in both OF and CF farm vegetables with a value of 2.14 mg/100 g and 3.18 mg/100 g, respectively (Table 3-1). Onion had the highest amount of P and K in organic farms, while spinach and lettuce had the highest, respectively in CF farms. The %S was not very high across the farms, though the least amount was found in lettuce, the highest (0.53%) was in cabbage across the farms, except in farm SF. The nutrient content varied significantly ($P < 0.05$) between vegetable species for each farm. The main effect of plant species was significant for the nutrient elements except for P (Supplementary Figure 3-1). Similarly, regardless of the plant species types, the nutrients were significantly (Mann U Whitney test, $P < 0.05$) different across OF and CF farms, except for B, N, Na, Mg and S. The main effect of organ types was statistically not significant ($P > 0.05$). There was a significant (ANOVA $P < 0.05$) interaction effect between plant species and fertilizer (organic or conventional) type except for Zn, while the interaction between plant species and organ type was found not to be significant ($P > 0.05$). Due to the unbalanced sample size across the plant species, the effects of the interactions between farms, plant species, and fertilizer type were not considered.

Table 3-1: Nutrient content of vegetable crops from different farm locations

			(mg/100g)											
	Farm	Plant	N	P	K	Ca	Mg	Na	Cu	Fe (mg/kg)	Zn	Mn	B	S (%)
Organic farm	BF	Cabbage	3.16±0.15 ^b	0.46±0.02 ^b	3.41±0.03 ^b	2.14±0.04 ^a	0.75±0.03 ^b	0.47±0.01 ^c	8.17±0.27 ^b	50.15±0.56 ^c	24.05±0.58 ^c	36.23±0.26 ^b	19.29±0.28 ^c	0.48±0.01 ^a
	BF	Onion	3.34±0.05 ^b	0.76±0.01 ^a	4.07±0.02 ^a	1.11±0.04 ^b	0.37±0.02 ^c	2.36±0.03 ^b	6.98±0.09 ^c	107.65±0.57 ^b	30.82±0.28 ^b	11.18±0.11 ^c	21.58±0.41 ^b	0.27±0.02 ^b
	BF	Spinach	4.31±0.07 ^a	0.33±0.02 ^c	3.33±0.07 ^b	0.69±0.05 ^c	1.10±0.01 ^a	3.26±0.06 ^a	28.53±0.51 ^a	140.56±0.52 ^a	62.54±0.51 ^a	49.04±0.67 ^a	28.51±0.49 ^a	0.27±0.02 ^b
	SF	Cabbage	2.14±0.02 ^c	0.27±0.02 ^b	2.73±0.05 ^c	0.50±0.01 ^c	0.21±0.01 ^b	0.35±0.01 ^d	3.55±0.05 ^d	87.32±0.37 ^c	19.69±0.3 ^c	16.18±0.28 ^c	16.24±0.47 ^b	0.33±0.02 ^b
	SF	Lettuce	2.33±0.08 ^b	0.49±0.01 ^a	3.51±0.03 ^b	0.35±0.03 ^d	0.17±0.01 ^c	0.98±0.02 ^b	7.48±0.08 ^b	106.0±1.00 ^a	45.0±1.00 ^b	32.15±0.26 ^b	11.52±0.08 ^c	0.12±0.03 ^d
	SF	Onion	1.33±0.03 ^d	0.27±0.02 ^b	1.57±0.02 ^d	0.57±0.02 ^b	0.21±0.01 ^b	0.73±0.01 ^c	4.92±0.39 ^c	88.27±0.21 ^b	18.6±0.37 ^d	31.48±0.51 ^b	16.77±0.26 ^b	0.18±0.01 ^c
	SF	Spinach	4.26±0.02 ^a	0.47±0.02 ^a	3.79±0.04 ^a	0.93±0.02 ^a	0.93±0.02 ^a	2.96±0.02 ^a	13.43±0.41 ^a	82.00±1.00 ^d	66.99±0.88 ^a	99.00±1.00 ^a	31.17±0.77 ^a	0.37±0.01 ^a
Conventional farm	JF	Cabbage	3.62±0.12 ^a	0.59±0.03 ^b	4.21±0.21 ^c	3.18±0.21 ^a	0.74±0.05 ^b	0.34±0.02 ^c	3.64±0.32 ^c	59.83±1.76 ^c	51.38±0.13 ^c	16.43±0.2 ^c	17.64±0.56 ^c	0.53±0.04 ^a
	JF	Lettuce	2.84±0.09 ^b	0.63±0.03 ^a	6.49±0.09 ^a	1.72±0.06 ^b	0.62±0.02 ^c	1.09±0.13 ^b	9.53±0.11 ^b	108.95±0.93 ^b	85.64±0.33 ^b	35.85±0.31 ^b	25.65±0.57 ^a	0.18±0.02 ^b
	JF	Spinach	3.61±0.11 ^a	0.52±0.04 ^b	5.89±0.04 ^b	1.32±0.05 ^c	1.06±0.04 ^a	2.21±0.03 ^a	11.92±0.35 ^a	172.32±2.11 ^a	178.0±0.55 ^a	65.18±0.76 ^a	23.74±0.28 ^b	0.16±0.01 ^b
	TF	Cabbage	2.54±0.11 ^b	0.46±0.04 ^b	3.76±0.21 ^c	0.55±0.04 ^d	0.23±0.02 ^c	0.57±0.04 ^c	3.49±0.04 ^c	73.65±0.31 ^c	24.52±0.5 ^c	41.65±0.31 ^c	13.01±0.05 ^d	0.43±0.06 ^a
	TF	Lettuce	2.16±0.11 ^c	0.33±0.02 ^c	4.2±0.05 ^b	1.36±0.04 ^a	0.43±0.02 ^b	1.72±0.03 ^b	5.16±0.30 ^b	126.31±1.43 ^b	20.0±1.00 ^d	111.17±1.76 ^b	20.32±0.59 ^b	0.13±0.05 ^c
	TF	Onion	2.14±0.07 ^c	0.49±0.03 ^b	3.4±0.12 ^d	0.60±0.01 ^c	0.23±0.02 ^c	0.59±0.02 ^c	5.21±0.29 ^b	75.16±1.04 ^c	39.23±0.44 ^a	30.34±0.82 ^d	15.74±0.71 ^c	0.29±0.01 ^b
	TF	Spinach	3.47±0.18 ^a	0.85±0.04 ^a	5.43±0.18 ^a	0.88±0.02 ^b	0.66±0.02 ^a	2.32±0.02 ^a	10.62±0.55 ^a	190.33±0.62 ^a	33.52±0.51 ^b	151.65±0.57 ^a	26.77±0.26 ^a	0.27±0.01 ^b

Values are mean ± SD (n = 5). Except for sulphur (S), which is in % proportion, the vegetable nutrient content was measured in mg/100 mg, except for Fe which was measured in mg/kg. CEC; cation exchange capacity, EC; electrical conductivity, OM; Organic matter and TOC; Total organic carbon. Values with different superscript letters for each farm (across the vegetables for each column) are significantly different based on the non-parametric Kruskal-Wallis test or the parametric ANOVA test. The Official Methods of Analysis of the Association of Official Agricultural Chemists (Bodily 1956).

3.3.2 Alpha diversity and endophytic bacterial structure in multivariate space

The alpha diversity, including Chao1, Shannon-Weiner, Simpson and Pielou's evenness significantly (Kruskal-Wallis test, t-test, Mann-Whitney U test, $P < 0.05$) vary across vegetable species (Figure 3-2), organ (Supplementary Figure 3-2, Supplementary Table 3-2), and fertiliser type (Supplementary Figure 3-2, Table 3-2). Results reveal that the abundance and richness of bacterial endophytes were lower in the leaves compared to the roots (Supplementary Figure 3-2). The phylogenetic diversity measure was not significantly different across the plant species (Supplementary Figure 3-3A) and organ types (Supplementary Table 3-2); however, the fertiliser types showed a significant difference with a higher mean observed in CF farms (Table 3-2). The core abundance was significantly different across the plant types (Supplementary Figure 3-3B). Onions had a higher abundance and richness compared to other plant species. The interaction effects of all fixed factors did not influence the differences in alpha diversity measures, except for the Shannon-Weiner index, which was found significant (general linear model (glm), $P = 0.0423$) (Supplementary Table 3-3).

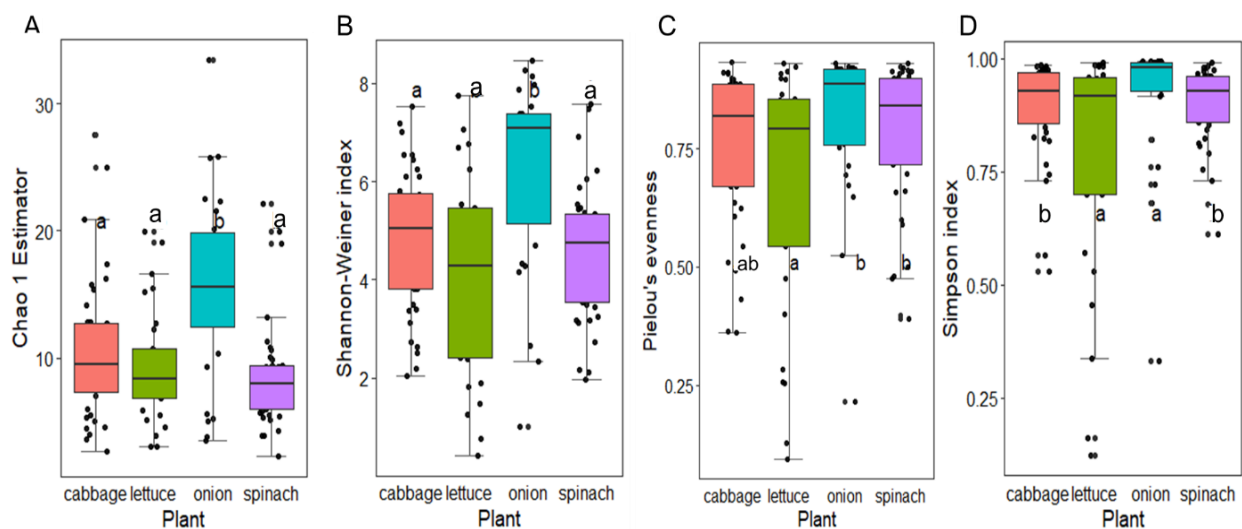


Figure 3-2: Alpha diversity indices measured across vegetable species. A) Chao1, B) Shannon-Weiner (H'), C) Pielou's evenness and D) Simpson index.

Table 3-2: Difference of alpha diversity measure across the fertiliser type factor.

Alpha diversity	Effect	Test statistic W and sig. P value	r (rank biserial) and 95% CI
Chao 1	positive	tiny; W = 2327.00, p = 0.906	r = 0.01, 95% CI [-0.18, 0.20])
Pielou's evenness	positive	small; W = 2636.00, p = 0.143	r = 0.15, 95% CI [-0.05, 0.33])
Shannon-Weiner	positive	Small; diff = 0.36, 95% CI [-0.24, 0.97], t (132.96) = 1.19, p = 0.234	Cohen's d = 0.21, 95% CI [-0.13, 0.54])
Inverse Simpson	positive	small; W = 2618.00, p = 0.166	r = 0.14, 95% CI [-0.06, 0.32])
Core abundance	positive	very small; W = 2423.00, p = 0.575	r = 0.05, 95% CI [-0.14, 0.24])
PD	positive	Large; W = 3009.00, p = 0.002	r = 0.31, 95% CI [0.12, 0.47])

Welch's t-test is for testing the group differences mean between the organic fertilised farms and conventional fertilised farms while the Wilcoxon rank sum exact test is for testing the difference in ranks between the OF and CF farms. Effect sizes were labelled following Cohen's (1988) recommendations. Sig. P value; significant P value. PD; phylogenetic diversity, CI; confidence interval. $P < 0.05$ is statistically significant. Shannon-Weiner was normally distributed and was tested with the Welch t-test.

The NMDS ordination plot reveals a substantial overlap in the bacterial communities of cabbage, lettuce and spinach, while onion samples somewhat cluster separately (Figure 3-3A and B). Despite reduced homogeneity of sample dispersion in the plant species group, onion remained significantly different from other plants. The PERMANOVA analysis suggests that plant species and organs had significant effects on the bacterial community composition (PERMANOVA; $P < 0.05$), with the dispersion test (PERMDISP, $P > 0.05$, Supplementary Table 3-4) that showed homogeneity for plant species, and thus, one can infer that the bacterial community variations are actually due to plant species type. However, the significant (PERMDISP, $P < 0.05$) dispersion for organ factor may have influenced the variations observed in the bacterial communities for this group. The only significant interaction effect was between plant species and organs for the unweighted unifracs (PERMANOVA $R^2 = 2.19\%$, $P < 0.036$; PERMDISP $P = 0.454$) (Figure 3-3B). In addition, some fixed factor interactions only accounted for variations in the bacterial community (PERMDISP, $P > 0.05$) without significantly impacting the community composition (PERMANOVA, $P > 0.05$, Supplementary Table 3-4).

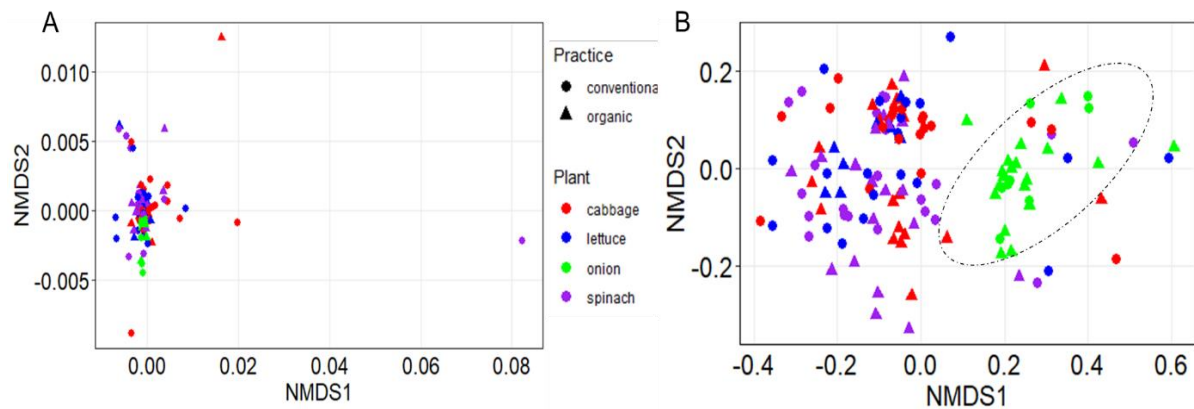


Figure 3-3: Unifrac distance dissimilarity between bacterial communities. (A) Weighted Unifrac and (B) unweighted unifrac non-metric multidimensional scaling (NMDS) plot. The dotted lines in the plot show the distance.

3.3.3 Comparative analysis of dominant and differentially abundant phylotype across fixed factors

The filtered reads generated were 1,678,081, with $12,339 \pm 1,893$ (mean $\pm$ SE) sequences per sample, which were clustered into 11,439 ASVs. The sequencing depth was confirmed adequate as indicated by the rarefaction curve (Supplementary Figure 3-4) and the Good's coverage index, which was 100% for all samples. Taxonomic assignment revealed the dominant ASVs (> 1% average relative abundance) belonged to 9 phyla and 25 genera (Figure 3-4A and B), with Proteobacteria and Actinobacteriota being the most relatively abundant across vegetable samples, representing about 80% of the total bacterial community at the phyla level. The genera *Pseudomonas*, *Pantoea*, *Bacillus*, *Serratia*, *Rahnella1*, *Rhodococcus*, *Xanthomonas* and *Streptomyces* were the most relatively abundant bacterial communities across vegetables (Figure 3-4B). However, their relative abundance varied, with onion, followed by cabbage having the highest bacterial community richness. Additionally, *Pseudomonas* (72%), *Pantoea* (22%), *Bacillus* (10%) and *Rahnella1* (5%) had the highest relative abundances in spinach, lettuce, onion, and cabbage, respectively. Across the plant organ and fertiliser types, Proteobacteria was higher in root and organic farms compared to Actinobacteria, which is higher in leaf and conventional

farms. The root and organic farms have a higher diversity at the genera taxa level (Supplementary Figure 3-5).

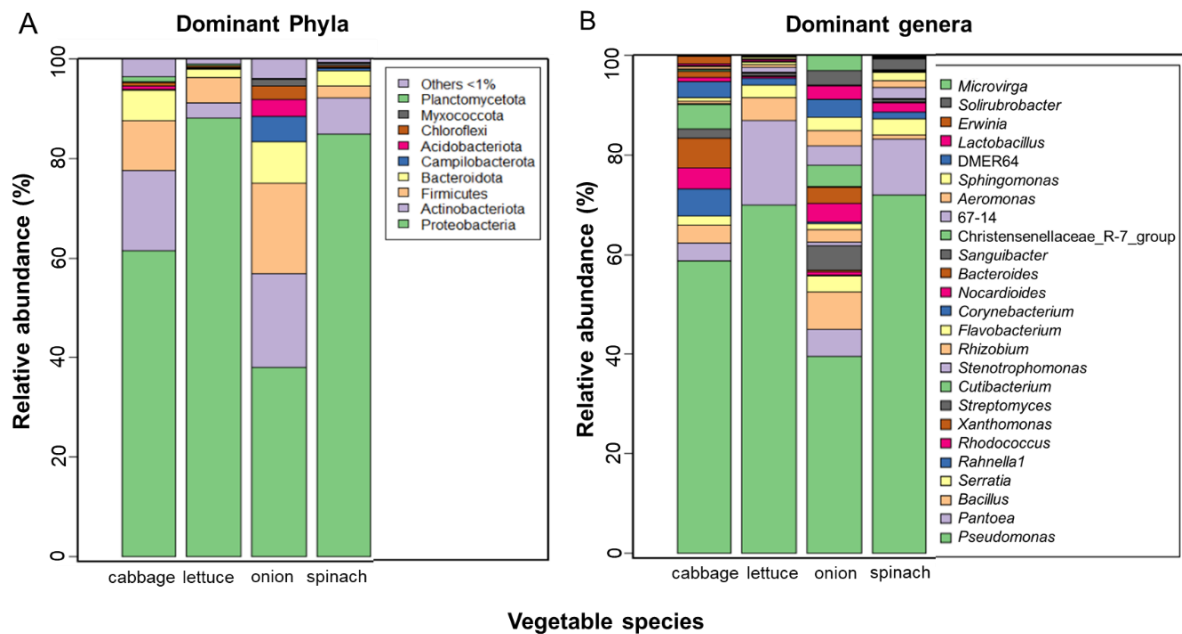


Figure 3-4: Relative abundance of bacterial communities (> 1%) across the vegetable species. (A) dominant phyla (B) dominant genera. The relative abundance at the genus level was computed amongst the bacterial communities with above 1% relative abundance after removing the unclassifiable and non-culturable bacterial communities from the data. Each taxon is represented by a different colour while the height represents the relative abundance of the taxon in each sample.

Following a sample-wide LDA Effect Size (LEFSe) analysis, a total of 131 discriminant features were significant among the vegetable species, however, only 33 of these features were significant after P value adjustment (FDR-adjusted $Q < 0.05$, LDA score > 3.02) (Supplementary Table 3-5). No discriminant features were found significant for fertiliser practice, while eight features (Figure 3-5B) were discriminant for plant organs after P value adjustment (FDR-adjusted $Q < 0.2$, LDA score > 3.74). The most discriminant features between plant species and organs are shown in Figure 3-5A-B. Cabbage had a higher abundance of *Galellales* and *Acidimicrobiia*, while lettuce and onion had a higher abundance of Gammaproteobacteria and Thermoleophilla, respectively. Spinach was found to have the family Enterobacteriaceae as the only discriminant feature. *Rhizobiales* and flavobacteriaceae were predominantly discriminant in the root and leaf, respectively.

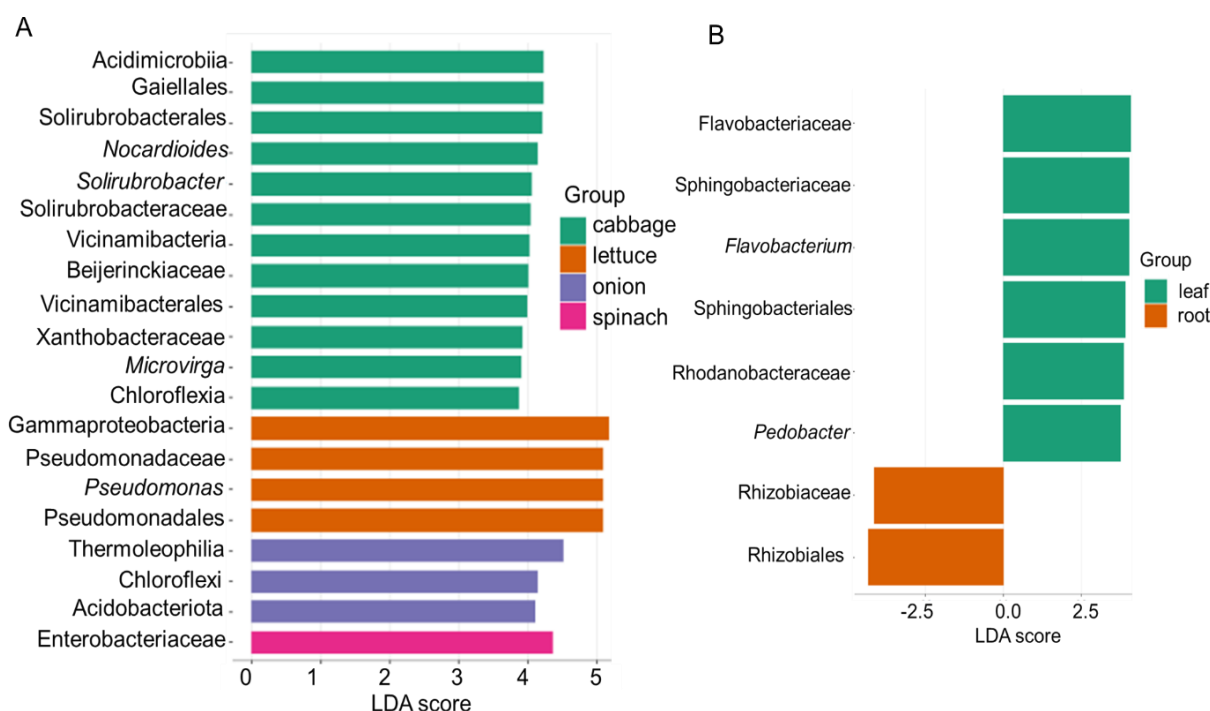


Figure 3-5: Significant discriminant features across the fixed factors. A) plant species and B) Plant organ types. The plot was generated using the microeco package in R studio.

3.3.4 Assessment of the diversity of unique and shared bacterial endophytes

Shared and unique bacterial communities vary among plant species, organs, and fertiliser types. The highest number of unique ASVs (35.6%) was observed in onion (Figure 3-6A), followed by cabbage, spinach, and lettuce, while the highest percentage of shared bacterial communities (2.7%) occurred between cabbage and spinach. Only 0.1% of the total bacterial communities (belonging to Proteobacteria, Firmicutes, Bacteroidota and Campylobacterota) were shared amongst all the vegetable species (Figure 3-6A) and may be regarded as the core microbiome of vegetables. The root had more unique ASVs compared to the leaf, with about 15% of shared communities between the leaf and root (Figure 6B). Although some ASVs (14.5%) were shared between OF and CF farms, a higher number of unique ASVs was observed in CF farms compared to OF farms vegetables (Figure 3-6C). The unique and shared ASVs are taxonomically diverse, with no dominant taxa identified between the groups. The classifiable shared bacterial communities among all the vegetable species were 14 ASVs and included important genera: *Pseudomonas*, *Bacillus*, *Escherichia-Shigella*,

Bacteroides, *Proteiniphilum*, *Bacteroides*, *Dyadobacter*, *Clade_Ie*, and *Saccharofermentans*.

Five other unclassified groups at the genus taxa level were identified in this group.

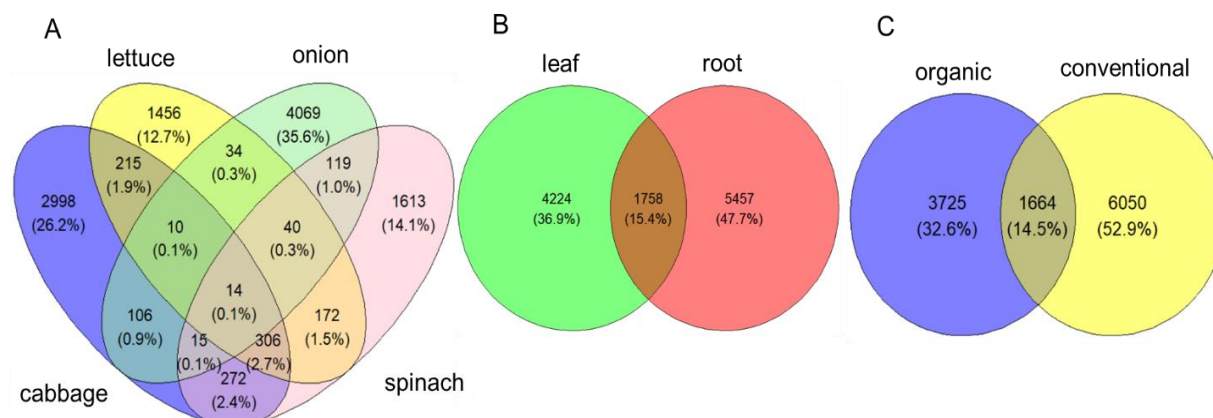


Figure 3-6: Unique and shared amplicon sequence variants (ASVs) across plant and fertiliser types (A) plant species (B) plant organs (C) fertiliser types

3.3.5 Analysing co-occurrence networks, patterns, and keystone taxa

The networks showed high power-law distribution for plant species (lettuce $R^2 = 0.86$; $P < 0.01$, onion $R^2 = 0.91$; $P < 0.05$, spinach $R^2 = 0.75$; $P < 0.01$), except for (cabbage $R^2 = 0.27$; $P > 0.05$). The high R^2 values and structural properties for some of the empirical networks compared to random networks (Table 3-3) suggest that the co-occurrence patterns are non-random. The empirical networks for the plant species had 38 to 103 nodes (corresponding to genera), indicating bacterial interactions, with each node having at least 1-21 edges. The network modularity index ranges from 0.235 – 0.396, suggesting a relative homogeneity of the bacterial community between vegetable species and fertiliser practice (values > 0.4 suggest a modular structure (Newman, 2006). Spinach had the highest number of positive associations between microbial communities while onion and cabbage only showed negative (Figure 3-5). The average network path distance between paired nodes was 2.42 edges across the plant species. Onion had the highest average path distance of 2.97, followed by spinach, cabbage, and lettuce. The node clustering coefficient

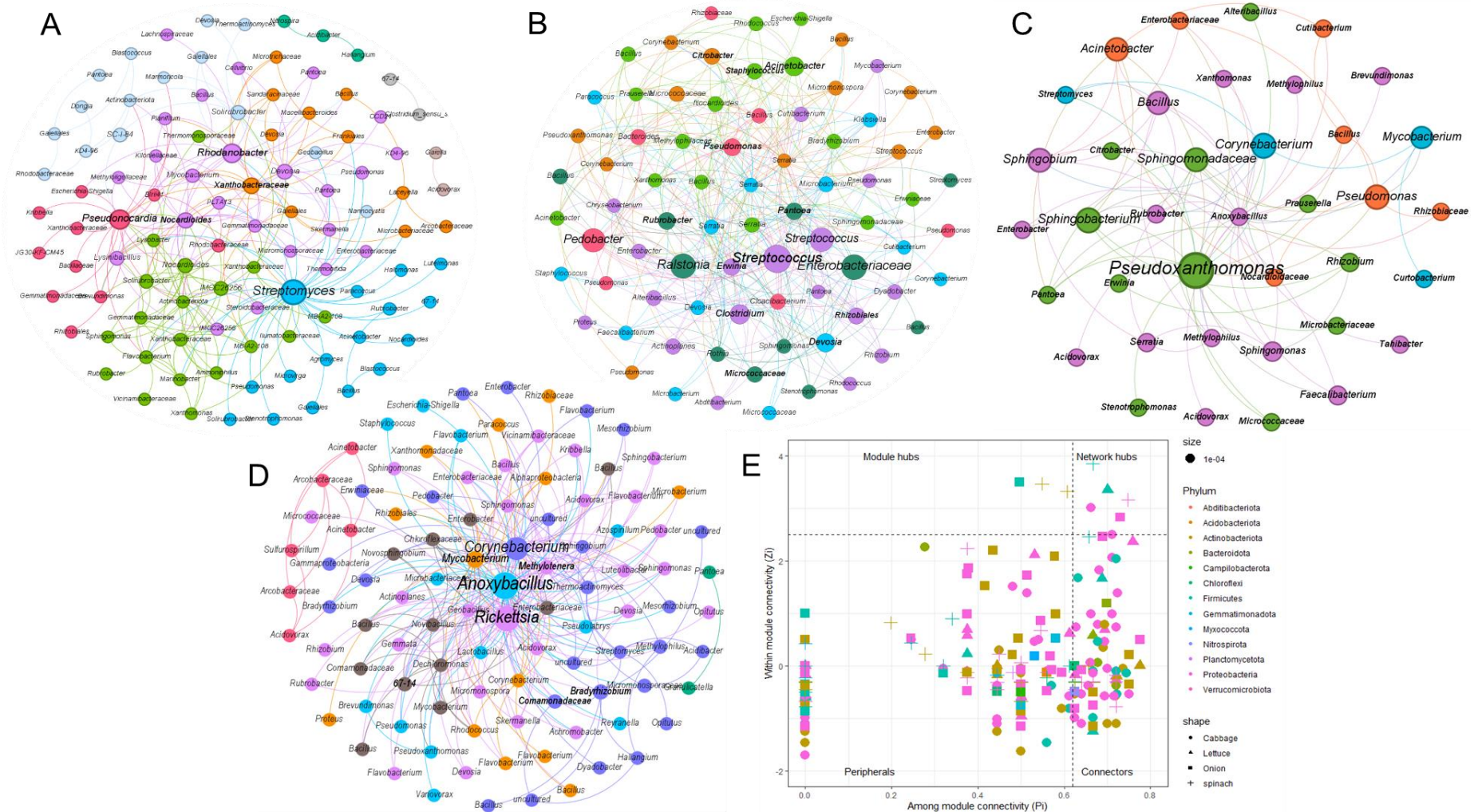
measuring how closely connected nodes are in a network ranged from 0.03 to 0.19 and was highest in lettuce compared to other plant species.

The networks showed low modularity, with onion (8 modules) having the highest number of modules followed by spinach, cabbage, and lettuce (Table 3-3). More than 45% of the nodes (> 1% relative abundance) were grouped as peripherals and less than 53% were categorized into connectors in each plant species network. The number of nodes grouped into network and module hubs was relatively small, with the highest percentage found to be only 3% and 2% in spinach, respectively. The lettuce network nodes are closer and more connected to their neighbours as revealed by their higher clustering coefficient and geodesic efficiency compared to other networks (Table 3-3).

Of all the plant species networks, onion has a more stable community due to its high modularity values compared to random chance alone (Figure 3-7). More than 60% of the core genera belonged to the generalist (peripheral), with the dominant being *Pseudomonas*, *Bacillus*, *Agromyces* and *Nocardioide*s as well as other unclassified genera in the phyla Proteobacteria, Firmicutes, and Bacteroidetes. Six bacterial communities: *Anoxybacillus*, *Serratia*, *Streptomyces*, *Pseudonocardia*, *Rickettsia*, and *Methylobacter* were identified as “network hub” taxa for the plant species factor. The module hub taxa are *Solirubrobacter* found in the onion network and *Corynebacterium* and *Mycobacterium* found in the spinach network (Figure 3-7).

Table 3-3: Structural properties of empirical and random networks between bacterial communities of vegetables

Network Indexes	Cabbage		Spinach		Onion		Lettuce	
	ENI	100 RNI	ENI	100 RNI	ENI	100 RNI	ENI	100 RNI
Average clustering coefficient (avgCC)	0.076	0.171 +/- 0.016	0.188	0.441 +/- 0.025	0.031	0.164 +/- 0.022	0.194	0.295 +/- 0.040
Average path distance (GD)	2.227	2.253 +/- 0.016	2.316	2.363 +/- 0.026	2.974	2.846 +/- 0.052	2.162	2.209 +/- 0.039
Geodesic efficiency (E)	0.501	0.497 +/- 0.002	0.473	0.461 +/- 0.003	0.396	0.396 +/- 0.005	0.514	0.509 +/- 0.005
Harmonic geodesic distance (HD)	1.997	2.012 +/- 0.009	2.113	2.170 +/- 0.016	2.524	2.525 +/- 0.032	1.945	1.967 +/- 0.019
Centralization of degree (CD)	0.209	0.209 +/- 0.000	0.476	0.476 +/- 0.000	0.355	0.355 +/- 0.000	0.586	0.586 +/- 0.000
Centralization of betweenness (CB)	0.101	0.093 +/- 0.013	0.232	0.310 +/- 0.018	0.360	0.375 +/- 0.024	0.515	0.504 +/- 0.029
Centralization of stress centrality (CS)	0.444	0.390 +/- 0.046	0.871	0.741 +/- 0.047	1.307	0.954 +/- 0.085	1.009	0.948 +/- 0.120
Centralization of eigenvector centrality (CE)	0.137	0.164 +/- 0.010	0.308	0.321 +/- 0.007	0.310	0.361 +/- 0.017	0.357	0.357 +/- 0.014
Density (D)	0.112	0.112 +/- 0.000	0.054	0.054 +/- 0.000	0.044	0.044 +/- 0.000	0.121	0.121 +/- 0.000
Reciprocity	1	1.000 +/- 0.000	1	1.000 +/- 0.000	1	1.000 +/- 0.000	1	1.000 +/- 0.000
Transitivity (Trans)	0.051	0.156 +/- 0.010	0.064	0.136 +/- 0.007	0.026	0.139 +/- 0.013	0.123	0.190 +/- 0.022
Connectedness (Con)	1	1.000 +/- 0.000	0.850	0.991 +/- 0.017	0.869	0.965 +/- 0.033	1	0.999 +/- 0.010
Efficiency	0.900	0.900 +/- 0.000	0.947	0.955 +/- 0.001	0.960	0.964 +/- 0.001	0.903	0.903 +/- 0.001
Hierarchy	0	0.000 +/- 0.000	0	0.000 +/- 0.000	0	0.000 +/- 0.000	0	0.000 +/- 0.000
Lubness	1	1.000 +/- 0.000	1	1.000 +/- 0.000	1	1.000 +/- 0.000	1	1.000 +/- 0.000
Modularity(fast_greedy)	0.235	0.244 +/- 0.009	0.313	0.287 +/- 0.008	0.396	0.377 +/- 0.009	0.259	0.283 +/- 0.016



3.3.6 Predicted functional structure and differentially abundant metabolic pathways

The PICRUST2 analysis revealed that about 98% of the total ASVs successfully mapped with the Kyoto Encyclopaedia Genes and Genomes (KEGG), and 1.6% of the gene sequences having above the maximum NSTI cut-off of 2 were expunged. A total of 8,185 KO terms, 447 metabolic pathways, and 2490 enzyme classification (EC) metabolic functions with species contributions were inferred from all ASVs. The pathways grouped into 39 secondary superclass 2 with the most abundant pathways (> 5% relative abundance) being cofactor carrier and vitamin (14.3%), aromatic compound degradation (10%), amino acid biosynthesis (8.7%), nucleoside and nucleotide degradation (6.7%), carbohydrate biosynthesis (5.4%), carbohydrate degradation (5.4) and fatty acid and lipid (5.2%). A higher abundance of biosynthesis and degradation/utilisation/assimilation pathways was observed for the bacterial endophytes functional profile in all the plant species except for onion (Figure 3-8).

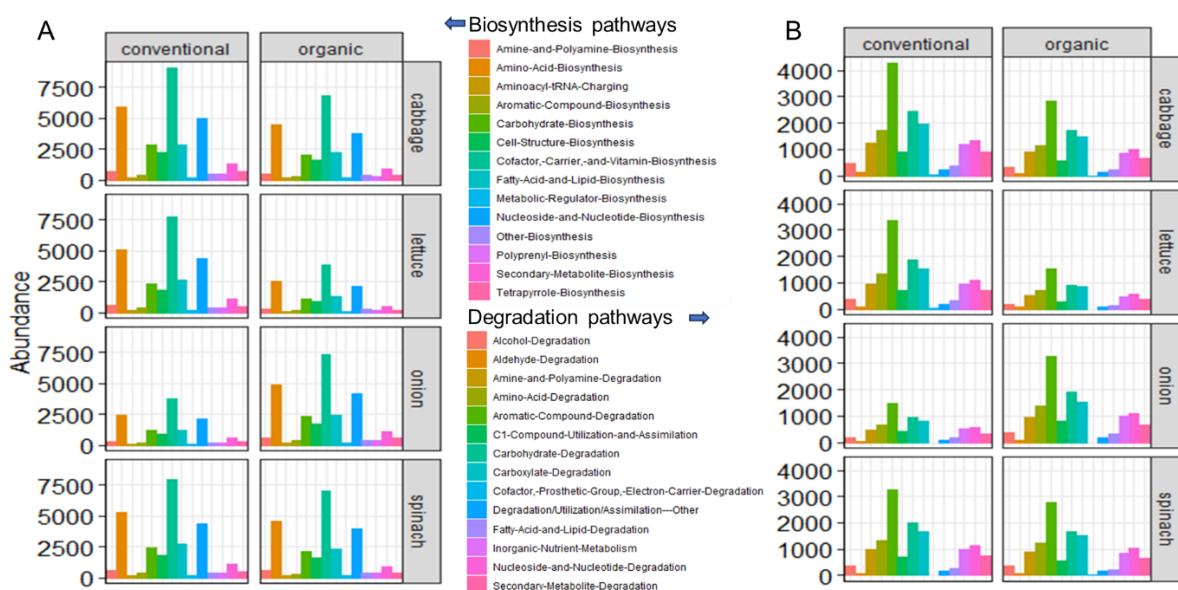


Figure 3-8: Predicted metabolic pathways for the endophytic bacteria across the plant species and fertiliser type (CF and OF) showing the comparative abundance of pathways belonging to super class 1. A) Biosynthesis and B) Degradation/Utilisation/Assimilation pathways.

Important pathways such as CALVIN-PWY and CODH-PWY (involved in CO₂-fixation) and PWY490-3 (nitrate reduction) contributing to ecological functions were not particularly

different across the OF or CF farms (Figure 3-9). However, CALVIN-PWY and PWY490-3 were highly prevalent among bacterial endophytes from OF farm SF under lettuce production while CODH-PWY was predominantly abundant in CF farm JF under cabbage production (Figure 3-9). Pathways associated with bacterial antibiotic resistance, including PWY-6470; β -lactam resistance and PWY-6471; peptidoglycan biosynthesis IV were predominant in CF compared to OF farms. whereas PWY0-1338; polymyxin resistance was higher in bacterial endophytes from vegetables in OF compared to CF farm (Figure 3-9). The pathways promoting bacterial virulence or pathogenicity such as PWY-6629; L-tryptophan biosynthesis, PPGPPMET-PWY; Guanosine 3'-diphosphate 5'-diphosphate biosynthesis, PWYG-321; mycolate biosynthesis, and PWY-7196; pyrimidine ribonucleosides salvage were highly predicted in OF farms, with farm SF having the highest abundance.

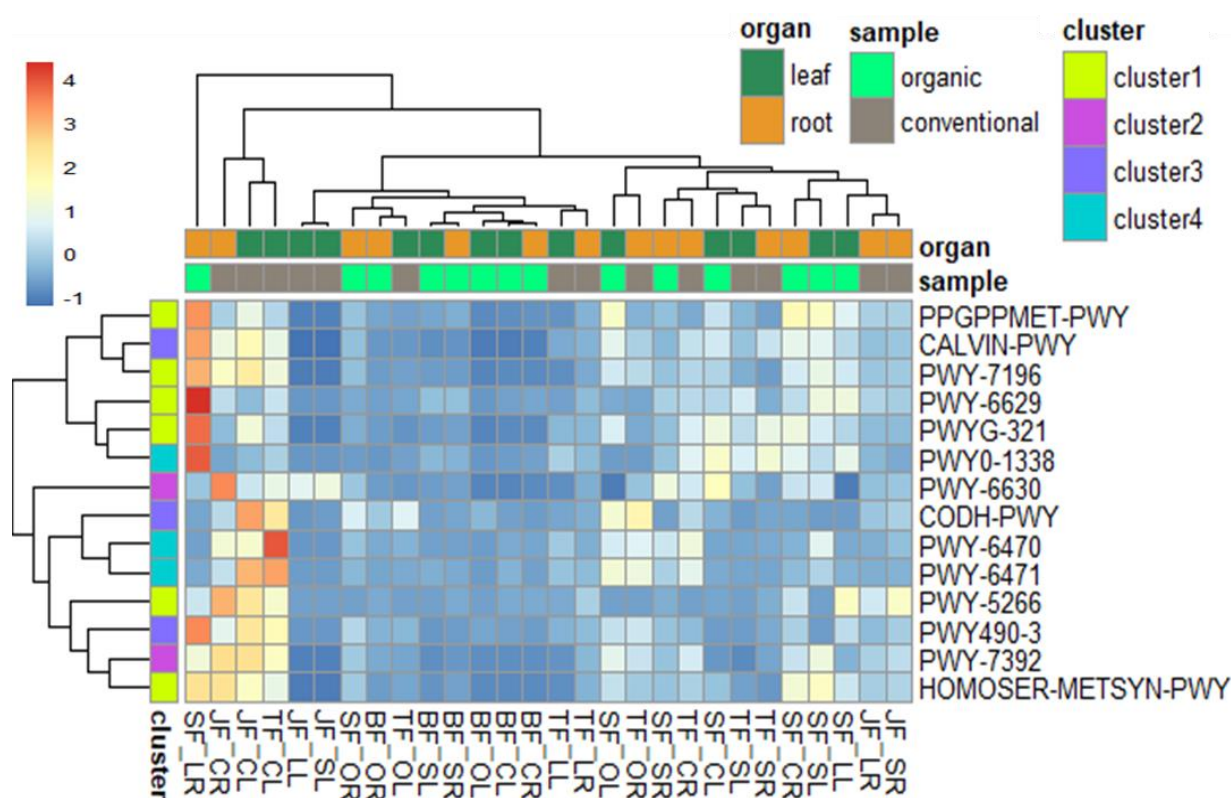


Figure 3-9: Relative abundance of selected economically important pathways across fertiliser type, plant species and organ types

The PWY-5266; p-cymene degradation was more abundant in CF compared to the OF farm and highly prevalent in bacterial endophytes from farm JF. The abundance of HOMOSER-METSYN-PWY; L-methionine biosynthesis I was comparatively similar across the farms. These pathways influence bacterial pathogenicity. Pathways of biotechnological importance such as PWY-7392; Taxadiene biosynthesis and PWY-6630; L-tyrosine biosynthesis were highly predicted in bacterial endophytes of vegetables from CF farms (Figure 3-9). Contrary to the fertilizer type factor, the organ types significantly ($P < 0.05$) affected the pathways. Only PWY0-1338, CODH-PWY, PWY490-3, and PWY-5266 were significantly affected by the plant types and the interaction between plant and fertilizer type significantly (ANOVA, $P < 0.05$) influenced the selected pathways.

Toxin-producing genes, such as leucocidin and hemolysin toxin family protein (K11038), membrane protein required for colicin production (K03558), and hemolysin A (K11005), and D (K11003), which may potentially affect plant nutritional values were assessed using their abundance across the fertiliser practice (Figure 3-10). Though these genes were not significantly different across the fertiliser practice, their mean abundance differed. For K11038, K11005 and K11003, the bacterial communities from vegetables cultivated under conventional fertiliser had higher abundance compared to that of organic farms.

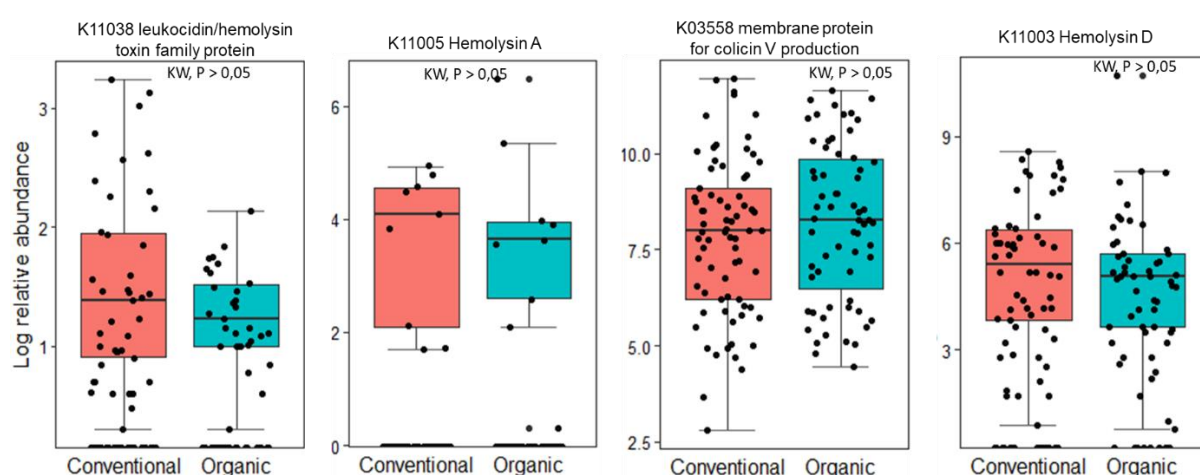


Figure 3-10: Selected toxin and haemolytic genes found among the bacterial endophytes of vegetables cultivated under organic and conventional fertilisers. A log abundance of four genes, which occurred among endophytic bacterial communities from organic and conventional fertilises. Significant value was determined with the Kruskal-Wallis test (KW).

Degradation/utilization/assimilation pathway was highly discriminant amongst communities from cabbage while aromatic compound degradation, amino acid biosynthesis and amine and polyamine degradation were predominantly discriminant in lettuce, onion, and spinach, respectively (Figure 3-11A). Fertiliser type factor may have influenced the abundance of nucleoside and nucleotide biosynthesis and generation of precursor metabolite and energy in CF farms. Mycolate, palmitoleate and oleate biosynthesis were highly discriminant in the OF farms (Figure 3-11B). In addition, glycolysis, cell structure biosynthesis and carbohydrate degradation were more discriminant in the leaf while aromatic, amino acid, fatty acid and lipid compound degradation, fatty acid and lipid biosynthesis and inorganic nutrient metabolism were highly discriminant in the root Figure 3-11C. The relative abundance differences (%), log₂ fold change and the adjusted p-values for the predicted metabolic pathway across the plant species and organ type are shown (Supplementary Figure 3-6).

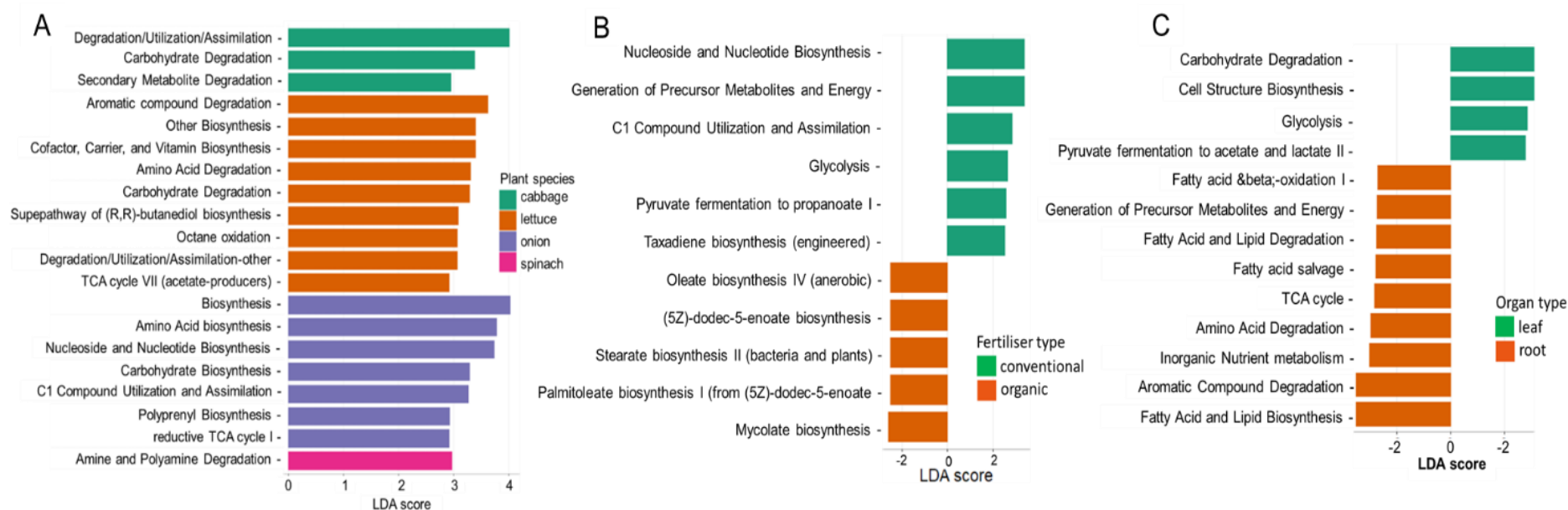


Figure 3-11: Discriminant metabolic pathways across the fixed factors A) Plant species B) Fertiliser type, C) Organ type. The plot was constructed in R using the micro-eco package.

3.3.7 Relationship between vegetable nutrient content, endophytic bacterial community and predicted metabolic pathways

The vegetable nutritional content had a significant ($r = 0.0798$, $P < 0.03$) and positively strong correlation with the predicted metabolic pathway abundance. The alpha diversity measured, Shannon, Chao1 and Pielou's evenness were significantly ($r = 0.053$, $P = 0.0348$) correlated to the nutrient content. In addition, the RDA analysis suggests the plant nutrient contents explain up to 15.7% and 16.9% of the total variance in the bacterial community composition (Figure 3-12) and the predicted metabolic pathways (Figure 3-13), respectively, as revealed by the significant (ANOVA, $P = 0.001$) model for the triplot. Amongst the explanatory variables assessed, P, Ca, S, Fe and B significantly impact the microbial community composition, while N, K, Na, and Zn, significantly influence the metabolic pathways. The Shannon-Weiner index had a negatively significant relationship with K in the OF farm and B in the CF farm (Supplementary Figure 3-7). Similarly, a significant negative relationship was observed for Chao1 and N, K, Mg, S, Cu and Zn in OF farms while only Mn, Na, Fe and B in CF farms (Supplementary Figure 3-8). A positive and significant association was observed between Pielou's evenness and Ca, Mg, S and B for the OF farm vegetable (Supplementary Figure 3-9).

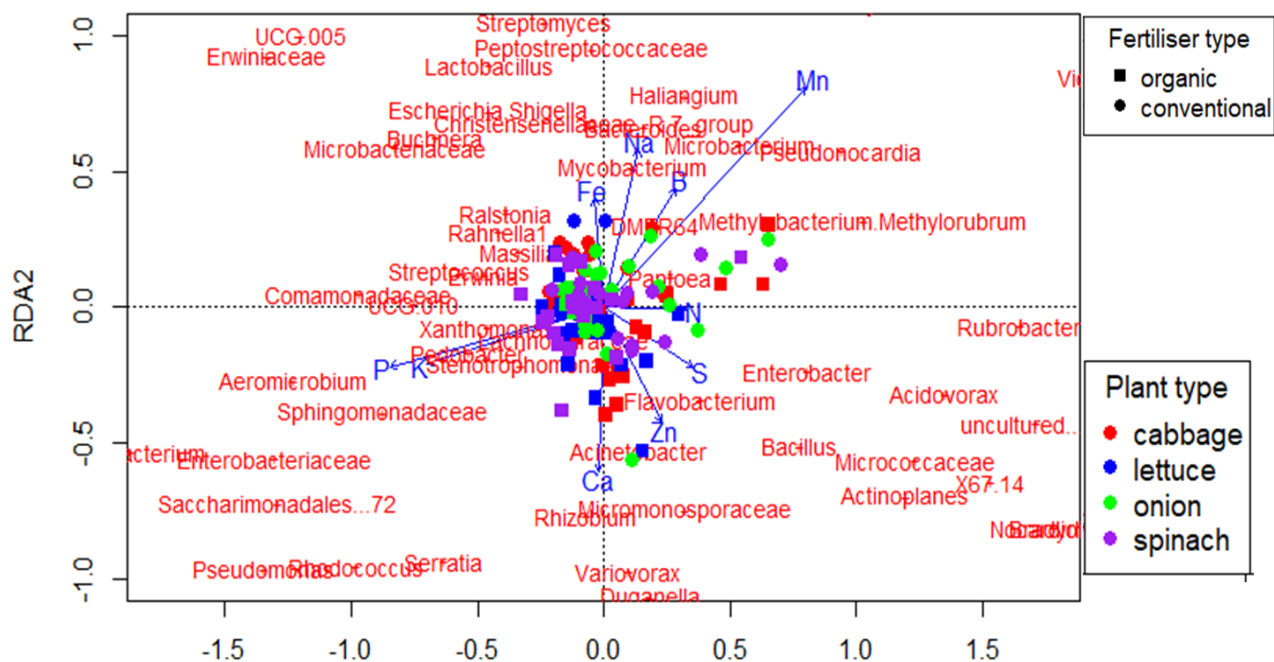


Figure 3-12: Triplot redundancy analysis (RDA) showing the correlation between vegetable nutrient content and bacterial endophyte communities.

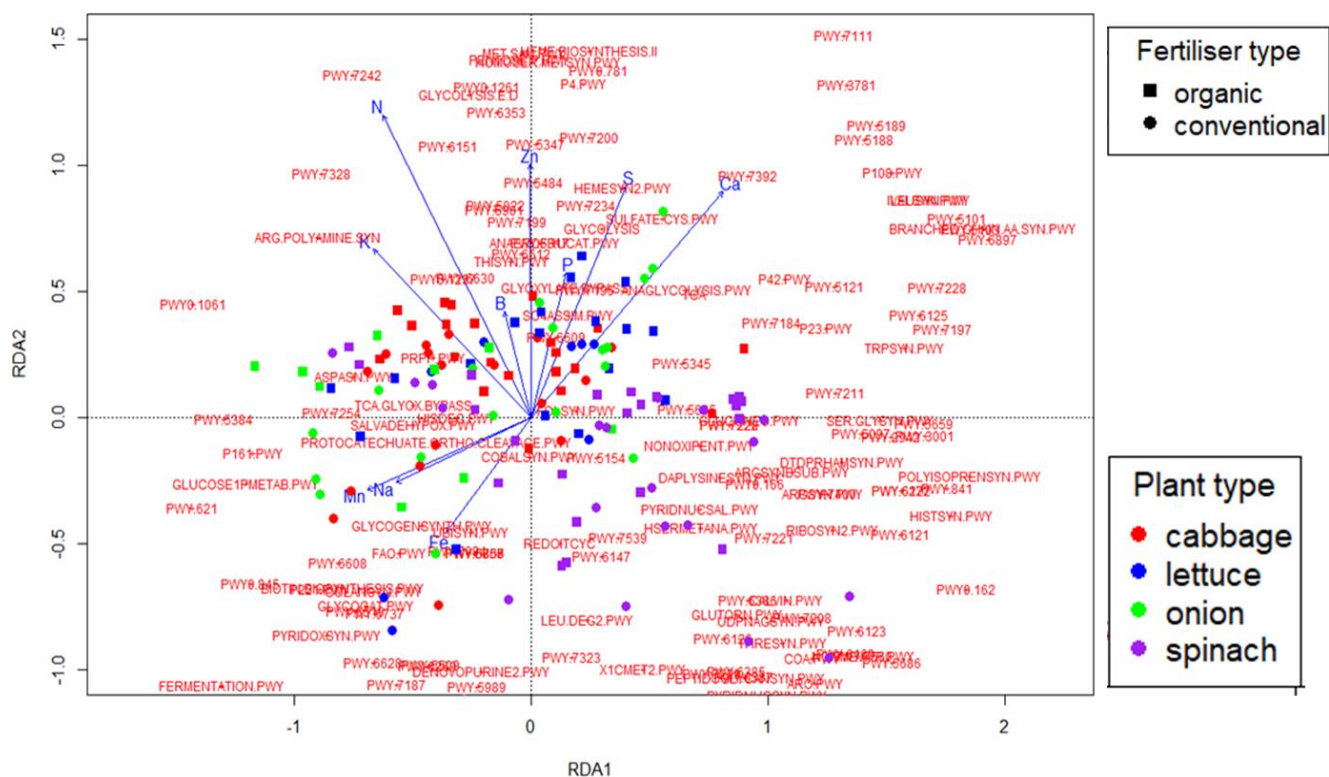


Figure 3-13: Triplot redundancy analysis (RDA) showing the correlation between vegetable nutrient content and metabolic pathways

3.4 Discussion

Plant microbiomes have been well studied for their ecological importance, especially as they influence plant health and productivity (Adeleke et al., 2019; Bulgarelli et al., 2013; Hardoim et al., 2015), creating awareness among ecologists to further comprehend the nexus between plant microbial communities and plant nutritional quality. Based on several studies, including the theory of community assembly, composition and interactions (Keddy, 1992; Pearson et al., 2018; Tabi et al., 2020), it has become imperative to understand how plant microbiomes impact human nutrition, especially when vegetables are consumed without cooking. There are different agronomic practices employed to improve soil and plant productivity; however, they have been implicated in altering the composition and functions of plant microbiomes (Bhattarai et al., 2015; Xue et al., 2018), which are essential to gut microbiome functioning. Thus, this study investigated endophytic bacterial communities of vegetables cultivated under organic or conventional fertilisers to gain insight into their community composition and functional profiles and how human diets, through plant nutritional quality, are impacted.

Vegetable nutrient content differ across crop type and bacterial endophyte diversity

Vegetable nutritional contents significantly ($P < 0.05$) differ across plant species and fertiliser types. A similar observation has been reported, with vegetable mineral composition variability related to soil fertility, climate and plant types (Han et al., 2011). Corroborating the findings in this study, a review of vegetable chemical contents reported spinach has the highest Fe content compared to other leafy vegetables (Butnariu and Butu, 2014). Vegetables, such as kale, spinach, cabbage, onion and lettuce have been reported with varying and essential nutrients for human benefits (Butnariu and Butu, 2014; Kumar et al., 2020). Several biological, chemical and physical factors affect the chemical composition of plant tissues, suggesting the reasons for the different published values for various vegetable nutrient content reported in the literature (Han et al., 2011; Titchenal and Joannie, 2005). These factors impact the differential nutrient composition in plants and other components of

the plant ecosystems such as the associated microbiomes and their functions (Ahkami et al., 2017; Leff and Fierer, 2013).

The endophytic bacterial community profiles of the vegetables were taxonomically and functionally diverse and are comparable to those reported in other studies (Jackson et al., 2013; Nithya and Babu, 2017; Pascazio et al., 2015). Microbial endophytes exist in all the plants evaluated to date (Verma et al., 2015; Xia et al., 2015), and corroborating the present study, all the vegetables examined had diverse bacterial endophytes (Figure 3-4). The phyllosphere has lower microbial complexity compared to the soil and rhizosphere communities (Vorholt, 2012), suggesting the relatively fewer bacteria that define its phylogenetic composition related to the roots (Figure 3-4). Proteobacteria, Actinobacteriota, Firmicutes and Bacteroidetes were the most relatively abundant (> 1%) phyla, with Proteobacteria dominating the phyllosphere bacterial communities. This is in agreement with the findings of other studies (Bulgarelli et al., 2013; Chimwamurombe et al., 2016). However, other studies have reported Firmicutes as the most abundant phylum in vegetables (Khalaf and Raizada, 2016; Xia et al., 2015), suggesting the effects of ecological factors such as location and crop types. Similar to other studies, bacterial genera such as *Pseudomonas*, *Bacillus*, *Pantoea*, *Serratia* and *Rahnella* were found to constitute the core phyllosphere bacterial communities (Bulgarelli et al., 2013; Lopez-Velasco et al., 2013). Corroborating this result, Hou and others isolated *Bacillus*, *Pseudomonas* and *Pantoea* from lettuce plants (Hou et al., 2013). Though the root and phyllosphere communities had a similar phylogenetic trend, the root had a higher bacterial community diversity compared to the phyllosphere (Supplementary Figure 5). The complex and high density of microbial communities in the soil and rhizosphere present a good proximity for potential endophytes to colonise the root, suggesting a higher diversity in the roots (Ahkami et al., 2017). More so, the phyllosphere is not protected from the daily and seasonal variations of heat, moisture and radiation, rainfall and winds, which impact plant physiological activities, including respiration, photosynthesis and pollination (Dastogeer et al., 2020). These factors affect microbial adaptation strategies,

and drive endophyte colonisation and consequently influencing the differential diversity and compositional profile (Dastogeer et al., 2020).

Furthermore, results revealed variations in the richness and diversity of the bacterial endophytes, consistent with the report in other studies (Xia et al., 2015; Zhang et al., 2019). The diversity varied across plant species, organ and fertiliser types, an indication of bacterial adaptation to ecological and biological factors, including plant genetic and physiological variation, although, only plant species and organs were significant ($P < 0.05$). The highest diversity in onions (Figure 3-2) may be attributed to the specificity and adaptability of the microbes to the anatomical and nutritional structure of the plant tissues (Gaiero et al., 2013). Higher variations in bacterial communities have been reported between samples from different plant species compared to similar species (Dastogeer et al., 2017; Lindow and Brandl, 2003). Similar to the results in this study, Leff and Fierer reported bacterial community composition and diversity vary significantly across plant species than farming practices (Leff and Fierer, 2013). According to Wagner and colleagues, plant leaves and roots harbour distinct bacterial communities across different genotypes of wild perennial plants (Wagner et al., 2016). On the other hand, an NGS-based metabarcoding technique, revealed plant organs as a major determinant of bacterial composition, the plant species-related bacterial community clustering was not detected across the three terrestrial orchard species studied (Alibrandi et al., 2020). A study reported distinct OTUs between different plant tissues, emphasizing the impact of species and tissue types on bacterial endophyte community composition (Zhang et al., 2019).

Soil nutrient management has been reported to significantly affect plant endophyte community composition (Gu et al., 2013; Wemheuer et al., 2017). There was no evidence of fertiliser type-specific clustering, as revealed by the NMDS (Figure 3-3), and this is contrary to the results in other studies. Ling et al. (2016) and Xia et al., (2015) reported a high endophytic bacterial diversity in crops cultivated under organic fertilisers. Fertilisation improves soil nutrient content and drives microbiome dynamics in the rhizosphere, a

dynamic region that harbours diverse potential endophytes, promoting root colonisation and differential abundance of endophytes across the organs. More so, soil nutrient management affects the physicochemical properties, including soil pH and moisture, which shape the soil microbiome, endophyte colonisation frequency and diversity (Hernandez et al., 2021). Compared to conventional fertilisers, a higher level of microbial composition and diversity are reported in organic fertilised soil (Koishi et al., 2020; Peltoniemi et al., 2021), contributing to the high endophytic bacterial colonisation in OF cultivated plants. Faith PD was significant for fertiliser type with a higher value in the CF farm compared to OF farms, suggesting a higher diversity in CF farms. Similarly, lettuce cultivated under OF farms was reported with a lower bacterial endophyte diversity compared to that cultivated under conventional fertilisers (Zhu et al., 2017). Various factors interact in complex ways to impact microbial community composition in plants, emphasising the importance of studying microbiomes under the influence of different ecological and agronomic factors to reveal a more reflective understanding compared to a singular factor effect alone. However, it is rarely possible to have several factors combined in a particular site, suggesting no particular set of factors can completely define how bacterial communities of a particular plant species vary in composition and diversity.

The results of this study revealed higher numbers of unique ASVs in CF farms, roots and onion plants, buttressing the fact that plant species, organ and fertiliser types impact endophytic bacterial richness in plants (Han et al., 2016; Zhang et al., 2022). The low numbers of ASVs shared amongst the plant species (Figure 3-6) imply a high genetic variability. Some of the bacterial endophytes are unclassified with low abundance, suggesting the need to further classify and understand their unique ecological roles. While community diversity may differ relative to plant species, there were shared communities among all vegetables (Figure 3-6). The unique communities may comprise the systemic endophytes, which naturally exist in the plant tissues, irrespective of species, ecological or geographical factors (Wani et al., 2015). Systemic endophytes enhance both induced and

acquired systemic resistance in the host plant (Hardoim et al., 2015) and largely contribute to plant health through the production of bioactive compounds that protect plants against diseases and pathogens (Liotti et al., 2018).

Endophytic bacterial functional profile impacting plant nutritional values

The metabolic pathway profiles were differentially abundant across the fixed factors, with a similar trend as the bacterial communities. An essential process that produces energy through cellular mechanisms is microbial metabolism, which enhances cell growth, activities and faster responses to ecological factors (Murray et al., 2013). Considering that nutrient levels regulate cell metabolism, the cellular physiological adaptations, including changes in cell protein contents influence the abundance of cell structure and cell wall biosynthesis (Beaufay et al., 2021). Similar to this, the higher relative abundance of biosynthesis pathways, mainly cell structure, vitamin, fatty acid and lipid and nucleoside and nucleotide biosynthesis observed in vegetables from CF farms compared to the OF farms may be related to the nutrient levels (Figure 3-8). CF farms had higher P, K, Ca, Fe, S and Zn, compared to OF farms, which may have promoted higher biosynthesis pathways. A study has reported a similar pattern in metabolic pathways relating to nutrient-specific abundance (Raes et al., 2021). Due to physiological and structural differences among plant species and organ types, the primary production differs, affecting the occurrence and relative abundance of unique pathways observed. Higher degradation pathways were differentially abundant in the leaves, which may have enhanced the high levels of cell structure biosynthesis due to increased metabolic activities. This result confirms the study's hypothesis that carbohydrate degradation pathways are more abundant in the phyllosphere, given that organic substrates containing high sugar and protein are mostly synthesised in the leaves compared to the roots (Rastegari, 2020). On the other hand, a high abundance of inorganic nutrient metabolism and lipids, amino and fatty acids degradation pathways were highly predicted in the root, signifying the importance of root systems, including the root bacterial endophytes, to supply the necessary and useable form of nutrients to the phyllosphere. The bacterial

functional profile result suggests that the PICRUSt2 prediction is a good resource for understanding microbial community responses to changes in ecological factors such as plant nutrient levels (Agrawal et al., 2019; Raes et al., 2021).

The present study revealed a vast functional profile for bacterial communities such as *Bacillus*, *Enterobacter* and *Pseudomonas*, contributing to ecosystem functions. Bacterial endophytes with CODH and CALVIN pathways participate in ecosystem sustainability by converting CO₂ to organic matter (Garritano et al., 2022; Hudson, 2023) and the nitrate reduction assimilatory pathway contributing to the N cycle and the N content in plant's biomass (Sparacino-Watkins et al., 2014) were highly abundant in CF farms. The contributions of bacterial endophytes with these pathways to plant organic matter buildup should be further investigated. Several studies have reported the importance of endophytes in biogeochemical cycling such as N, C and S, which is a key process that maintains and regulates the natural elements necessary for living beings (Gupta et al., 2012; Prasad et al., 2019). In addition, taxadiene and L-tyrosine biosynthesis contributing to bioactive compound syntheses suggest endophytic bacteria have huge therapeutic potential in human health management (Flores-Bustamante et al., 2010; Juminaga et al., 2012). These pathways were higher in CF farms, suggesting it is crucial to understand the agronomic practices that drive the production of such important pathways to enhance their sustainable production.

Furthermore, several millions of microbial cells get into the human gut when vegetables are consumed without heat processing (Rincón and Neelam, 2021) and considering some of the functional profiles of endophytes are related to plant nutritional quality, human health may be affected. The bacterial endophytes having a high abundance of PPGPPMET, PWY-6629, PWYG-321 and HOMOSER-METSYN-PWY pathways, which enhance microbial virulence of great human health concerns when consumed (Mu et al., 2023; Quémard, 2016; Shaun Lott, 2020). Similarly, pathways including, p-cymene degradation and mycolate biosynthesis, which enhance bacterial pathogenicity (Marchese et al., 2017; Quémard, 2016) were differentially predicted in OF farms compared to CF farms, suggesting microbial communities

with such pathways might pose a health risk, especially in immunocompromised individuals. Despite the health benefits of vegetables, there are growing concerns about their safety and quality due to several factors contributing to their contamination (Kljujev et al., 2018; Zhu et al., 2017). Bacterial endophytes cannot be eliminated from the plant tissues by surface washing, disinfecting, or cooking in some instances, and could become integrated into the human gut microbiome when plant materials are consumed as salads (Berg et al., 2014a), suggesting vegetables may be a reservoir of opportunistic pathogens to human (Hölzel et al., 2018; Rincón and Neelam, 2021; Tatsika et al., 2019). In similar studies, human opportunistic pathogens have been reported in vegetables, such as onions and carrots (Nithya and Babu, 2017; Tatsika et al., 2019), however, these studies did not investigate the possible source of the pathogens. Organic substrates used as fertilisers may be a primary source of the pathogens and antibiotic-resistant genes in the vegetables. Similar to the result obtained, lettuce cultivated under OF had a significantly higher amount and abundance of antibiotic-resistant bacteria and genes compared to CF practice (Zhu et al., 2017). *Salmonella* sp. and *Escherichia coli*, which are common pathogens inhabiting vegetables were highly abundant in lettuce (Hölzel et al., 2018; Rincón and Neelam, 2021). Animal manure used as organic fertilisers is a major source of pathogens and antibiotic-resistant genes due to their antibiotic residues, which accumulated from the chemical compounds used for treating diseases and promoting animal growth (Wang et al., 2015). Other sources of ARGs and ARMs could be attributed to wastewater treatment plants, sewage sludge compost and contaminated rivers used for irrigation (Chen et al., 2016; Zhu et al., 2017). Thus, the quality and sources of organic substrates and water used for fertilisers and irrigation should be properly examined before use to reduce their negative impact

Co-occurrence patterns and their influence on keystone taxa

Co-occurrence patterns of microbial communities are essential for assessing the ecological interactions among microbial taxa (Deng et al., 2012; Zhou et al., 2022). From the network analysis, the results showed that the plant species affected the vegetable endophytic

bacterial association with a high proportion of negative associations among the communities (Figure 3-7), suggesting the impact of competition among the endophytic communities (Goberna and Verdú, 2022). Competition may have developed due to nutrient demand caused by functional redundancy (Sasaki et al., 2015). The RMT-based method was used to establish the reference point, allowing for automatic threshold selection to reduce ambiguity in the constructed networks. According to Deng et al. (2012) and Zhou et al. (2022), multiple factors drive the relationship between specific agricultural practices and network topological features. They found that organic fertilisers caused an increase in the subnetwork size in the subsoil, facilitating the migration of microbes across soil profiles. The plant species networks formed analogous numbers of clusters, with each cluster comprising different taxa, suggesting the functions of the clusters in each plant network differed in response to the plant's physiological structure or tissue nutrient content. In addition, the identification of generalists reveals more insight into microbial community structure and their differential interactions which have a practical role in microbial interactions (Johnson, 2000). This study identified module hub taxa such as *Solirubrobacter* in onion, which has a unique potential for establishing a niche for the survival of the bacterial community group, have been reported as keystone taxa in soil ecosystems (Xun et al., 2021). The identification of generalists using the among/within module connectivity approach provides a comprehensive insight into microbial interactions, presenting a more representative network modularity and the role of the nodes among and within the overall structure (Li et al., 2018).

3.5 Conclusion

This study highlights the importance of endophytic bacterial communities of leafy vegetables and considers the impact of agronomic factors such as plant species and fertiliser types, mainly organic and conventional fertilisers, on their diversity and functional profiles to gain better insight into the cumulative interactions between these factors and the effects on crop health and nutritional quality. The results showed a differential composition and diversity of bacterial endophytes between the vegetable types, with about 80% of the phyla belonging to

Proteobacteria and Actinobacteriota. Endophytic bacterial communities of vegetables are influenced by plant species, organ and fertilizer types. There is a high abundance of pathways promoting microbial pathogenicity and virulence in OF farms, which may have implications for plant health and nutritional quality, posing health risks to humans, especially when vegetables are eaten raw by immunocompromised individuals. Future studies should focus on isolating individual endophytic bacteria and evaluating their plant growth-promoting attributes *in vitro* and the field. The predicted metabolic functions were based on the 16S rRNA marker gene sequencing profile, further studies are necessary to assess the presence and expression of ecologically important functions of endophytic bacteria. In addition, the influence of specific farm practices, such as soil tillage, types and sources of organic substrates and geographical locations, should be correlated with endophytic bacterial community dynamics. Similarly, the impact of these factors on plant regions, e.g., the rhizosphere and the consequential impact on the plant and its bacterial endophytes should be understood. The outcome may provide more information about the specific farm techniques that significantly influence the endophytic community associations.

The plant regions such as the rhizosphere and rhizoplane greatly influence plant-associated microbiomes, including the endophytic microbial communities. The rhizosphere is a complex region and a densely populated zone with food web interactions that significantly impact carbon flow and transformation. It is a hot spot for many microorganisms that may eventually colonise the plant under favourable conditions. Exploring the plant rhizosphere to understand the nexus between microbial endophytic communities and rhizosphere microbial communities of plant may define unique agronomic practices or strategies that are most appropriate for selecting and/or maintaining an ecologically beneficial endophytic community composition within plant tissues. Therefore, the next chapter investigates the vegetable rhizosphere and compares the bacterial communities between the two environments to ascertain the impact of vegetable rhizosphere on the endophytic bacterial community composition and diversity.

3.6 References

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CHAPTER 4 :

SOIL NUTRIENT MANAGEMENT INFLUENCES DIVERSITY, COMMUNITY ASSOCIATION AND FUNCTIONAL STRUCTURE OF RHIZOSPHERE BACTERIOME UNDER VEGETABLE CROP

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4.1 Introduction

Soil microbes participate in nutrient cycling, organic matter decomposition and energy flow, which are crucial in sustaining soil ecosystem functions (Ling et al., 2022). However, soil nutrient management, soil properties and climatic conditions, which vary across geographic locations, alter how soil ecosystem function through a possible shift in microbial diversity and functions (Ahkami et al., 2017; Ge et al., 2008; Yang et al., 2021). Substantial responses to changes in land use and soil nutrient build-up have been reported for both plant and soil microbial communities, with potential consequences for ecosystem functioning (Nielsen et al., 2015). Soil microbial diversity is vast and its interactions with other ecosystem components (e.g. vegetation and soil parameters) are complex, which significantly impacts the correlation between soil microbial diversity and ecosystem functions in response to disturbances (Garnica et al., 2020). Thus, insights into soil bacteriome structure and how it is affected by agronomic practices are key to making informed decisions that will promote agroecosystem sustainability.

Currently, organic and conventional fertilisers are the main agro-inputs for improving soil nutrient content (Musyoka et al., 2017). Improved soil nutrient content enhances overall plant health by altering the soil parameters and predisposing the selection of certain rhizosphere bacterial communities with unique ecosystem functions. Consequently, the plant and

microbes interactions in the soil, along with the above-ground productivity, are affected by the type of fertiliser applied (Ahkami et al., 2017). Using phospholipid fatty acids, a study has reported that organically managed soil enhanced bacterial and fungal biomass, with an increase in total microbial metabolic activity and soil organic matter. Thus, soil nutrient management could drastically impact soil ecosystem functioning, through the imbalances caused by fertiliser application and nutrient deposition (Martínez-García et al., 2018).

Furthermore, plant species or genotypes influence the synthesis of unique exudates and extracellular enzymes, which impact microscale spatial patterns in soil bacterial communities and differentially stimulate the growth of specific microbial groups under different ecosystems across geographic locations (Hoch et al., 2019; Yan et al., 2021). Usually, plants exert a selective influence on their rhizo-microbiome to acquire key beneficial traits (Jaramillo et al., 2016), indicating rhizosphere microbial communities are a reflection of plant species and plant-beneficial genetic functions. For example, legumes harbour bacteria with nitrogenases that enhance their N-fixing ability (Lugtenberg, 2015). Similarly, plant-associated microbes, which perform functions such as nitrate reduction, denitrification, nutrient solubilisation, and production of phytohormones such as indole acetic acid, ethylene and siderophore are a reflection of the host plant's needs (Raimi et al., 2017). These microbial functions influence plant productivity and health, which in turn drive soil bacterial diversity and ecosystem functions (Mendes et al., 2013).

Soil parameters vary by geographic location and primarily provide similar conditions that affect the activities of plants and their associated microbiome to soil microbes (Mendes et al., 2013). Studies have shown that factors such as soil pH, carbon, organic matter and nutrient content drive spatial patterns of soil microbial diversity within an ecosystem (Bardgett and Caruso, 2020; Nan et al., 2020). Spatial patterns of soil microbial community due to plant species and fertiliser types and how these relate to bacterial taxonomic and functional profiles remain largely underexplored. An increase in land-use intensification is reported to alter soil microbial composition (Felipe-Lucia et al., 2020) and keystone taxa,

which are crucial for microbial community structure and ecosystem functioning (Banerjee et al., 2018). Due to the importance and unique role of keystone taxa, their interactions or loss can trigger a shift in microbiome structure (Yang et al., 2021). Interactions between microbial community members have been widely assessed using microbial co-occurrence network analysis (Li et al., 2018; Xu et al., 2022). Thus, co-occurrence patterns and microbial networks are key to understanding how bacterial community interactions and keystone taxa distribution and functions change under different agronomic practices (Hernandez et al., 2021; Huang et al., 2019).

Consequently, this study assessed the effects of fertiliser type (organic versus conventional) and plant species type on the microbial dynamics (including keystone taxa) and ecosystem functions of vegetable rhizosphere soil across different farms. It is hypothesised that fertiliser type in each of the farming systems predisposes the selection of certain rhizobacterial communities, which possess diverse but unique functional gene repertoire of agronomic importance. Results from this study could provide baseline information useful in predictive modelling for manipulating soil bacterial communities to improve agroecosystems. Similarly, the approach used in this study could unravel taxa with unique biomarker potential for monitoring soil health and productivity in a given nutrient management system.

4.2 Experimental Procedures

4.2.1 Description of the study sites and rhizosphere soil sampling

Rhizosphere soils were collected from four vegetable farms located in the North West (farms B and S) and Gauteng (farms J and T) provinces of South Africa. The description of the location and climate characteristics of the farms have been comprehensively described in section 3.2.1. Sampling was conducted in the summer (October-February) of 2021. Healthy vegetable crops of the same age and size including, *Allium cepa* (onion), *Brassica oleracea* (cabbage), *Lactuca sativa* (lettuce) and *Spinacia oleracea* (spinach), were collected from each farm as described in section 3.2.1. The rhizosphere soil collection followed the method

described by Barillot *et al.*, (2013), with some modifications. Briefly, vegetables were uprooted and shaken vigorously to remove bulk soil from the roots. Then, the rhizosphere soil was collected by hand-shaking several undamaged roots to release the adhering soil into a sterile beaker. Five replicate samples were collected for each plant per farm and an equal quantity (50 g each) of the soils from the same vegetable for each farm was pooled to obtain a composite sample. An additional sample of the rhizosphere soil was collected from each farm; a different vegetable type was sampled at each farm, bringing the total number of samples to 20. Samples were immediately placed in sterile zip-lock bags, transported to the laboratory on ice and stored for further analysis.

4.2.2 Physicochemical parameters and enzyme analyses of rhizosphere soil

The soil pH was measured from a 1:2.5 soil-water suspension using a pre-calibrated pH meter (HQ40d Hach, USA), while the electrical conductivity (EC) was determined with an EC meter. The amounts of macronutrients (N, P, K and Ca), micronutrients (Mn, Fe, Zn, and Cu), heavy metals (Pb, Cd, Cr, Hg, and As) and exchangeable cations (Ca^{2+} , Mg^{2+} , K^{+} , and Na^{+}) in the soil were determined using the inductively coupled plasma mass spectrometry (ICP-MS), following standard procedure (Bulska and Wagner, 2016). Other physicochemical properties, such as soil texture (sand, silt, and clay proportion), cation exchange capacity (CEC), moisture and organic matter (OM) content, and the soil enzyme activities, including acid and alkaline phosphatases, β -glucosidase, urease and dehydrogenase activity were analysed at the Eco-Analytical, North-West University, South Africa, following standardized methods described by the Soil Science Society of South Africa (SSSSA, 1990)

4.2.3 High-throughput sequencing of 16S rRNA gene

Soil community DNA was extracted using Power Soil DNA extraction kits (Qiagen, Hilden, Germany) following the manufacturer's instructions. The DNA concentration was measured with a Qubit fluorometer (Invitrogen, Carlsbad, CA, USA) and the integrity was checked using gel electrophoresis with a 1% agarose gel. Extracted DNA was normalised to

equimolar concentrations (5 ng/μl) using 0.1 M Tris-HCl (pH 8.5) and the partial 16S rRNA gene (V3-V4 region), a universal barcode for the characterisation of bacteria, was amplified in a polymerase chain reaction (PCR) using Illumina-barcoded 341F and 805R primers (Klindworth et al., 2013). The 16S rRNA gene library was prepared as described by van Wyk *et al.*, (2017). Paired-end (2 × 300 bp) sequencing of the gene libraries was performed on the Illumina MiSeq sequencer using the Nextera v3 kit (Illumina Inc., San Diego, CA, USA). Sequencing was performed at the sequencing facility of the Unit for Environmental Sciences and Management, North-West University, Potchefstroom, South Africa.

4.2.4 Bioinformatics

The 16S rRNA gene sequence reads were demultiplexed and trimmed of barcodes using MiSeq Reporter software (Illumina Inc, San Diego, CA, USA) and then quality checked with FastQC (v. 0.11.5) (Babraham Bioinformatics, UK). Low-quality reads were trimmed with trimmomatic software (v. 0.38) (Bolger et al., 2014) and reads with mean nucleotide base quality score of less than 20 (Phred Q score) were removed. Reads were analysed using the Quantitative Insight into Microbial Ecology v2 platform (QIIME 2, Release 2020.11; Bolyen et al. 2019). Quality-filtered reads were denoised and clustered into amplicon sequence variants (ASVs) using DADA2 denoiser (Callahan et al., 2016) with a pre-trained classifier of the SILVAngs rRNA gene reference (release 138) (Quast et al., 2013). After singleton removal, the ASV table was rarefied to an even depth before the taxonomic assignment and diversity analyses in QIIME 2. Alpha diversity indices such as the Shannon-Wiener index, inverse Simpson index, Chao1 richness estimator and phylogenetic diversity were analysed in R v 4.1.1 (R Development Core Team, 2018).

4.2.5 Predicted metagenomic profile of rhizosphere bacterial communities.

The functional metagenomic profile of the absolute abundance of microbial genera was predicted using the Tax4Fun2 package (Wemheuer et al., 2020) in R (R Development Core Team, 2018). Firstly, the ASVs were searched against the 16S rRNA gene reference

sequences using the basic local alignment search tool (BLAST) via the *runRefBlast* function. Thereafter, the functional prediction was evaluated using the *makeFunctionalPrediction*, where the ASVs were summarised based on the results of the next neighbour search (Wemheuer et al., 2018). Predicted profiles were annotated based on the Kyoto Encyclopaedia of Genes and Genomes (KEGG) orthology (KO) pathways (Kanehisa et al., 2012). KO terms and metabolic pathways were generated for the ASVs and the relative abundance of the functional genes was calculated. To gain insights into the influence of agronomic practices on bacterial community functional genes, only a few of the KO terms contributing to major ecosystem functions (e.g., organic matter decomposition and biogeochemical cycling), plant growth and nutrient metabolism were investigated.

4.2.6 Co-occurrence network analysis

Bacterial community co-occurrence patterns were analysed by constructing ecological networks using the Random matrix theory (RMT)-based method in the molecular ecological network analysis (MENA) pipeline (<http://ieg4.rccc.ou.edu/mena>) (Faust and Raes, 2016). To assess the impact of fertiliser type on soil bacterial structure, organic and conventional farm networks were constructed. Network complexity was reduced by using only ASVs present in at least 40% of the samples across the organic and conventional farms and the Pearson correlation coefficient was employed for data transformation. Using a multilevel modularity optimisation algorithm, bacterial community groups were identified by clustering networks into modules (Blondel et al., 2008). Node connectivity was established based on among-module connectivity (P_i) and within-module connectivity (Z_i); creating four sub-categories of nodes (a) peripheral ($Z_i < 2.5$; $P_i < 0.62$), (b) connectors ($Z_i < 2.5$; $P_i > 0.62$), (c) module hubs ($Z_i > 2.5$; $P_i < 0.62$) and (d) network hubs ($Z_i > 2.5$; $P_i > 0.62$) (Guimera and Amaral, 2005). The networks were visualised in Gephi v 0.9.2.

4.2.7 Statistical analyses

Unless otherwise stated, all statistical analyses were performed in R software (v.4.1.1). The significance for all tests was set at $P < 0.05$ and the data was tested across fixed factors: farms, fertiliser types (organic versus conventional farms) and vegetable species. Data normality was tested using the Shapiro-Wilk test and non-normal data was transformed with $\log_{10}$, square root or sine to fit a normal distribution. Normal or non-normal data were analysed using parametric or non-parametric tests, respectively. Correlation between soil physicochemical and enzyme activity data was tested using Pearson or Spearman rank correlation for normalised or non-normal datasets. The community structure across the farms, and between organic and conventional farm soil was visualised with a nonmetric multidimensional scaling (NMDS) plot and a cluster dendrogram using *vegan* and *dendextend* (v. 1.12.0) (Galili, 2015) packages in R studio. Differences in multivariate space across the fixed factors were performed with Bray-Curtis dissimilarity using permutational multivariate analysis of variance (PERMANOVA) and Permutational test for homogeneity of multivariate dispersion (PERMDISP). Linear Discriminant Analysis (LDA) Effect size (LEfSe) (Segata et al., 2011) was performed to detect differentially abundant genera (Mann-Whitney U test or Kruskal-Wallis test, P -value < 0.05 ; LDA score > 2) between the farms, organic and conventional farms and plant species using *microeco* package in R software (Liu et al., 2021). Subsequently, the LEfSe results were visualised as bar plots. An indicator species functional analysis was performed for predicted KO terms to detect the most discriminatory KOs between the fixed factors. KO terms with false discovery rate (FDR)-adjusted $P < 0.05$ and an indicator value of > 0.1 were taken to be discriminant for the fixed factors. Redundancy analysis (RDA) was performed in R software to show the physicochemical parameters that best explain the variations in the microbial community composition.

4.2.8 Data availability

The 16S rRNA gene sequence data for this study are available in the sequence read archives (SRA) of the National Centre for Biotechnological Information under a BioProject with SRA accession no PRJNA904574 (<https://www.ncbi.nlm.nih.gov/bioproject/904574>).

4.3 Results

4.3.1 Rhizosphere soil physicochemical properties

The soil physicochemical parameters varied across the fixed factors with no particular trend. A near-neutral pH range was observed across the farms, with the conventional farms having a higher pH than the organic farms (Table 4-1). The moisture and OM content ranged from 1.06%–1.42% and 4.98–5.89%, respectively in both farms. Organic farms largely had a higher CEC and OM content than conventional farms. Farms mostly had low quantities of microelements and heavy metals, except for Pb and As in farm J (Table 4-1). Soil texture was mainly classified as sandy-clay-loam for organic farms and sandy-loam for conventional farms. Some of the soil physicochemical properties significantly (Kruskal-Wallis test, $P < 0.05$) differed across the farms (Table 4-1), while only the differences in OM, clay proportion, CEC, NH_4 , P and Cu were statistically significant (Mann-Whitney or t-test, $P < 0.05$) across the fertiliser types.

Table 4-1: Selected soil physicochemical properties across farms and fertiliser types

Parameters	Organic		Conventional	
	Farm B	Farm S	Farm J	Farm T
pH (H ₂ O)	7.05 ± 0.15 ^a	7.52 ± 0.13 ^a	7.62 ± 0.6 ^a	7.68 ± 1.1 ^a
Moisture (%)	1.27 ± 0.2 ^a	1.06 ± 0.12 ^a	1.42 ± 0.4 ^a	1.39 ± 0.2 ^a
OM (%)	5.89 ± 0.12 ^a	5.37 ± 0.11 ^b	4.98 ± 0.19 ^b	5.13 ± 0.19 ^b
TOC	1.62 ± 0.21 ^c	2.44 ± 0.02 ^b	2.99 ± 0.48 ^a	1.68 ± 0.11 ^c
EC (mS cm ⁻¹)	0.44 ± 0.0 ^b	0.44 ± 0.0 ^b	0.22 ± 0.0 ^c	0.70 ± 0.0 ^a
CEC (cmol [+]) kg ⁻¹)	11.68 ± 0.21 ^b	29.29 ± 1.62 ^a	8.42 ± 1.12 ^c	10.24 ± 1.42 ^{bc}
NO ₃ (mg l ⁻¹)	135.82 ± 1.72 ^a	65.34 ± 1.53 ^c	35.96 ± 3.52 ^d	125.96 ± 4.39 ^b
PO ₄ (mg l ⁻¹)	10.52 ± 0.44 ^b	11.03 ± 1.25 ^{ab}	10.45 ± 1.28 ^b	14.19 ± 1.82 ^a
NH ₄ (mg l ⁻¹)	1.04 ± 0.1 ^b	1.12 ± 0.09 ^b	1.53 ± 0.1 ^b	4.28 ± 0.44 ^a
SO ₄ (mg l ⁻¹)	35.54 ± 1.32 ^c	70.13 ± 2.17 ^a	15.37 ± 2.17 ^d	53.79 ± 3.03 ^b
HCO ₃ (mg l ⁻¹)	15.25 ± 0.48 ^b	42.71 ± 1.41 ^a	36.61 ± 3.13 ^a	36.61 ± 3.94 ^a
P (mg kg ⁻¹)	42.5 ± 3.27 ^b	14.3 ± 1.73 ^c	72.2 ± 3.25 ^a	40.2 ± 2.34 ^b
K (mg l ⁻¹)	17.2 ± 0.97 ^{bc}	19.55 ± 0.94 ^{ab}	16.42 ± 1.81 ^c	53.56 ± 1.81 ^a
Na (mg l ⁻¹)	16.78 ± 0.68 ^b	16.78 ± 0.87 ^b	10.28 ± 1.56 ^c	30.81 ± 2.53 ^a
Ca (mg l ⁻¹)	32.86 ± 2.61 ^b	33.26 ± 1.89 ^b	13.63 ± 3.56 ^c	51.7 ± 3.61 ^a
Mg (mg l ⁻¹)	18.21 ± 1.02 ^a	17.26 ± 0.89 ^a	6.2 ± 1.24 ^b	17.5 ± 2.20 ^a
Cl (mg l ⁻¹)	42.19 ± 2.1 ^b	30.14 ± 3.09 ^c	12.76 ± 2.6 ^d	99.62 ± 3.76 ^a
Fe (mg l ⁻¹)	0.51 ± 0.02 ^a	0.0 ± 0.00 ^c	0.01 ± 0.0 ^{bc}	0.03 ± 0.0 ^b
Cu (mg l ⁻¹)	0.03 ± 0.00 ^c	0.05 ± 0.00 ^b	0.19 ± 0.00 ^a	0.05 ± 0.00 ^b
Zn (mg l ⁻¹)	0.0 ± 0.00 ^c	0.0 ± 0.00 ^c	0.14 ± 0.00 ^a	0.01 ± 0.00 ^b
Pb (mg/kg)	5.46 ± 0.18 ^d	53.29 ± 0.91 ^b	91.27 ± 0.24 ^a	8.25 ± 0.10 ^c
As (mg/kg)	2.68 ± 0.16 ^c	3.69 ± 0.0b	12.35 ± 0.61 ^a	3.47 ± 0.0 ^b
Hg (mg/kg)	0.04 ± 0.0 ^b	0.11 ± 0.0 ^b	2.91 ± 0.18 ^a	0.05 ± 0.0 ^b
Pd (mg/kg)	0.26 ± 0.02 ^b	0.37 ± 0.0 ^a	0.17 ± 0.0 ^c	0.18 ± 0.0 ^c
Cd (mg/kg)	0.03 ± 0.0 ^c	0.085 ± 0.0 ^b	0.16 ± 0.0 ^a	0.02 ± 0.0 ^c
Particle Size (>2 mm)	3.8 ± 0.78 ^c	29.9 ± 2.43 ^a	5.2 ± 0.68 ^b	0.4 ± 0.00 ^d
Clay (%)	23.1 ± 0.75 ^a	27.4 ± 2.56 ^a	12.6 ± 0.86 ^c	17.9 ± 1.76 ^b
Sand (%)	65.4 ± 2.72 ^b	64.0 ± 3.91 ^b	76.3 ± 2.66 ^a	65.8 ± 4.3 ^b
Silt (%)	11.5 ± 0.86 ^b	8.6 ± 1.84 ^a	11.1 ± 1.72 ^b	16.3 ± 1.8 ^c

Values are mean ± SD (n = 5). EC, electrical conductivity; CEC, cation exchange capacity; OM, Organic matter, and TOC; Total organic carbon. Values with different superscript letters for each farm (across rows) are significantly different based on the non-parametric Kruskal-Wallis H test or the parametric analysis of variance (ANOVA) test.

4.3.2 Soil Enzyme Activities

The soil enzyme activities were significantly (Kruskal-Wallis H test, $P < 0.05$) different across the farms and fertiliser type, except for alkaline phosphatase, which was not influenced by the fertiliser type. Mostly, the enzyme activities were higher for organic farms compared to conventional farms (Table 4-2). The alkaline and acid phosphatases had a very low range of activities across the farms. Comparing among farms, farm B had the highest dehydrogenase and β -glucosidase activities, while farm S had the highest activity for urease. Farm J had the lowest urease activity, which is approximately five, 12 and 23 folds lower compared to farms B, T, and S, respectively (Table 4-2). The main effect of crop type and the interactions between the fixed factors on soil enzyme activities were not statistically significant (Kruskal-Wallis H test, $P > 0.05$).

Table 4-2: Important rhizosphere soil enzyme activities across the farms

Parameters	Organic		Conventional	
	Farm B	Farm S	Farm J	Farm T
Dehydrogenase	249.4 $\pm$ 7.09 ^a	156.28 $\pm$ 6.61 ^c	171.17 $\pm$ 2.1 ^b	89.39 $\pm$ 0.78 ^d
β -glucosidase	140.98 $\pm$ 2.41 ^a	124.53 $\pm$ 0.47 ^b	108.09 $\pm$ 1.19 ^c	83.28 $\pm$ 0.22 ^d
Alkaline phosphatase	0.26 $\pm$ 0.01 ^b	0.52 $\pm$ 0.04 ^a	0.52 $\pm$ 0.02 ^a	0.26 $\pm$ 0.02 ^b
Acid phosphatase	0.51 $\pm$ 0.02 ^a	0.52 $\pm$ 0.02 ^a	0.52 $\pm$ 0.00 ^a	0.26 $\pm$ 0.01 ^b
Urease	10.83 $\pm$ 0.48 ^c	49.81 $\pm$ 1.74 ^a	2.17 $\pm$ 0.06 ^d	25.99 $\pm$ 0.24 ^b

Values are mean $\pm$ SD (n=5) followed by superscript letters across the rows. The superscripts are significant differences based on the non-parametric Kruskal-Wallis H test or parametric independent sample ANOVA test across the farms. The units of measurement for the enzyme are INF $\mu\text{g g}^{-1} 2\text{h}^{-1}$ (dehydrogenase), P-nitrophenol $\mu\text{g g}^{-1} \text{h}^{-1}$ (β -glucosidase, acid and alkaline phosphatase) and $\text{NH}_4\text{-N } \mu\text{g g}^{-1} 2\text{h}^{-1}$ (urease).

4.3.3 Diversity and community structure of bacterial amplicon sequence variants

A total of 1,933,349 16S rRNA gene reads was obtained from all samples, with a mean read count of 96,667 per sample. Quality-filtered reads were clustered into 28,604 ASVs after

pruning low count and low variance features. The ASVs were rarefied to an equal depth of 74,170 (rarefaction curve in Supplementary Figure 4-1) before examining the treatment effects. The distribution and richness of ASVs differed substantially across the farms, with farm S having the highest number of unique ASVs (Figure 4-1A). Observed ASVs were higher in organic compared to conventional farms and 276 ASVs were shared amongst all the farms (Figures 4-1A & B). Comparing the shared ASVs in the organic (Supplementary Figure 4-2A) with the conventional (Supplementary Figure 4-2B) farms, 1222 and 313 ASVs were exclusive to the organic and conventional farms, respectively (Figure 4-1B). Similarly, ASV richness differed across the rhizosphere of each vegetable species. Unique ASVs were higher in cabbage, followed by lettuce and onion rhizosphere, while spinach and lettuce rhizosphere share more ASVs (Supplementary Figure 4-3).

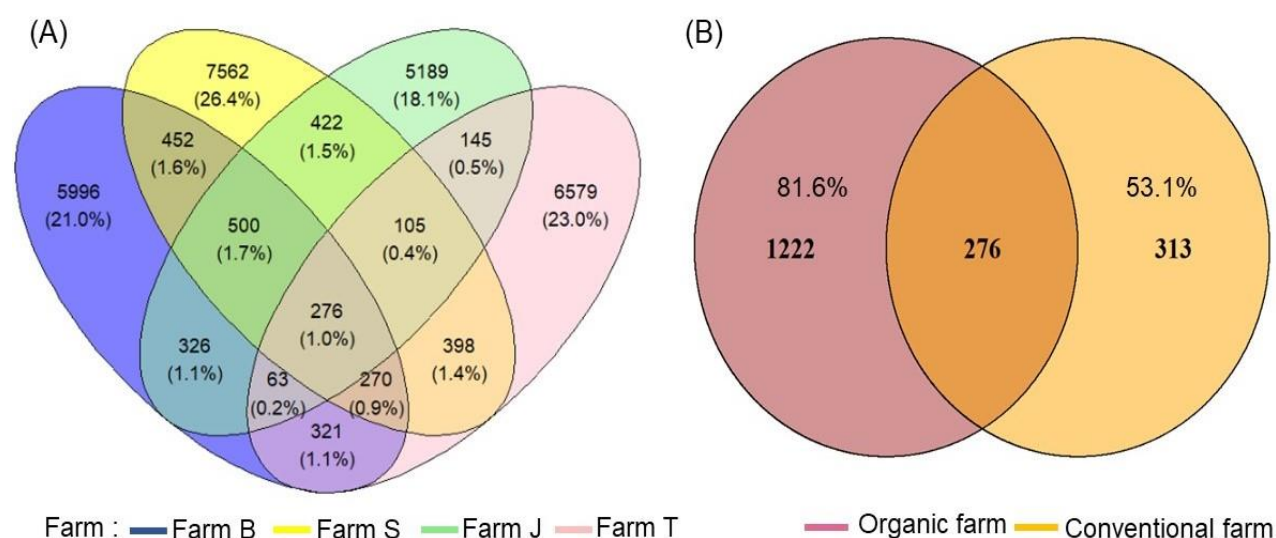


Figure 4-1: Comparison of unique and shared amplicon sequence variants (ASVs) richness
 A) ASV richness across the farms, B) Unique ASVs between organic and conventional farms

The alpha diversity indices measured were not significantly influenced by the main effect of fertiliser type and plant species, except for the inverse Simpson index, which was significant (ANOVA, $F = 3.65$, $P = 0.035$) across the farms, with the pairwise comparison (Tukey HSD,

P-adjusted = 0.0218) showing differences between farms T and S. In addition, the combined effects of the fixed factors (fertiliser type and plant species) and their interactions had no significant effect (general linear model, $P > 0.05$) on the alpha diversity indices measured.

Bacterial community structure was highly differentiated across the farms and between the organic and conventional farms in multivariate space. Although the bacterial community structure in the organic farms (farms B and S) are somewhat differentiated, they are jointly less similar to conventional farms (farms J and T) (Figure 4-2A); the community structural pattern is also supported by the hierarchical clustering (Figure 4-2B). Moreover, the bacterial community composition was significantly different between the farms (PERMANOVA, $P = 0.001$) and across organic and conventional farms (PERMANOVA, $P = 0.009$), contributing about 39.01% and 11.74% of the variations in the models and a PERMDISP of 0.073 and 0.269, respectively. Significant variation was observed between farms B and T, J and T, and S and T. However, the plant species had no significant (PERMANOVA, $P = 0.568$) effect on the bacterial community structure, which was not clearly differentiated (Supplementary Figure 4-4). The non-significant and low dispersion rate in the bacterial community across the farms (Supplementary Figure 4-5A) suggests the results depicted by the NMDS plot in Figure 4-2A are reliable. The stress plot run for the NMDS plot is below 0.2 (Supplementary Figure 4-5B). The effect of fertiliser type and plant species interaction on the bacterial community structure was not significant (Supplementary Table 4-1).

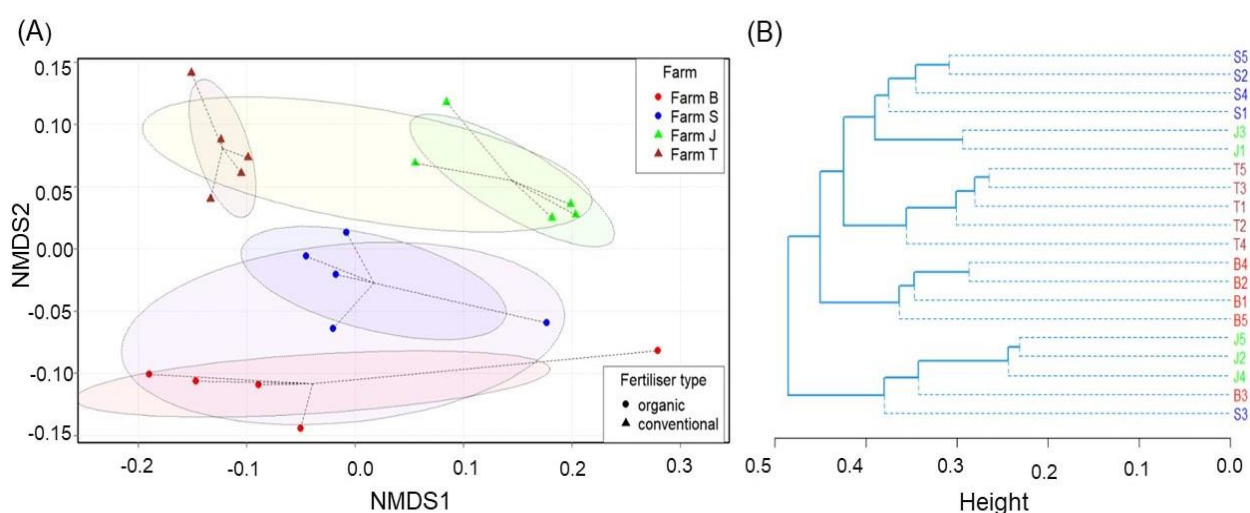


Figure 4-2: Bray-Curtis dissimilarity between bacterial communities. (A) Comparison of observed ASVs between organic and conventional fertilisation and across farms based on non-metric multidimensional scaling (NMDS). (B) Unweighted paired group mean arithmetic (UPGMA) hierarchical cluster dendrogram. The eclipses in the NMDS plot show 95% confidence intervals (standard error) in multivariate space within the group centroids, while the dotted lines indicate the distance of each sample to its group centroid in multivariate space. The stress plot (Supplementary Figure 4-5B) for the NMDS showed that the original dissimilarities are well preserved (stress = 0.0165047) in the reduced number of dimensions.

4.3.4 Dominant and differentially abundant rhizosphere bacterial communities

Across all datasets, the relatively more abundant classifiable ASVs belonged to nine phyla and 19 major genera. Across the farms, the most relatively abundant (>5%) phyla were Actinobacteria (32.3%), Proteobacteria (25.4%), Acidobacteria (8.4%), Firmicutes (7.6%), Chloroflexi (7.6%), and Planctomyces (5.7%) (Figure 4-3A). The relatively more abundant (> 5%) genera across the farms are *Bacillus*, *Rubrobacter*, *Gemmatimonas*, *Solirubrobacter*, RB41, *Nocardioides* and *Bryobacter* (Figure 4-3B). *Bacillus* and *Nocardioides* were relatively more abundant in conventional farms, with their highest abundance found in farm J, while *Rubrobacter* and *Solirubrobacter* were more abundant in organic farms, mainly in farms B and S, respectively. Across the plant species, the bacterial community composition at the phylum and genus taxa levels were not particularly different; however, at the phylum level, Chloroflexi, Planctomycetes and Gemmatimonadetes were relatively most abundant in cabbage, whereas Actinobacteria, Proteobacteria and Firmicutes were the most abundant phyla in onion (Supplementary Figure 4-6A) compared to other vegetables. Cabbage had

the highest relative abundance of *Bacillus* and *Rubrobacter*, whereas onion had the highest relative abundance of *Nocardioides*, *Streptomyces* and *Solirubrobacter* (Supplementary Figure 4-6B).

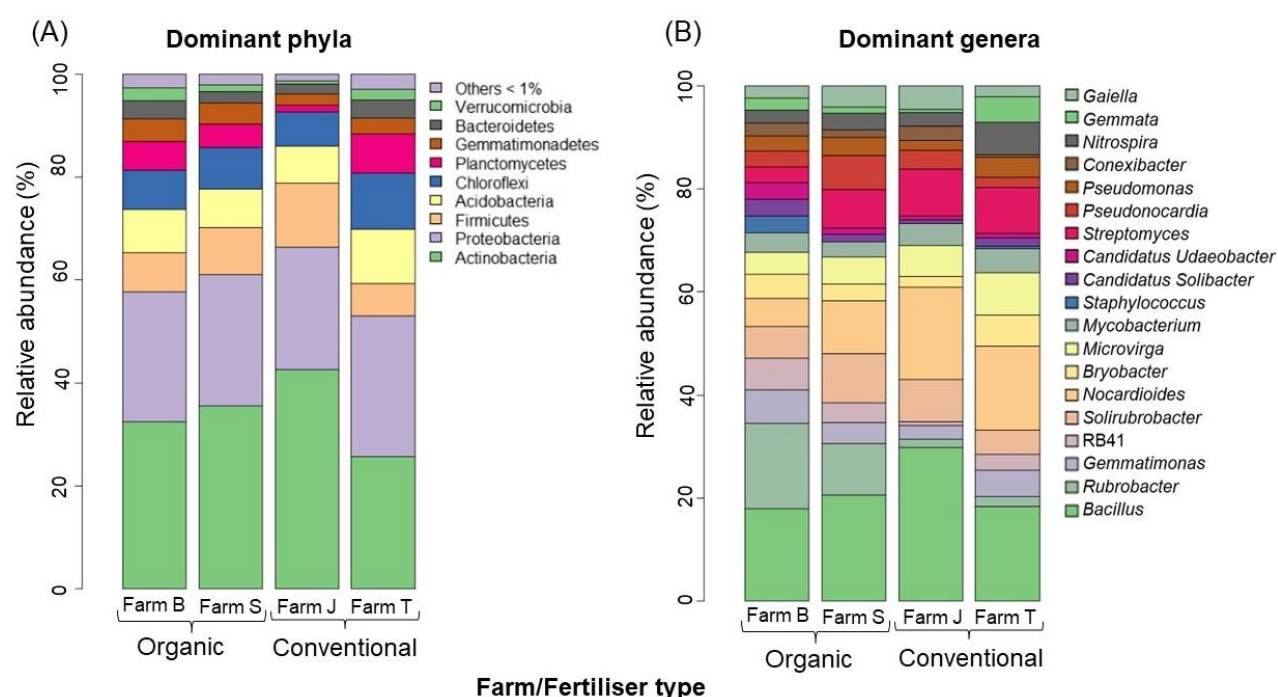


Figure 4-3: Average relative abundance (> 5%) of dominant phylotypes across the farms. (A) Dominant Phyla (B) Dominant Genus taxa. The phylotypes with average relative abundance below 1% and the unculturable and unclassified at the genus taxa level were expunged from the plot. The bar plots were constructed based on the average relative abundance per farm site in R studio.

Linear discriminant analysis (LDA) effect size (LEFSe) showed that a total of 205 features were discriminant across the farms (Kruskal-Wallis H rank-sum test, $P < 0.05$; LDA score > 2.0 ; Supplementary Table 4-2) while only 18 features were discriminant for organic farms versus conventional farms (LDA score > 2.4 ; Supplementary Table 4-3). No taxa was discriminant across the plant species after multiplicity adjustment (Kruskal-Wallis rank-sum test; $P > 0.05$). The genera *Rubrobacter* and *Microlunatus* were highly discriminant in organic farms (Figure 4-4A), while *Nocardioides*, *Ilumatobacter*, *Lysobacter* and *Marmoricola*, were significantly discriminant in conventional farms (Figure 4A). Some of the

bacterial communities that were significant and differentially abundant (FDR-adjusted $P < 0.05$, LDA score > 2.0) at the genus taxa across the farms are shown in Figure 4-4B. *Rubrobacter* and uncultured Conexibacteraceae were more discriminant in farm B, while Pira4lineage and uncultured bacterial Clone C112 were discriminant in farm T. In farm S, Uncultured Actinomycetales, Thermoactinomyces, and *Tumebacillus* were found to be discriminant. The discriminant features in conventional farms, *Marmoricola* and *Lysobacter*, were mainly from farm J. Before multiplicity adjustment of the LEFSe results for plant species type, *Pseudomonas*, *Paracoccus* and *Candidatus Udaeobacter* were discriminant ($P > 0.05$, LDA score > 2.0) and abundant in cabbage, onion, and spinach, respectively (Figure 4-4C), while lettuce rhizosphere soil showed no discriminant feature.

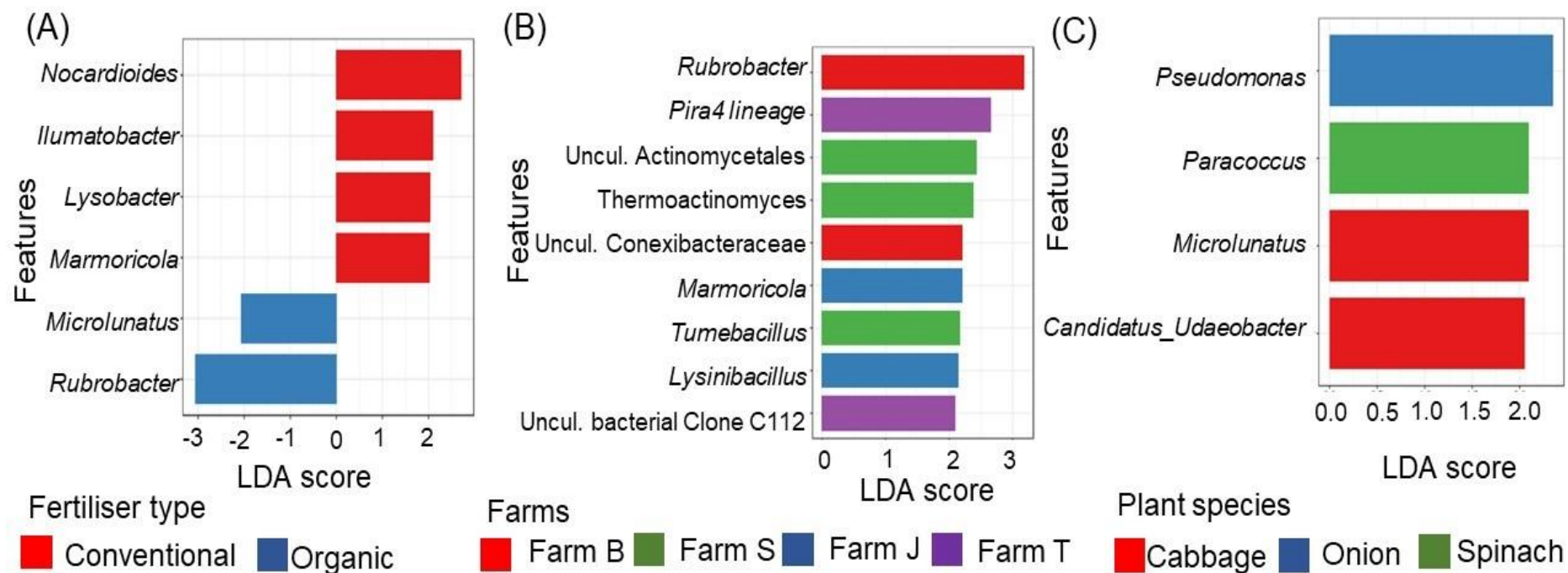


Figure 4-4: Differentially abundant genera of the rhizosphere bacterial communities(A) Top 6 statistically significant discriminant (FDR-adjusted P-value < 0.1, LDA score > 2.4) genera between organic and conventional farms (B) Top 9 discriminant (FDR-adjusted P-value < 0.05, LDA score > 2.0) between the farms. (C) Differentially abundant genera (FDR-adjusted P-value < 0.1, LDA score > 2.0) between the plant species. The bar plot was generated using the Linear Discriminant Analysis (LDA) Effect size (LefSe) in the R using the microeco package. Uncul.; uncultured

4.3.5 Bacterial functional profile and differentially abundant agroecological important enzymes

The KO terms and pathways predicted for all the ASVs were 6,429 and 279, respectively. After multiplicity adjustment, 17 of the predicted pathways were significant for fertiliser type factor (Kruskal-Wallis rank sum test, $P < 0.05$; LDA > 0.181 ; Supplementary Table 4-4), while no significant pathway was observed for the plant species factor. Across the farms, a total of 161 functions were significant (Kruskal-Wallis rank sum test, $P < 0.05$; LDA > 0.274) after multiplicity adjustment (Supplementary Table 4-5). Seventeen abundant KO terms, contributing to important agroecological processes such as the synthesis or metabolism of N, P, C, S and Fe compounds were compared across the farms (Figure 4-5). Predicted KO terms, including nitrogenase, acid and alkaline phosphatase, ferric chelate reductase and aminocyclopropane-1-carboxylate (ACC) deaminase, had low relative abundance in organic compared to conventional farms. Based on the Bray-Curtis distance, the predicted pathways in organic farms and conventional farms clustered separately as shown by the hierarchical cluster dendrogram plot in Figure 4-5.

A higher relative abundance of K01505, K02217, K07405, K01187 and K03332 was predicted in the rhizosphere of cabbage relative to other plant species. Similarly, K00368, K02585, K01183, K07406 and K02013 were higher for ASVs from spinach than in other plant species, while onion had the least abundance of predicted KOs (Supplementary Table 4-6). The major functional profile group of the bacterial communities at taxa rank level 1 is metabolism, followed by environmental information, and genetic and cellular processes. Using LEfSe, it was revealed there are differences in the abundances of key enriched pathways across the fixed factors (Kruskal-Wallis rank-sum test, $P < 0.05$, LDA > 0.2). Organic farms had more differentially abundant pathways compared to conventional farms. Amino acid, arginine, proline metabolism, C-fixation and citrate cycle pathways were discriminant in the organic farm (Supplementary Table 4-4), while sulphur, phosphonate and phosphinate metabolism were more abundant in the conventional farms. Farm T, followed by

farm J had the highest number of important enriched pathways (Supplementary Table 4-5). Xenobiotic degradation and terpenoid and polyketide metabolism were differentially abundant in farm J while bacterial secretion, translation and genetic information system were more abundant in farm T (Supplementary Table 4-5).

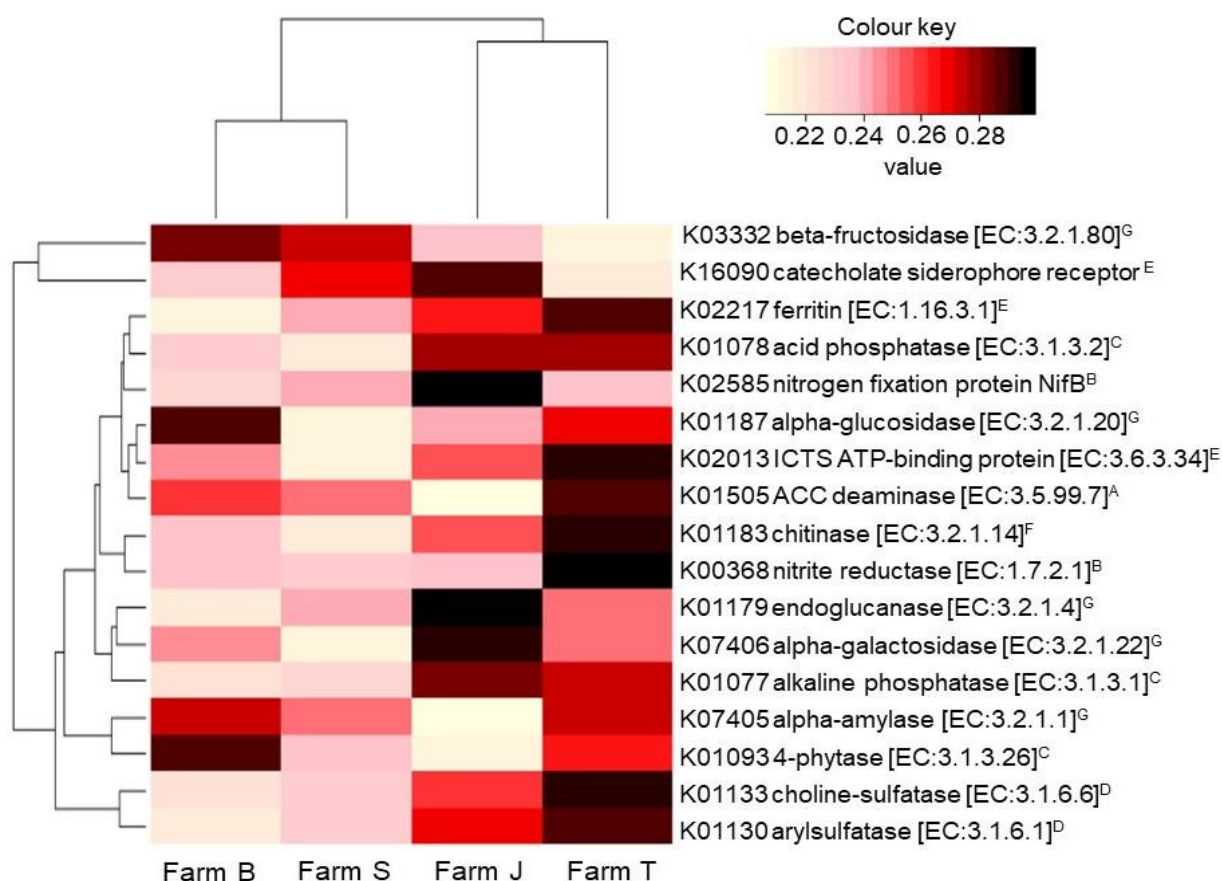


Figure 4-5: Relative abundance of KEGG Orthology terms contributing to important agroecosystem functions. The hierarchical clustering dendrograms are based on the mean Bray-Curtis distance between the farms. The colour range for the relative abundance is scaled across the farms. The superscripts by the enzyme commission (EC) number indicate (^A); ACC, (^B); N-fixation, (^C); phosphorous mineralisation, (^D); sulphur mineralisation (^E); soil iron balance, (^F); biocontrol, and (^G); soil C breakdown. ICT; iron complex transport system.

4.3.6 Relationship between biological and physicochemical properties

Although there were no significant correlations between some of the soil enzyme activities and physicochemical parameters (Supplementary Table 4-7), beta-glucosidase had a

significantly positive correlation with pH ($r = 0.32$, $P = 0.001$) and PO_4 ($r = 0.12$, $P = 0.045$) but negative correlation with OM ($r = -0.69$, $P = 0.015$), NH_4^+ ($r = -0.72$, $P = 0.005$) and K ($r = -0.30$, $P = .019$). Both alkaline and acid phosphatase significantly ($P < 0.05$) correlated with TOC, positively and Cl, negatively. Moreover, alkaline phosphatase correlated positively with NO_3 while acid phosphatase correlated positively with pH and EC. In addition, dehydrogenase had significant correlations that were negative with pH, PO_4 , NH_4 and K but positive with OM and TOC. Although there exists either a positive or negative correlation between the soil enzymes and moisture content, Ca and sand, the correlations were not significant. Similarly, though not significant, there were correlations between the species function percentage of bacterial community with the soil parameters (Supplementary Figure 4-7).

The RDA plot showed that the soil physicochemical properties contributed 34.5% (R-squared adjusted value) of the variation in the bacterial community structure. Farms and fertiliser type-specific clustering were observed, especially for the organic farms (Figure 4-6). Amongst the physicochemical parameters in the RDA model, TOC, OM, EC, and CEC were statistically significant (ANOVA, $P < 0.05$) (Supplementary Table 4-8). Similarly, there were significant correlations between the environmental variables and the distance matrix (Mantel, $P < 0.05$) (Supplementary Table 4-8). Overall, the plot showed that soil physicochemical properties largely influenced the variations observed in the bacterial community (Figure 4-6). PO_4 , Na and NH_4 had more impact on the bacterial community structure in farm T, while moisture, TOC, sand, and pH, had more influence on bacterial community structure in the organic farms (farms B and S).

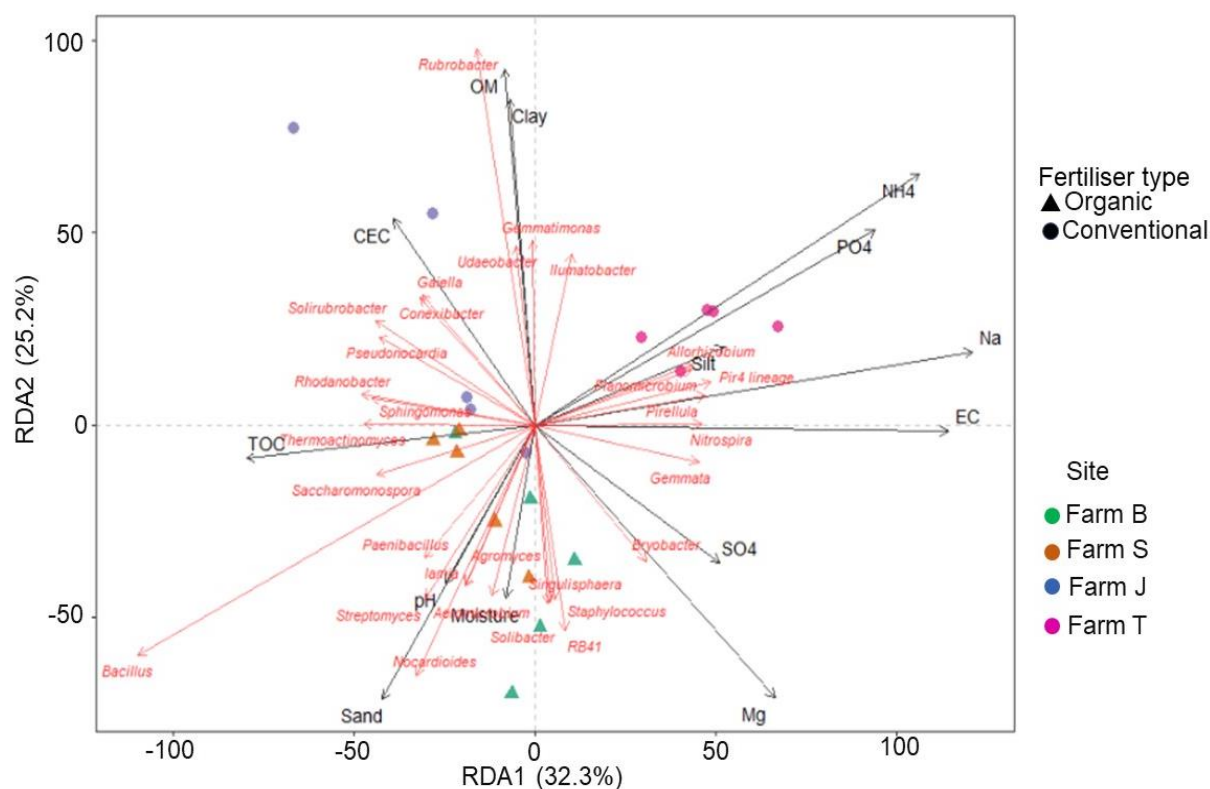


Figure 4-6: Redundancy analysis (RDA) triplot showing the correlation between bacterial community relative abundance and explanatory variables. The first 30 major bacterial genera were used and the variables were the physicochemical factors. The first species axis (RDA) is significant ($P < 0.05$)

4.3.7 Co-occurrence analysis

Some of the ASVs had significant ($r = 0.70$, $P < 0.05$) positive correlations with the environmental traits, which produced a significant (Mantel, $P = 0.001$) effect on the network connectivity. The significant ASVs and node connectivity were found to be significant for two major phyla: Actinobacteria (Mantel, $r = 0.120$, $P = 0.022$) and Acidobacteria (Mantel, $r = 0.344$, $P = 0.009$). From the topological metrics (Table 4-3), both organic and conventional farm networks were non-random as inferred from the significant power-law distribution for each network ($R^2_{\text{organic}} = 0.88$, $P < 0.05$; $R^2_{\text{conventional}} = 0.84$, $P < 0.05$) and the higher values of some of the structural features in the empirical compared to the random networks (Table 4-3). The empirical network consisted of 247 nodes and 704 edges (Figure 4-7A) for the organic farms and 284 nodes and 1027 edges for the conventional farms (Figure 4-7B).

Table 4-3: Topological properties of empirical and random networks between bacterial communities

Network indices	Organic		Conventional	
	ENI	100 RNI	ENI	100 RNI
Average clustering coefficient	0.129	0.043 ± 0.006	0.216	0.309 ± 0.011
Average path distance	6.183	3.467 ± 0.038	2.791	2.545 ± 0.027
Geodesic efficiency	0.215	0.321 ± 0.003	0.456	0.435 ± 0.003
Harmonic geodesic distance	4.648	3.115 ± 0.025	2.191	2.300 ± 0.017
Centralization of degree	0.073	0.073 ± 0.000	0.326	0.326 ± 0.000
Centralization of betweenness	0.122	0.074 ± 0.008	0.067	0.090 ± 0.010
Centralization of stress centrality	17.228	0.299 ± 0.028	2.725	0.590 ± 0.055
Centralization of eigenvector centrality	0.210	0.184 ± 0.015	0.175	0.173 ± 0.003
Density	0.019	0.019 ± 0.000	0.050	0.050 ± 0.000
Reciprocity	1.000	1.000 ± 0.000	1.000	1.000 ± 0.000
Transitivity	0.192	0.061 ± 0.006	0.131	0.254 ± 0.005
Connectedness	0.801	0.958 ± 0.021	0.471	0.980 ± 0.017
Efficiency	0.981	0.984 ± 0.000	0.900	0.952 ± 0.001
Hierarchy	0.000	0.000 ± 0.000	0.000	0.000 ± 0.000
Lubeness	1.000	1.000 ± 0.000	1.000	1.000 ± 0.000
Modularity	0.676	0.406 ± 0.006	0.210	0.166 ± 0.004

ENI: Empirical Network Indices, RNI: Random Network indices

A higher level of positive (~ 72%) associations occurred amongst the bacterial communities in the organic farms compared to conventional farms, which had a higher negative (~ 33%) association. Though community segmentation was apparent in both networks, the organic farms had a more profound fragmentation. Moreover, a higher network complexity was observed for organic farms compared to conventional farms, which had more numbers of smaller modules and edges than organic farms. The module hubs were not of similar

parameters, with organic farms (Figure 4-7A) having more bacterial communities compared to conventional farms (Figure 4-7B). Actinobacteria, Acidobacteria, Proteobacteria and Chloroflexi were dominant in the keystone nodes. No network hub was observed for both networks. While Actinobacteria occurred as a major node that highly connects modules in both networks (Figure 4-7), Bacteroides, Proteobacteria, Acidobacteria and Chloroflexi are other connectors in the organic farm network. The keystone nodes comprised the genera *Bacillus*, *Nocardiodites*, *Blastocatella* and *Saccharomonospora*. A major connector in conventional farms is *Agromyces* while organic farms are dominated by the family Micrococcaceae, Roseiflexaceae, Blastocatellaceae, Xanthobacteraceae and Chitinophagaceae (Figure 4-7).

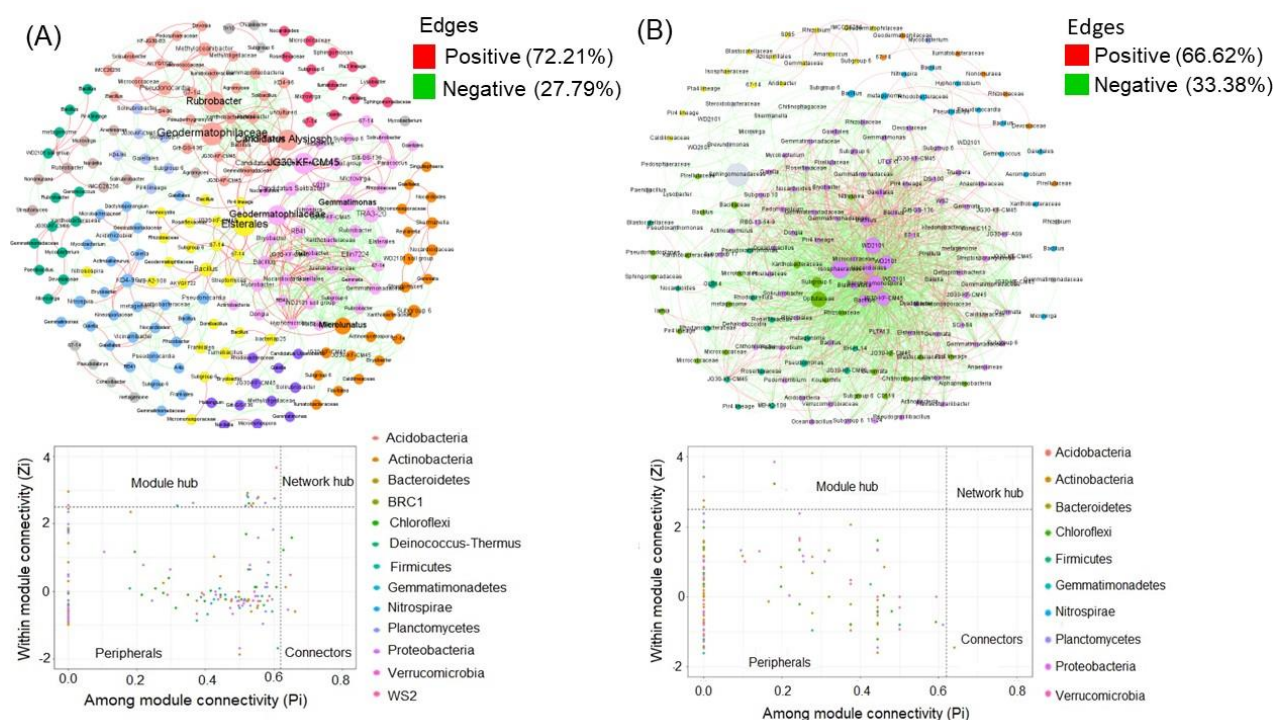


Figure 4-7: Co-occurrence networks of bacterial communities and classification of nodes for detection of keystone taxa within ecological networks in (A) organic and (B) conventional farm rhizosphere soil. The nodes represent bacterial species (round shape), coloured according to the community modularity class. Node sizes are proportional to their degree of distribution, and nodes having less than two connections have been removed. Edges are network connections signifying significant (FDR-adjusted $P < 0.01$) associations between nodes. Positive associations are coloured red, while negative associations are blue. Detected module hubs in the two farm networks are linked with Acidobacteria, Actinobacteria, Proteobacteria and Chloroflexi, while the connectors are affiliated with Actinobacteria for both organic and conventional farms. The co-occurrence network statistics are presented in Table 4-3

4.4 Discussion

Soil microbes significantly contribute to ecosystem functions through organic matter decomposition, nutrient cycling, and mineralisation. These functions and plant ecosystems are influenced by agronomic practices such as soil nutrient management and cropping systems. Thus, this study provided insights into the rhizosphere soil bacterial community structure and functions of vegetable crops cultivated under organically and conventionally managed soil in different farms. Functional profiling and differentially abundant taxa of rhizosphere bacteria allowed for the identification of rare microbial communities with unique potentials in agroecosystems, emphasising the benefits of understanding soil microbiome dynamics to reveal specific patterns, functions and strategies used by microbes under different agronomic conditions.

Soil physicochemical parameters influence nutrient richness.

The fact that soil nutrients and structure drive plant growth and soil microbial diversity is well established (Bach et al., 2010; Xu et al., 2022). Some of the soil parameters, including pH, EC, moisture content, NH_4^+ and NO_3^- were not significant across the farms, However, except for pH and P, the measured values were relatively not within the optimal range required for the productivity of most crops, including vegetables (Warncke et al., 2009; Rosen and Eliason, 2005; Liu and Hanlon, 2018). Contrary to our results, low moisture content has been reported in chemically fertilised soils (Rachwał et al., 2021) and a higher pH in organically managed soil (Aziz et al., 2017; Diacono and Montemurro, 2010). Higher levels of organic matter increase soil water-holding capacity through soil aggregation, signifying soil organic matter and moisture content are strongly correlated (Rajkai et al., 2015). Although organic farms typically have higher organic matter content and soil texture type that support high moisture content, our results showed that conventional farms had higher moisture content, possibly due to other factors such as time of sampling and irrigation system. Compared to conventional farms, soils in the organic farms largely had higher clay content, CEC and OM content; this is in agreement with the observations from other studies (Han et al., 2016;

Rigane et al., 2020). Among the farms, farm S, which is organically managed, had the highest particle size (Table 4-1). Soil particle sizes impact soil aeration and structure, while high clay content and CEC strongly drive the fixation of key soil nutrients (Kome et al., 2019; Ramos et al., 2018). Being negatively charged, clay particles adsorb positive ions such as Ca^{2+} , Fe^{3+} , K^{+} and NH_4^{+} which are key indicators of soil nutrient richness (Tomašić et al., 2021). In contrast, our results show that in the organic farms (Farm B and S), which had higher clay content, some of the positive ions, including NH_4^{+} , K^{+} and Ca^{2+} were relatively lower suggesting other factors, including irrigation, could have influenced the soil nutrient levels.

Soil enzyme activities in organically and conventionally managed soil

High activity of soil enzymes, including dehydrogenase, β -glucosidase and urease activities have been reported in organic farms (T. Hernandez et al., 2021; Pittarello et al., 2021). Extracellular enzyme production has been positively correlated with high microbial biomass and diversity in the community. Such reports are consistent with our results that showed a high activity of β -glucosidase across organic farms, validating the comparatively higher C mineralisation in organic farms compared to conventional farms; compost and manure applied in organic farms are rich in carbon substrate (Cordero et al., 2019; Zang et al., 2018). Acid and alkaline phosphatase activities are optimal at pH 3.0–5.5 and 8.5–11.5, suggesting the low activities across the farms may be linked to the soil neutral pH (Neina, 2019). Soil enzyme activities dynamically respond to soil nutrient management, which influences soil parameters, particularly pH, OM, moisture content and nutrient content (Koishi et al., 2020; Pan et al., 2013). A significant correlation between soil pH and dehydrogenase, β -glucosidase and acid phosphatase suggests pH is a key factor driving enzyme activities (Pan et al., 2013). The observed positive correlation of β -glucosidase with OM indicates the specificity of the enzyme to soil C, which is a fraction of the total soil OM (Meena and Rao, 2021). Corroborating with our result of reduced enzyme activities in the conventional farm, excessive use of chemical fertilisers has been reported to hinder soil

enzyme activities (Pittarello et al., 2021; Rachwał et al., 2021). Thus, the types of fertilisers used in plant cultivation affect changes in soil conditions, such as enzyme activities which may cause variations in the dynamics of the associated soil microbial community (Liu and Hanlon, 2018; Ullah et al., 2019).

Rhizosphere bacterial community diversity and structure

Soil properties, especially organic matter drive bacterial growth and metabolism, suggesting the bacterial richness across the farms is related to soil conditions (Peltoniemi et al., 2021; Rachwał et al., 2021). This was evidenced in the RDA triplot (Figure 4-6), which revealed a significant correlation between the soil parameters and some bacterial communities. Soil physical and biological parameters, including OM, moisture and nutrient content, pH, dehydrogenase, β -glucosidase, and urease, support improved soil and plant health, which in turn influence the rhizosphere bacteria composition (Yan et al., 2021). Rhizosphere microbiome has some analogous traits with its associated plants, suggesting plant selective impact on rhizosphere microbes and consequently shaping rhizobacterial assemblage (Ling et al., 2022). Similar to other studies, though only the inverse Simpson diversity index was significant, the differences in alpha diversity across the farms suggest fertiliser types influence bacterial community richness (Acharya et al., 2021; Hu et al., 2011). According to Hermans et al. (2017) and Kamaa et al. (2011), bacterial communities respond to prevailing soil nutrient management types, which vary across landscapes; this is in agreement with our results which revealed differences in bacterial communities across the different farms, as depicted by the NMDS plot (Figure 4-2A). However, the bacterial community structure of soils from similar fertiliser types showed some differences, an indication that other site-specific factors, such as the source and type of seeds, fertilisers, and irrigation systems not considered in this study, may be responsible.

Important bacterial phyla, including Actinobacteria, Proteobacteria, Acidobacteria, Firmicutes and Chloroflexi, which were relatively abundant across the farms have been reported in

similar studies (Ezeokoli et al., 2020; Zhang et al., 2022). These phyla usually exist in C-rich niches, such as the rhizosphere, which supports high metabolic activities and fast microbial growth. The variation in taxa abundance across crop species may be due to differences in root architecture and exudates, which differentially stimulate the growth of rhizosphere microbial communities (Hoch et al., 2019; Yan et al., 2021). Similarly, this may have influenced the higher ASV abundance observed in cabbage compared to other crop species, suggesting the need to further explore factors influencing rhizobacteria diversity variability in different crop species under similar conditions. Several studies have identified core bacterial microbiomes primarily using taxonomic assignment; however, for detailed knowledge of microbial dynamics, it has become imperative to further identify soil microbes using key functions that are commonly expressed (Ling et al., 2022). For instance, Gemmatimonadetes, a recently classified phylum found among the major phyla in the cabbage soil have species that contribute to C-fixation through chlorophototrophy (Thiel et al., 2018). In addition, predominant genera, such as *Bacillus*, *Nocardioides*, *Pseudomonas*, *Rubrobacter* and *Lysobacter*, which are constantly enhanced in the rhizosphere, contribute to key microbial functions and processes, such as biogeochemical cycling, organic matter decomposition, mineralisation and processes that are crucial to agroecosystem sustainability (Adeleke et al., 2019; Raimi et al., 2017).

Ecological functions of differentially abundant rhizosphere bacterial community

The differentially abundant *Rubrobacter* in organic farms is underexplored despite its ecological restoration and engineering potential, which is attributed to its ability to survive in low-nutrient soil and under intense desiccation (Baubin et al., 2021). Similarly, *Lysinibacillus* and *Pseudomonas* predominant in organic farm soils are well-known plant growth-promoting rhizobacteria involved in N-fixation, phytohormone production and P solubilisation (Li et al., 2017; Shahwar et al., 2023; Vignesh et al., 2021). *Candidatus Udaeobacter*, a differentially abundant genus in the spinach rhizosphere, exhibits multidrug resistance and the ability to evade the harmful effects of antimicrobials (Willms et al., 2020). The unique properties of

these genera suggest they are suitable indicator species for assessing the soil nutrient status. In contrast to the organic farms, conventional farms had differentially abundant *Norcardioides*, *Lysobacter*, *Ilumatobacter* and *Marmoricola*. *Norcardioides* participate in bioremediation, especially organochlorine degradation such as lindane pesticide and chloroaromatics, making them a crucial candidate for soil remediation strategy (Ito et al., 2019; Singh and Singh, 2019). *Lysobacter* is a well-known biocontrol agent (Lin et al., 2021) while *Ilumatobacter* and *Marmoricola* participate in C and N cycling, respectively (DeLorenzo et al., 2012; Liu et al., 2023). Although soil microbes have been extensively classified, the high proportion of unclassified sequences in this study gave credence to the fact that the majority of soil microbiomes are yet to be fully identified and characterised (Delgado-Baquerizo, 2019; Furtak et al., 2020). However, metagenomics, a leading and relatively recent technique for effective analysis of diverse environments has advanced microbial diversity knowledge, offering valuable strategies for optimising the cultivation of yet uncultured species (Kulski, 2016; Mendes et al., 2017).

Nitrogenase and NifT play key roles in the N-cycle, while cellulases break down cellulose and polysaccharides, and chitinase degrades chitins, contributing to C and N levels in the ecosystem (Glick, 2012). Corroborating the report by Ling et al., (2022), alpha-glucosidase and beta-fructokinase were highly predicted in organic farms, possibly due to the high level of C supplied by compost and animal manure, suggesting a more sustainable soil microbial community may be achieved through organic amendments that increased these key enzymes. Bacterial communities such as *Bacillus*, *Enterobacter*, *Citrobacter* and *Pseudomonas* detected have been previously reported with N-fixing ability due to their nitrogenases (Herna et al., 2017; Li et al., 2017). On the contrary, nitrite reductase genes driving the denitrification pathway in the N-cycle were significantly abundant in Farm T, which is a conventional farm, establishing the fact that these genes play a crucial role in preventing groundwater pollution caused by nitrate compounds due to excessive chemical fertiliser application (Liu et al., 2020). Arylsulfatase and choline-sulfatase drive the sulfur

content in the soil and are usually activated under sulfur-deficient conditions (Cregut et al., 2013; Sánchez-Romero and Olguin, 2015). Surprisingly, these genes were highly prevalent in conventional farms (Farm T and J) (Figure 4-5), suggesting other factors, including available sulphur, forms of sulphur compounds and other nutrient complexes in the soil may have influenced the prevalence of these enzymes (Siwik-Ziomek et al., 2016). In addition, the high abundance of enzyme coding genes predicted in the rhizosphere of cabbage compared to other vegetables could enhance soil nutrient richness and in turn, drive increased soil microbial composition and diversity (de Bruijn, 2015; Xu et al., 2022). While variations across geographic locations drive changes in soil physicochemical parameters (Moore et al., 2022), the changes may in turn influence soil microbial and enzyme activities (Meena and Rao, 2021), suggesting the reason for some of the variations observed across the farms. Thus, it may be recommended towards best practices to always understand agronomic soil parameters for appropriate selection of efficient soil nutrient management.

Microbial co-occurrence network and keystone taxa

Microbial co-occurrence structure greatly impacts community assembly, abundance and diversity (Pan et al., 2021). Soil nutrient management (types of fertiliser applied) and plant species have been reported to influence the network structure of soil bacterial communities (Xu et al., 2022; Xue et al., 2018). The network analysis showed that conventionally managed soil had high numbers of bacterial communities with negative links, which may indicate weak connections among microbial associations. This observation was corroborated by Huang *et al.*, (2019), who attributed the distinctly weakened ecological interactions between soil microbes to long-term chemical fertilisation. In contrast, the higher level of positive interactions in the organic farms may signify greater ecological cooperation among the microbial communities for diverse ecosystem functions and consequently; a network structure with high stability and high-order level complexity (Gu et al., 2019; Xue et al., 2018). Profound community fragmentation was noticed for organic farm networks. According to Hernandez et al., (2021b), ecological stress greatly influences the complexity and stability

of microbial networks, indicating fragmentation may not necessarily be due to fertiliser types. Thus, other factors such as soil types, irrigation and land preparation that impact soil microbial diversity may be investigated.

In microbial networks, modules comprise species, which are interconnected with more frequent and intensive interactions than other parts of the community (Ling et al., 2022). Compared to conventional farms, organic farms had high numbers of complex topological structures and modules, which suggests high niche differentiation in organic farms (Figure 4-7; Table 4-3). Unique microbes play key roles in the functioning of bacterial communities by contributing to the information current across the entire network (Banerjee et al., 2018). Similar to our observation, Gu et al., (2019) reported no network hubs in their study, suggesting unique phylotypes for specific functions are absent. However, some generalists, including *Bacillus*, *Nocardioides*, *Agromyces* and *Blastocatella* were detected, signifying diverse keystone species that could drive the soil microbial communities in each of the farms (Gu et al., 2019). Module hub, network hub and connectors harbour keystone communities whose identification further improves the understanding of microbial community structure and interactions (Pan et al. 2021). In this study, *Bacillus* found in organic farms is a unique generalist, participating in nutrient solubilisation and mineralisation; thus, can generate a niche for other plant growth-promoting rhizobacteria. However, the conventional farm network has *Agromyces* as a major connector, which is involved in xylan degradation; thus, may create a niche for microbial populations that cannot degrade polysaccharides (Rivas et al., 2004).

To further improve our agroecosystem management knowledge, it is recommended that the impact of soil nutrient management on soil microbial diversity and functional structure be investigated across seasons. Data on crops cultivated in the previous seasons, irrigation systems and sources of fertilisers were not available, contributing to some of the limitations in this study.

4.5 Nexus of vegetable rhizosphere bacterial communities and phyllosphere endophytic bacterial communities

Some of the bacterial communities observed in the rhizosphere were also found in the endophytic bacterial communities of vegetable phyllosphere, suggesting rhizosphere is a reservoir of potential endophytes, which may colonise plant tissues under favourable conditions. Actinobacteriota, Proteobacteria and firmicutes were the most abundant phyla found in both ecosystems, similar to the observations of other studies (Jia et al., 2020; Jin et al., 2014), although Actinobacteriaota was dominant in the rhizosphere while Proteobacteria was dominant in the plant phyllosphere bacterial communities (Figure 4-8). At the genus taxa, *Pseudomonas*, *Bacillus*, *Flavobacterium*, *Rhodococcus*, *Nocardioides*, *Microbacterium*, *Rubrobacter*, and *streptomyces* were found in the plant tissues and the rhizosphere (Figure 4-9). However, they differ in richness across the samples. In addition, *agromyces* and *Nocardioides* were major generalists in both plant and rhizosphere co-occurrence networks, suggesting the organisms may be performing key roles in both environments. Unique properties of rhizosphere bacteria have also been reported in the leaf microbial communities (Rastegari, 2020). A proteogenomic analysis revealed the presence of genes such as nitrogen fixation, methanol dehydrogenase and formaldehyde-activating enzymes in the rhizosphere and phyllosphere samples of rice (Knief et al., 2012).

Dominant phyla

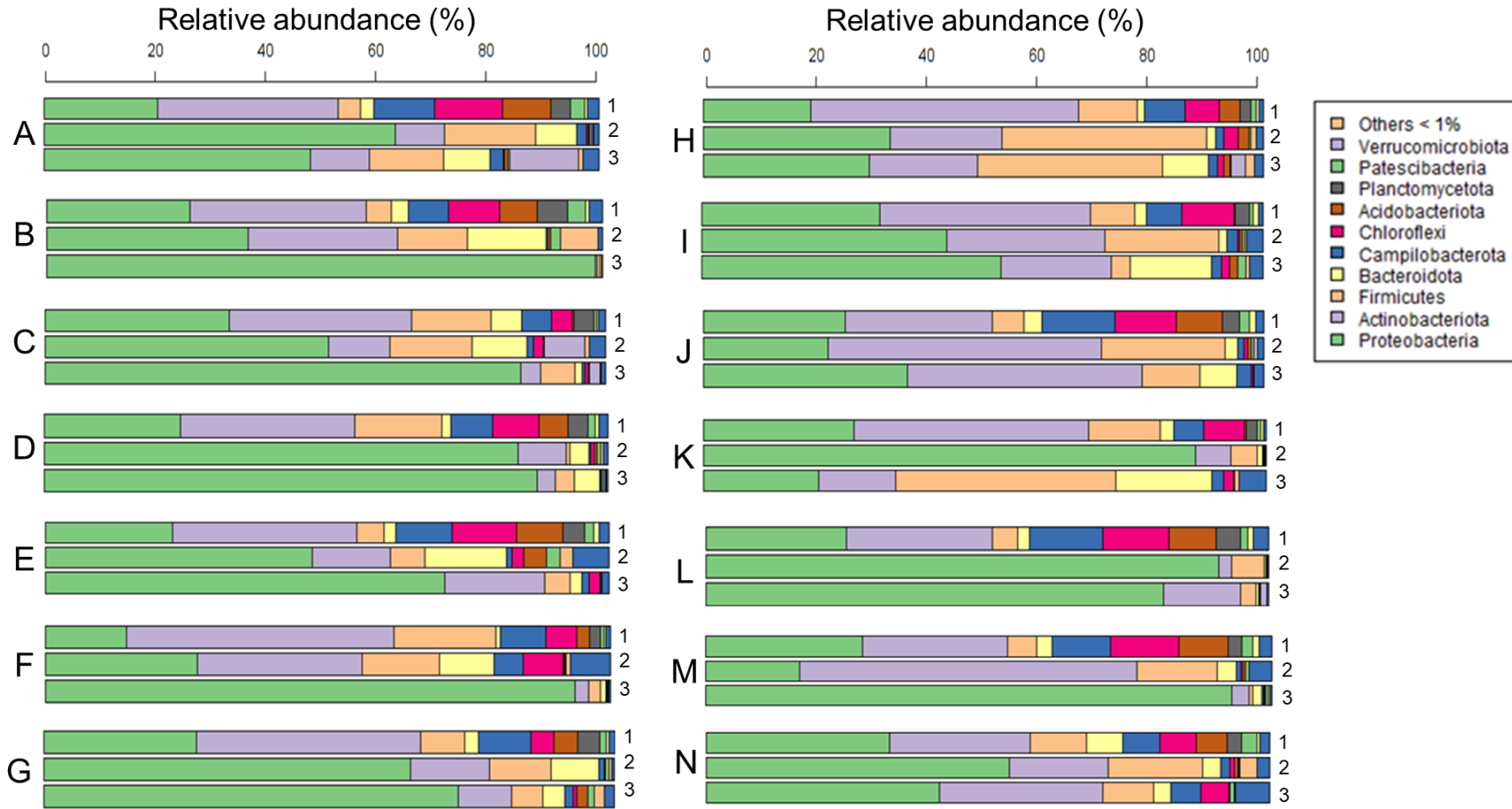


Figure 4-8: Comparison of the rhizosphere bacterial communities and phyllosphere endophytic bacterial communities at the phylum taxa level. The vegetable phyllosphere bacterial endophytes (above 1% relative abundance) were compared with its corresponding rhizosphere bacterial communities. 1; rhizosphere soil, 2; plant root and 3; plant leaf. A; BF cabbage, B; BF spinach, C; BF onion, D; JF lettuce, E; JF cabbage, F; SF cabbage, G; JF spinach, H; SF onion, I; SF lettuce, J; TF cabbage, K; SF spinach, L; TF onion, M; TF lettuce and N; TF spinach.

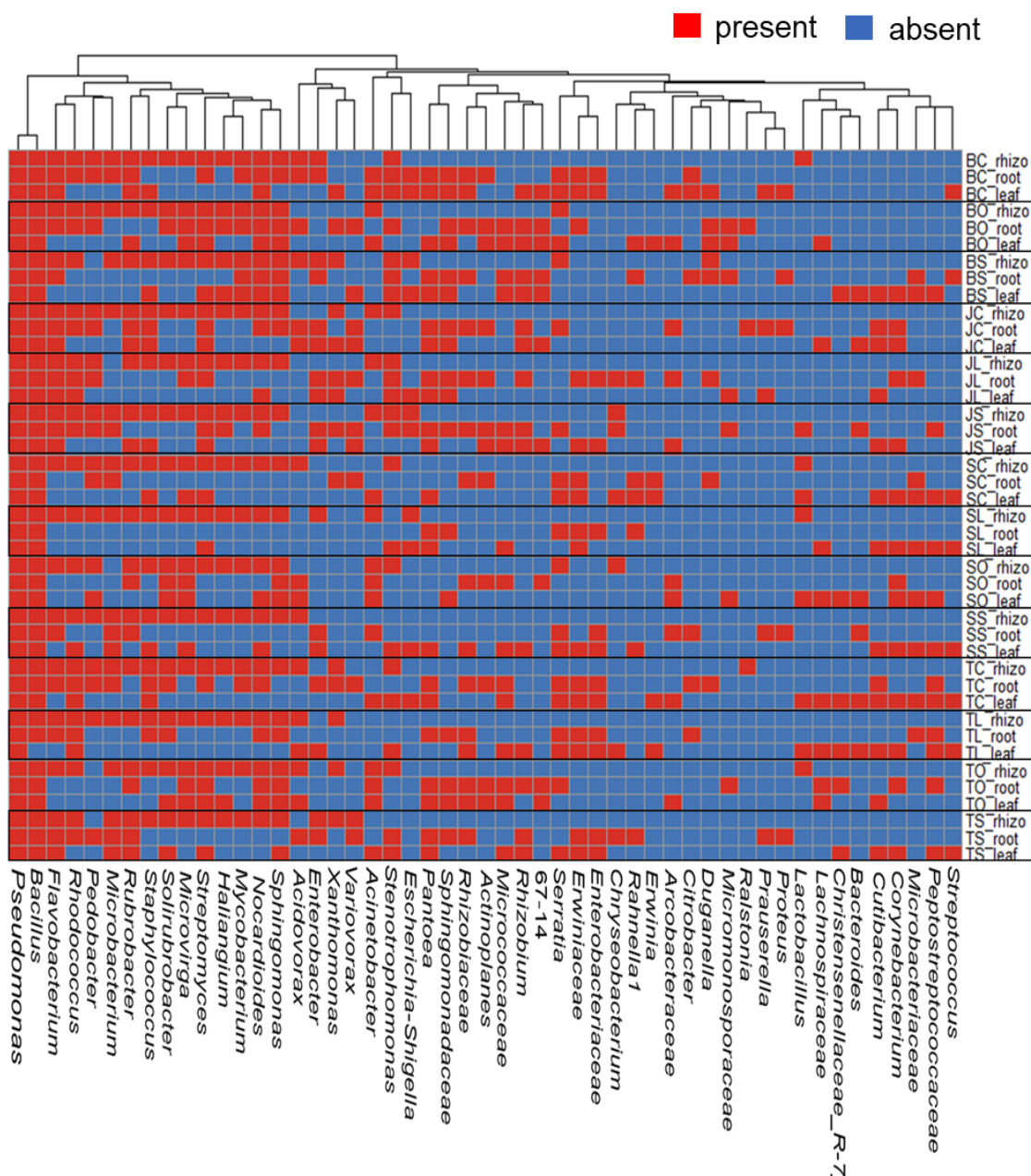


Figure 4-9: Comparison of the rhizosphere bacterial communities and phyllosphere endophytic bacterial communities at the genus taxa level. The heatmap was made using data for the presence or absent of the first 50 most abundant phylotype (above 1% relative abundance) in the phyllosphere compared with that of the rhizosphere. The bacterial communities of the rhizosphere and the phyllosphere of each vegetable in each farm were compared. The first letter of the sample codes B, S, J and T represents farms B, S, J and T, while the second letter stands for the vegetable code (C; cabbage, L; lettuce, O; onion, S; spinach).

4.6 Conclusions

This study revealed that the soil bacterial community diversity and functions are influenced by farm location and fertiliser types. Unique bacterial communities, which contribute to ecosystem functions were differentiated across these fixed factors. To a large extent, the

discovery of unique bacterial communities such as *Bacillus* and *Rubrobacter* with plant growth-promoting and niche-creation potential across the farms could improve the understanding of soil microbial dynamics for enhancing soil and plant productivity. Conventional farms had reduced keystone taxa compared to organic farms, signifying fertiliser types impact the abundance of unique microbial taxa, which is a key factor in ecosystem functioning. This study provided comprehensive details on rhizosphere soil bacterial diversity and functions, which could be useful in the identification of biomarker species for monitoring soil productivity in a particular nutrient management system. Ecologically important taxa, such as *Bacillus* and *Agromyces* are good sources of microbial resources for bioformulation production. Overall, the findings provided a baseline for understanding how fertiliser types as organic and conventional fertilisers, impact soil bacteria community structure and related ecological functions. Such knowledge may be useful for monitoring invasive species or formulating a strategy for replenishing extinct beneficial species following land-use intensification or a particular agronomic practice.

The high-throughput sequencing used in chapters 3 and 4 provided robust information on the bacterial community composition, diversity and functional profiles. However, it is important to isolate, culture and investigate the bacterial endophytes for their plant growth-promoting properties in real situations. Before an organism is considered to perform a particular function, it has to be investigated *in vitro* by evaluating the biological phenomena under different conditions to ascertain the best combination of conditions under which the traits investigated can be expressed effectively. The *in vitro* results can effectively be harnessed in biotechnology and field applications. Importantly, the presence of a particular gene in a cell may not necessarily confer the expression of related functions. Therefore, the next chapter will isolate and characterise the PGP abilities of the bacterial endophytes.

4.7 Reference

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CHAPTER 5 :

16S rRNA PROFILING AND PLANT GROWTH-PROMOTING POTENTIAL OF CULTIVABLE BACTERIAL ENDOPHYTES OF VEGETABLE CROPS

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5.1 Introduction

Lately, the growing awareness of plant growth-promoting (PGP) microorganisms, especially those colonising healthy plant tissues, termed endophytes, has caused an increase in the exploitation of their potential in sustainable agriculture (Sturz et al., 2000). Some of the primary goals of sustainable agriculture include efficient use of resources, reducing agrochemical inputs such as chemical fertilisers and pesticides, and maintaining closed nutrient cycles within the farm systems (Raimi et al., 2017). Interestingly, harnessing endophytic bacteria in bioinoculant technology offers an excellent opportunity to realise some of these goals (Bakhtiyarifar et al., 2021; Gaiero et al., 2013). Over centuries, plants have established an intimate association with diverse endophytic microbes, including bacteria, viruses, and fungi, as part of their developmental process and ecological adaptation (Hardoim et al., 2015). Endophytes live within apparently healthy plant internal tissues- roots, stems, leaves, flowers, and seeds - without causing any harm to the host, exchanging chemical compounds from physiological and metabolic processes (Varma et al., 2017). Plants constitute a unique ecological niche for endophytic microbes, while endophytic microbes impact the host's health, adaptation, disease resistance and tolerance to abiotic and biotic stresses (Bakhtiyarifar et al., 2021; Singha et al., 2021). Thus, the frequency of occurrence, community composition and diversity of endophytes are critical factors in understanding plant health, physiological state, and productivity (Vardharajula et al., 2017). Consequently, bioprospecting endophytic microbes with unique PGP

and biocontrol attributes offers excellent potential for the discovery of novel and competent bacterial species that may be useful in the formulation of bioinoculants for improving plant productivity.

Endophytic bacteria improve plant productivity by enhancing soil nutrient richness through N-fixation, nutrient mineralisation and solubilisation, especially of phosphorous (P) and potassium (K) elements (Bakhtiyarifar et al., 2021; Mashiane et al., 2018). Endophytes influence plant growth and biomass by producing phytohormones such as indole acetic acid (IAA) and cytokinin, which regulate plant metabolism and performance under stressful environmental conditions (Puri et al., 2018; Vardharajula et al., 2017). Different studies have revealed that plants treated with PGP inoculant had increased resistance to drought, salinity, soil acidity and pathogens (Aydi et al., 2016; Díaz Herrera et al., 2016). Endophytic bacteria may enhance plant defence mechanisms by producing metabolites such as siderophore, hydrogen cyanide (HCN), and 1-aminocyclopropane-1-carboxylate (ACC) deaminase or by competing with undesirable microbes for nutrients and host colonisation (Ali et al., 2014; Rana et al., 2020). These benefits of endophytic bacteria in sustainable agriculture have recently caused increased attention amongst agronomists and biotechnologists.

Various soil bacterial species are well-known microbial resources in bioinoculant production (Adeleke et al., 2019a; Raimi et al., 2017). Unfortunately, most exotic inoculants for improving crop productivity have been reported with low success (Herrmann et al., 2015; Raimi et al., 2019) due to soil microbial complexity and the challenges of integrating non-resident bacterial species into already established communities (Forchetti et al., 2007). A major ecological risk associated with bioinoculant is the disruption of soil microbiome diversity and functioning caused by the foreign strains, which generate invasive and competitive responses (Soumare et al., 2020). However, one strategy that may help overcome this challenge is to harness efficient PGP endophytic bacteria that have established beneficial associations with the host plants (Gaiero et al., 2013; Passari et al., 2016). Thus, agronomists should now consider endophytic

microbes as an essential agent for improving plant growth and health, suggesting the need to explore more endophytes for biotechnological applications.

Endophytic bacteria are ubiquitous and exist within all plant species investigated up to date (Santoyo et al., 2016). Several agronomically important crops, including maize, rice, soybean, pepper, sugarcane and a host of others, have been well studied for their endophytic microbes and possible applications (Hardoim et al., 2015; Jasim et al., 2013; Rai et al., 2007). However, endophytic bacteria of vegetable crops remain underexplored, especially for their PGP attributes, creating a setback for their application in bioinoculant production. Therefore, this study investigated the endophytic bacteria of vegetable crops to discover microbial candidates with efficient PGP capability. In addition, the study intends to improve the understanding of how endophytic bacterial colonisation, diversity and community composition differed across vegetable species, organs and geographical locations. Aside from providing insights into the potential PGP ability of the recovered bacterial endophytes, this study reveals possible bacterial candidates useful for developing bioinoculant formulations.

5.2 Materials and Methods

5.2.1 Study location and sampling of plant materials

The sampling sites were two vegetable farms located in Potchefstroom, North West Province (26°42'55.0"S 27°04'59.6"E) and Betrams, Gauteng Province (26°11'40.1"S 28°03'58.3"E) in South Africa. The geographical location and climate parameters of the farms have been described in section 3.1.1. Vegetable farming is well-practised in these provinces, with the average annual production in North West greater than that of the Gauteng province (Macaskill, 2016). The farm located in the North West province practices organic farming, while the Gauteng province practices conventional farming. The Gauteng province farms practise conventional farming, using chemical inputs, including fertilisers, pesticides and fungicides, whereas the farms in the North West province practise mainly organic farming, utilising plant and animal waste as major agricultural inputs (see section 3.1.1). For data analysis, the former

was designated as farm S and the latter as farm J. Vegetable farming is a major agricultural production in the two provinces, with higher production in the North West compared with the Gauteng province (Macaskill, 2016). Cultivation of vegetables, such as beetroot, carrot, cabbage, lettuce, onions, and spinach has been ongoing for over 2 years on the farm.

Sampling was conducted in the summer (October- February) of 2021. Based on visual inspection, healthy cabbage (*Brassica oleracea*), lettuce (*Lactuca sativa*) and spinach (*Spinacia oleracea*) plants were sampled from the farms as earlier discussed in section 3.1.1. Five replicates of each vegetable were randomly selected and carefully uprooted with a sterile trowel without damaging the tissues. The samples were packed in well-labelled sterilised zip lock bags and transported to the laboratory on ice packs without compromising the biological properties. Tissue washing, sterilisation, and microbial cultivation were done within 48 h.

5.2.2 Sterilisation procedure for eliminating non-endophytes

Vegetable tissues were sterilised to eliminate epiphytes and non-endophytes following a modified protocol of McKinnon (McKinnon, 2016). Briefly, the samples were washed under running tap water to remove sticky dirt and humus on the tissue surfaces. The tissues were cut into fragments (~10 cm) with a sterile scalpel, placed in a permeable container, and rinsed in freshly prepared Tween 80 (0.05% v/v) to break the surface tension and disperse air bubbles. While ensuring total immersion of tissues during incubation, the sterilisation followed the procedure in Supplementary Table 5-1. The sterilised tissues were air-dried under a laminar flow chamber and stored at -20°C for further analysis. The efficiency of the tissue sterilisation process was tested by plating the water from the last rinse on a Tryptone Soy agar (TSA) and incubating at 30°C for 3-5 days. The absence of microbial growth on the agar plates confirmed efficient sterilisation.

5.2.3 Endophytic bacterial cultivation, isolation, and enumeration

For isolation and enumeration, the sterilised tissues were macerated with a sterile mortar and pestle. Ten-fold serial dilution was made using 1 g of macerated tissues diluted up to 10^{-8} in 9 ml

of sterile saline solution (0.85% w/v). A known volume of each dilution was plated on a TSA or NA medium. Tissue fragments (~ 2-3 cm) were plated to calculate the colonisation rate. Single colonies were counted after 48-72 h of incubation at $30 \pm 2^{\circ}\text{C}$, and morphologically distinct colonies were selected, purified and aseptically transferred to fresh Tryptone Soy Broth (TSB) in McCartney bottles.

5.2.4 DNA isolation, PCR-based fingerprinting and molecular identification

Total genomic DNA was extracted from a 24 h bacterial colony grown on TSA medium using the microwave method described in Raimi et al. (2020). The partial 16S rRNA gene (v3-v4 region) of the bacterial DNA was amplified in a polymerase chain reaction (PCR) using the universal primer set 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTAC GACTT-3'). The amplification was performed in a T100™ (BioRad, USA) thermal cycler with a 25 µl reaction volume containing 12.5 µL of one Taq 2x Master Mix with Standard Buffer (New England, Biolabs Inc. USA), ~50 ng DNA template, 10 µM each of the forward and reverse primers and nuclease-free PCR-grade water to a final volume. The thermocycling conditions were initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 sec, annealing at 53°C for 30 sec and extension at 72°C for 1 min and final extension of 72°C for 1 min. The amplicon integrity and sizes were analysed by electrophoresis using 1% agarose gel in 1× TBE buffer. The gel was run at 80 V for 45 min and visualised with a Gel Doc (BioRad Laboratories, USA).

5.2.5 PCR product clean up and Sanger sequencing

The PCR products were first cleaned using Agencourt AMPure XP beads (Beckman Coulter, Life Sciences, USA) and 80% ethanol and eluted with 10 mM Tris. Subsequently, the products were amplified using a T100™ (BioRad, USA) thermal cycler in a 20 µl reaction volume comprising 4 µl Ready Reaction Mix (Thermo Scientific, USA), 2 µl Big Dye™ (ThermoFisher Scientific, USA), 3.2 pmol of primer, ~ 30 ng template and PCR-grade water to final volume. The sequencing PCR conditions were initial denaturation at 96°C for 1 min, 25 cycles of

denaturation at 96°C for 10 sec, annealing at 50°C for 55 sec and extension at 60°C for 4 min. A second clean-up was performed on the sequencing PCR product using CleanSEQ beads (Beckman Coulter, Life Sciences, USA) and 85% ethanol following the protocol by RAMAC (RAMAC, 2013).

The PCR products were sequenced in a SeqStudio™ Genetic Analyser (ThermoFisher Scientific, USA) employing the Sanger method (BigDye™ Terminator Cycle Sequencing). Sequences were analysed for quality and chimeras using BioEdit Sequence Alignment Editor software (version 7.2.5.0). Twenty-three isolates with low ammonia production were not identified. Bidirectional sequences were merged, and the contiguous sequences generated were compared with sequences in the National Centre for Biotechnology Information (NCBI) GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm (<http://blast.ncbi.nlm.nih.gov/>). Using Mothur software (version 1.35.1), sequences were clustered into 21 OTUs, and the most similar relatives ($\geq 97\%$ similarities) to the OTU representatives were mined from the database. A phylogenetic tree was constructed in Molecular Evolutionary Genetics Analysis (MEGA) software version 11 (Tamura et al., 2021).

5.2.6 Functional characterisation of recovered bacterial endophytes

Endophytic bacteria were evaluated for PGP potential, including N-fixation, P-solubilisation and production of IAA, siderophore, HCN and hydrolytic enzymes (amylase, cellulase, gelatinase, lipase and protease). The N-fixing ability was evaluated by growing the endophytes in Nitrogen-free bromothymol blue (Nfb) medium (5.0 g DL-malic acid, 0.5 g K₂HPO₄, 0.2 g MgSO₄·7H₂O, 0.1 g NaCl, 0.02 g CaCl₂·2H₂O: 2 mL bromothymol blue solution (0.5% in 0.2N KOH), 4.0 mL Fe (III) EDTA (1.64%), 1.0 mL vitamin solution (contains 0.1 g/L biotins and 0.02 g/L pyridoxine), and 1 L distilled water, pH 6.8) for 4 days at 30°C. The NH₄⁺ produced indicates the isolate's N-fixing potential and was evaluated using the indophenol blue colourimeter method (Keeney and Nelson., 1982). Endophytes that can solubilise P were identified using the Pikovskaya medium. The formation of clear halos around colonies after incubation for 7 days at 30°C is a positive result for P-solubilising ability (Jayakumar and Natarajan, 2013). Quantitative

analysis of soluble phosphate produced by the isolates in the National Botanic Research Institute's phosphate (NBRIP) medium was measured using the molybdenum blue technique (Deaker et al., 2011).

The production of IAA was evaluated using the Salkowski reagent (2 ml 0.5 M FeCl₃, 49 mL 70% HClO₄ solution, and 49 mL of sterile distilled H₂O) following the method of Szymańska et al. (2016). For siderophore production, the method described by Loudon et al. (2011) using colour indicator hexadecyltrimethylammonium bromide (HDTMA) and Chrome azurol S (CAS) was employed. The coefficient of activity (W_{act}) was determined using the formula $W_{act} = S_h^2 / (S_c \times t)$, where S_h - diameter of hydrolysis zone, S_c - colony diameter, and t - time of incubation (Hryniewicz et al., 2010). The ACC deaminase production was screened using sterile minimal DF (Dworkin and Foster) salts media following the method described by Penrose and Glick (2003). Test isolates were inoculated on the DF salts media plates and incubated for 3 days at 28 °C. Isolates growing on the plates are positive for ACC deaminase production. Hydrocyanic acid production was determined using nutrient agar amended with glycine [0.44% (w/v)]. A Whatman filter paper no. 1 saturated with a 2% sodium carbonate in a 0.5% picric acid solution was placed in the Petri dish upper lid and sealed with parafilm. An uninoculated plate was used as the control. After incubation, the filter paper with deep-orange to brown colouration is positive for HCN production (Ali et al., 2014).

Furthermore, endophytic bacteria were assessed for their ability to synthesise hydrolytic enzymes — amylase, cellulase, lipase, gelatinase and protease. Amylolytic and cellulolytic enzymes were evaluated following the method of Gopinath et al. (2005). Lipase activity was assessed using lipase medium (10 g peptone, 5 g NaCl, 0.1 g CaCl₂ · 2H₂O, 20 g) containing 1% Tween 20 as substrate (Jayakumar and Natarajan, 2013). A visible residue around the colonies due to complete degradation of the fatty acid salt confirms lipase activity. For gelatinase, Nutrient agar and 8% gelatin (Sigma, USA) solution sterilised separately were added in a ratio of 20 to 1. Inoculated plates were incubated at room temperature for 48 -72 h. A clear zone around the colony is positive for gelatinase production. Proteinase activity was evaluated on

Mueller-Hinton agar supplemented with 3% (w/v) Skimmed milk (Oxoid, UK). The paracasein precipitation zone around the colonies within 48-72 h is positive for proteolytic activity (Kannan et al., 2015).

5.2.7 Statistical analyses

Except otherwise stated, all the data obtained were analysed in R software version (3.6.2) (R Development Core Team, 2018). The endophytic bacterial colony count and PGP traits data were presented as mean values of replicate results with their standard deviation. Data variables were checked for normality using the Shapiro-Wilk normality test. If the data did not meet homogeneity of variance criteria, they were subjected to the nonparametric tests; otherwise, the parametric tests were used to determine statistically significant differences between variable group means. Pairwise comparisons were analysed using Tukey's Honest Significant Differences (Tukey HSD) post hoc test to examine the significantly different groups at $P < 0.05$. Species composition similarity was analysed with the Jaccard dissimilarity index and Sørensen index (S) using the Estimate S Win version 9.1.0 (Colwell and Elsensohn, 2014). The alpha diversity indexes, including Shannon, Simpson and Chao1, were analysed using the vegan package in R studio. A Venn diagram was constructed to show species occurrence and shared across the vegetable samples and farm locations (Heberle et al., 2015). The colonisation frequency was evaluated using the number of fragments with at least a colony on the growth medium divided by the number of fragments analysed.

5.3 Results

5.3.1 Frequency and viable count of cultivable endophytic bacteria across the tissues and location

A total of 105 morphologically distinct endophytic bacteria were recovered from the vegetables: cabbage, lettuce and spinach (Figure 5-1). More endophytic bacteria were isolated from the

lettuce compared to the cabbage and spinach in both farm locations (Supplementary Table 5-2). The total endophytic bacteria from farm S were 49, with lettuce and spinach accounting for 35% and 33%, respectively. In farm J, the same number of isolates were recovered from lettuce and spinach, while cabbage had the least, about 25% of the total recovered isolates. The number of isolates recovered from the organs was highest in the stem tissues, followed by the root and the leaf across the farms.

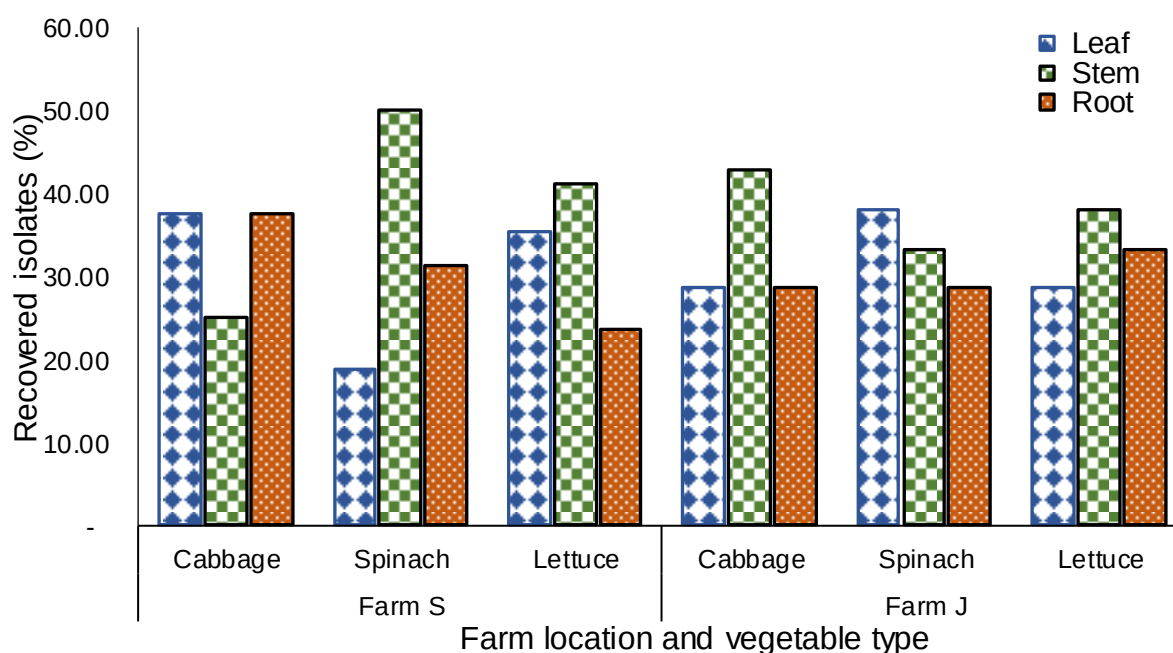


Figure 5-1: Endophytic bacteria of vegetable crops across farm locations. The endophytic bacteria were recovered from the leaf, stem and root tissues of cabbage, spinach and lettuce from farm S and J .

The endophytic bacterial population in the vegetables ranged from 3×10^4 to 4×10^7 CFU g⁻¹ across the farms (Table 5-1). The bacterial population for cabbage ranged from 3×10^4 to 59×10^5 CFU g⁻¹, while that of spinach ranged from 3×10^4 to 6×10^6 CFU g⁻¹. Lettuce had the highest bacterial count, ranging from 12×10^4 to 4×10^7 CFU g⁻¹ across the farms. The mean bacterial cell count was significantly ($P < 0.05$) different across farm locations (Supplementary Figure 5-1) and vegetable species (Supplementary Figure 5-2). In farm S, there were more bacterial cells across the vegetable leaf tissues except for cabbage, which had more count in the stem tissues (Supplementary Figure 5-3). In contrast, vegetables in farm J had a high bacterial cell count in the stem tissues except for lettuce, with a high count in the root tissues

(Table 5-1). The endophytic bacterial cell counts were not significantly ($P > 0.05$) different across the organs. However, the combined effect of plant species and organs were significantly ($P < 0.05$) different (Figure 5-3).

Table 5-1: Total recovered and viable cell count of endophytic bacteria in vegetables (x 10^4 CFU/g)

Vegetable organs	Farm S			Farm J		
	Cabbage	Lettuce	Spinach	Cabbage	Lettuce	Spinach
Recovered isolates	16	17	16	14	21	21
Leaf	32 ± 1.0	4000 ± 11.0	602 ± 8.6	16 ± 2.0	12 ± 1.7	8 ± 2.0
Stem	598 ± 7.2	82 ± 2.6	21 ± 2.0	81 ± 3.0	16 ± 2.0	13 ± 2.0
Root	17 ± 2.0	98 ± 2.6	15 ± 1.7	3 ± 1.0	405 ± 4.4	3 ± 1.0

Viable cell count is presented as the mean of triplicate data with standard deviation ($\pm$ SD). The isolates are the total morphologically distinct bacterial endophyte bacteria from each plant.

5.3.2 Phylogenetic analysis, endophytic bacterial identification, and operational taxonomic units

The identified endophytic bacteria belonged to four major classes: Actinobacteria, Bacilli, Betaproteobacteria and Gammaproteobacteria, comprising 15 core genera and representing 39 dominant species (Table 5-2). The genera and species composition differed across the vegetable species and organs. Proteobacteria, followed by Firmicutes and Actinobacteria, were the most abundant phyla. *Pseudomonas* was the most predominant genera in nearly all the vegetable species and organs in farm S but was exclusively found in lettuce from farm J (Supplementary Table 5-2). *Bacillus* was the second most abundant genera, mainly found in farm J, followed by *Pantoea*, recovered in vegetables from both farms (Table 5-2). *Staphylococcus* and *Robertmurraya siralis* were only detected in lettuce roots from farm J. Similarly, *Enterobacter* and *Comamonas* recovered from cabbage, and *Robertmurraya siralis* and *Leclercia* mainly found in lettuce and spinach, respectively, were found in farm J. *Serratia* were primarily found in the cabbage stems and lettuce roots in farm S (Supplementary Table 5-2).

Table 5-2: Taxonomic classification of endophytic bacteria recovered from vegetable crops

Phylum	Class	Genus	Species in farm J	%	Accession	Species in farm S	%	Accession
Actinobacteria	Actinobacteria	<i>Glutamicibacter</i>	<i>G. mysorens</i>	99.7	OL635003	<i>G. uratoxydan</i>	100	OL634984
		<i>Microbacterium</i>	<i>M. paraoxydans</i>	100	OL634985	<i>M. liquefaciens</i>	100	OL635005
Firmicutes	Bacilli	<i>Bacillus</i>	<i>B. cereus</i>	100	OL634993			
			<i>B. haynesii</i>	100	OL897283			
			<i>B. mycoides</i>	98.5	OL634994			
			<i>B. pumilus</i>	98.5	OL634996			
			<i>B. nakamurai</i>	100	OL634995			
			<i>B. safensis</i> subsp. <i>safensis</i>	100	OL634997			
			<i>B. velezensis</i>	100	OL634998			
			<i>B. subtilis</i>	100	OL897282			
			<i>Robertmurraya siralis</i>	100	OL897284			
		<i>Priesta</i>				<i>P. megaterium</i>	100	OL860938
		<i>Staphylococcus</i>	<i>S. cohnii</i>	100	OL635014			
Proteobacteria	Betaproteobacteria	<i>Comamonas</i>	<i>C. terrigena</i>	99.7	OL634999			
	Gammaproteobacteria	<i>Acinetobacter</i>				<i>A. haemolyticus</i>	100	OL634983
		<i>Enterobacter</i>	<i>E. bugandensis</i>	100	OL635000	<i>E. cloacae</i>	100	OL860939
			<i>E. roggenkampii</i>	100	OL635001			
			<i>E. sichuanensis</i>	98.9	OL635002			
		<i>Leclercia</i>	<i>L. adecarboxylata</i>	100	OL635004			
		<i>Pantoea</i>	<i>P. agglomerans</i>	99.6	OL635006	<i>P. conspicua</i>	100	OL860940
			<i>P. eucalypti</i>	99.1	OL635007			
			<i>P. eucrina</i>	100	OL635008			
			<i>P. vagans</i>	99.8	OL635009			
		<i>Pseudomonas</i>	<i>P. agarici</i>	99.7	OL635010	<i>P. gessardii</i>	100	OL634987
			<i>P. azotoformans</i>	100	OL634986	<i>P. kairouanensis</i>	99.8	OL634988
			<i>P. atacamensis</i>	99.4	OL635011	<i>P. reinekei</i>	100	OL634989
			<i>P. syringae</i>	99.6	OL635012	<i>P. vancouverensis</i>	100	OL634990
		<i>Raoultella</i>	<i>R. terrigena</i>	100	OL635013			
		<i>Serratia</i>				<i>S. liquefaciens</i>	100	OL634993
		<i>Stenotrophomonas</i>	<i>S. maltophilia</i>	99.6	OL635015	<i>S. maltophilia</i>	100	OL634991
		<i>Xanthomonas</i>				<i>X. fragariae</i>	100	OL634992

The total bacterial endophytes were clustered into 21 major OTUs and used for the evolutionary analysis of the taxa in a phylogenetic tree (Figure 5-2). The root tissues yielded 14 different endophytic bacterial species belonging to eight genera, while the leaf tissues had 17 species from four genera. The stem tissues had more species composition, with 18 bacterial species from 13 genera (Supplementary Table 5-2).

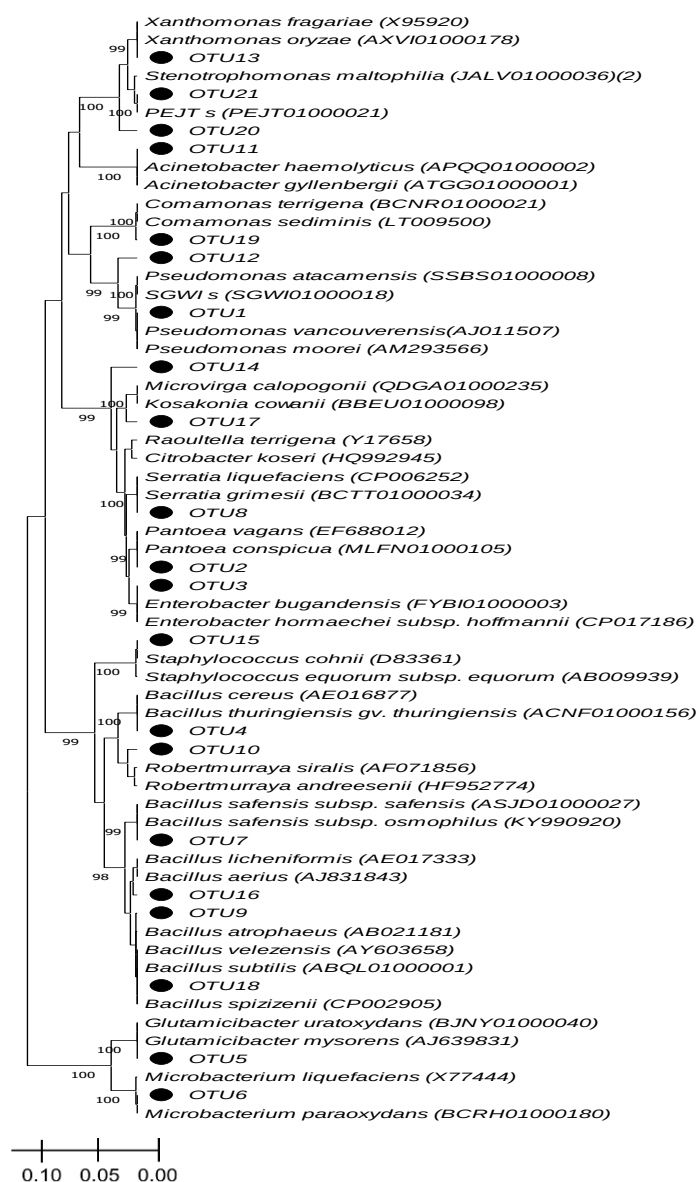


Figure 5-2 Phylogenetic tree showing evolutionary relationships of endophytic bacterial taxa. The evolutionary history was inferred using the unweighted pair group method with the arithmetic mean (UPGMA). The percentage of the replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown below the branches. Evolutionary distances were computed with the Kimura 2-parameter method and are in the unit of the number of base substitutions per site. The site variation rate was modelled with a gamma distribution (shape parameter = 5). This analysis used the pairwise deletion option, involving 61 nucleotide sequences with 305 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al, 2021).

Lettuce had the highest abundance of recovered endophytic bacteria across the farms, while cabbage with seven genera and 14 species had the least endophytic bacterial abundance.

Stenotrophomonas maltophilia was the only species common to the two farms. However, at the genus taxa level, *Glutamibacter*, *Microbacterium*, *Stenotrophomonas*, *Pseudomonas* and *Pantoea* were common to both farms.

The vegetables from farm J had a higher number of OTUs compared to farm S (Figure 5-3A), with the highest number of OTUs observed in spinach (10 OTUs), followed by lettuce and cabbage with 8 and 6 OTUs, respectively. There are no shared OTUs amongst the three vegetables from farm J, but at least one unique OTU existed between two vegetables. In farm S, two OTUs were unique to all the vegetables, while one OTU was shared between cabbage and lettuce (Figure 5-3B). The total number of OTUs observed for lettuce and cabbage was considerably similar for both farm locations. However, there was a considerable difference in the number of OTUs observed for spinach (farm S = 10 and J = 21) across the farms (Figure 3C).

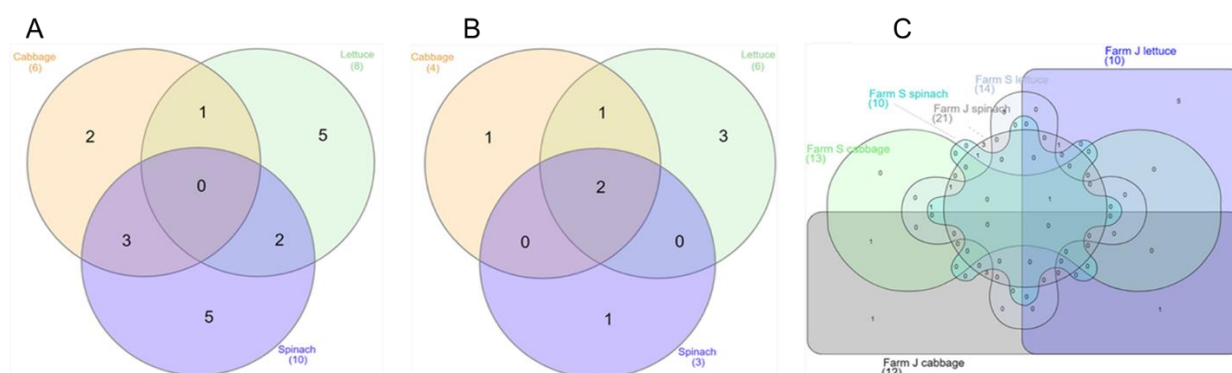


Figure 5-3 Venn diagram showing Operational Taxonomic Units (OTUs) composition of recovered endophytic bacteria in vegetables. (A) farm J and (B) farm S, and (C) farm J and S. The vegetables investigated are cabbage, lettuce and spinach. Values in brackets under each vegetable name are the total number of OTUs recovered from each vegetable. The InteractiVenn was used for constructing the Venn diagram (Heberle et al, 2015).

5.3.3 Endophytic bacterial colonisation and diversity

The endophytic bacterial colonisation rate was at a maximum (100%) in all the assessed tissues. Each plated tissue (~36) produced at least a colony on the media. The Jaccard similarity (J) and Sørensen (S) indices for species composition comparison were of the same trend for the farm locations (Supplementary Table 5-3 and 5-4). The calculated Jaccard similarity index was relatively low, ranging from 4.5% to 33.30%. The maximum similarity was between lettuce and spinach from the same farm (Supplementary Table 5-3). Spinach and lettuce from farms J and S had somewhat species composition similarity ($J = 4.8\%$). Among the vegetable organs, the spinach stem in farm J had the highest diversity indices for Simpson, Shannon-Wiener and Chao1 (Table 5-3). The lowest species diversity was in spinach stem from farm S, with the least Shannon and Simpson indices values. Chao1 was very low in cabbage root from farm J and in spinach and cabbage stem from farm S (Table 5-3).

Table 5-3: Diversity indices of endophytic bacteria of vegetable crops from two farm locations

Diversity index	Farm J									Farm S								
	Spinach			Lettuce			Cabbage			Spinach			Lettuce			Cabbage		
	Leaf	Stem	Root	Leaf	Stem	Root	Leaf	Stem	Root	Leaf	Stem	Root	Leaf	Stem	Root	Leaf	Stem	Root
Total species	8	7	6	6	8	7	4	6	4	3	5	8	6	4	7	6	6	4
Shannon	1.24	1.95	1.49	1.04	1.04	1.10	1.10	1.01	0.64	1.04	0.56	0.69	1.33	1.04	1.33	1.04	0.63	1.39
Simpson	3.0	7.0	4.0	2.7	2.7	3.0	3.0	2.6	1.8	2.7	1.6	2.0	3.6	2.7	3.6	2.7	1.9	4.0
Chao 1	7.0	28.0	6.5	3.5	3.5	6.0	6.0	3.0	2.0	3.5	2.0	3.0	5.5	3.5	4.3	3.5	2.0	10.0

5.3.4 Functional diversity

All the functional traits assessed were not statistically significant (ANOVA, $P > 0.05$) across the vegetable types, organs, and locations, except for siderophore and soluble PO_4 production, which were influenced by the farm location (Supplementary Table 5-5). The most predominant biological functions were IAA and soluble PO_4 production, exhibited by 90% and 91% of the endophytes, respectively.

5.3.4.1 Indole acetic acid production and phosphate solubilisation potential

The IAA obtained ranged from 0.14 ± 0.1 to 44.48 ± 2.9 $\mu\text{g/mL}$ and was higher amongst isolates from spinach leaf in both farms (Supplementary Table 5-2). In farm S, *Stenotrophomonas maltophilia* had the highest amount of IAA (33.14 $\mu\text{g/mL}$), followed by *Pseudomonas conspicua* (21.84 $\mu\text{g/mL}$) and *Pseudomonas azotoformans* (18.29 $\mu\text{g/mL}$), whereas in farm J, *Bacillus cereus* produced the highest amount of IAA (33.14 $\mu\text{g/mL}$) followed by *Robertmurraya spiralis* (14.91 $\mu\text{g/mL}$) and *Enterobacter bugandensis* (12.60 $\mu\text{g/mL}$) (Supplementary Table 5-2). Irrespective of the farm location, vegetable type and organ, there were no significant ($p > 0.05$) differences in the IAA production amongst the endophytes. About 52% of the endophytic bacteria could solubilise P and produced soluble PO_4 in the range of 3.35-38.78 mg/L across the vegetable type, organ and farm location. The soluble PO_4 produced by isolates from the stems ranged from 3.35-38.78 mg/L; the root ranged from 7.17–33.40 mg/L, while the leaf endophytes had between 7.27–36.25 mg/L. Amongst the genera, *Serratia*, followed by *Pseudomonas*, *Bacillus*, *Pantoea*, *Enterobacter*, *Stenotrophomonas*, *Microbacterium*, *Raoultella* and *Robertmurraya* exhibited high PO_4 solubilisation ability (Supplementary Table 5-2). About 55% of the endophytic bacteria in farm J had an average of 30 mg/L soluble PO_4 produced. In contrast, though farm S had a higher percentage of isolates with high solubilisation traits, the average soluble PO_4 produced was below 20 mg/L. No significant ($P > 0.05$) differences were observed for

soluble PO₄ concentrations across the vegetable types and organs, except for farm location (Supplementary Table 5-5).

5.3.4.2 Nitrogen fixation and ammonia production

Amongst the endophytic bacteria recovered, only 62% displayed N-fixing potential, with *Bacillus* and *Pseudomonas* genera showing a higher potential. Ammonia production was highest in *Pseudomonas*, *Bacillus* and *Pantoea*. *Pseudomonas azotoformans* from farm S produced a high amount of NH₄⁺ (Supplementary Table 5-2). In farm J, *P. atacamensis* from lettuce had the highest amount of NH₄⁺ (93.24 mg/L). Other bacterial genera, including *Bacillus*, *Glutamicibacter*, *Pantoea*, *Serratia*, *Acinetobacter* and *Xanthomonas*, also produced a relatively high amount of NH₄⁺ above 35 mg/L (Supplementary Table 5-2). However, the farm location, vegetable types and organs had no significant ($p > 0.05$) effect on NH₄⁺ production of the endophytic bacteria (Supplementary Table 5-5).

5.3.4.3 Siderophore, ACC deaminase and hydrogen cyanide production

Some of the endophytic bacteria, about 30%, possess high siderophore production ability (Figure 5-4A). The amount of siderophore produced was higher for isolates in lettuce and spinach from farm S compared to those of farm J. *Pseudomonas* showed high siderophore production potential across both farms. In farm S, *P. azotoformans* and *Stenotrophomonas maltophilia* had the highest siderophore production (39.67 mm), followed by *Serratia liquefaciens* (38.33 mm), *Pantoea conspicua* (24.67 mm), and *Glutamicibacter uratoxydans* (18.3 mm) (Supplementary Table 5-2). In farm J, *Bacillus mycoides* and *B. cereus* had ~ 27 mm, while *B. safensis* subsp. *safensis*, *Enterobacter bugandensis* and *Pseudomonas atacamensis* had below 21 mm. Siderophore production amongst the endophytic bacteria was significantly different across the farm location, while vegetable types and organs had no significant effect (Supplementary Table 5-5). The coefficient of activity was evaluated for seven high siderophore-producing strains over 84 h period. Though *Serratia liquefaciens*

had the most increased activity between 12 to 60 h, *P. azotoformans* maintained the highest activity after 84 h (Figure 5-5).

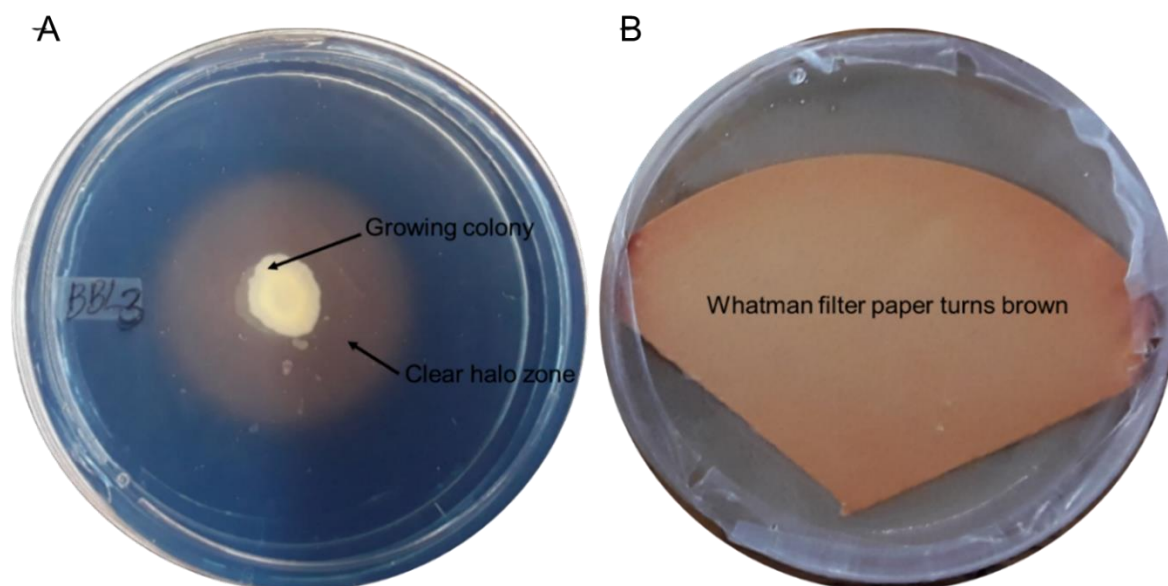


Figure 5-4: Siderophore and hydrogen cyanide production by endophytic bacteria. (A) CAS blue agar plate showing siderophore activity of bacterial endophytes. The iron-chelating agent, siderophore, produced by the growing endophytic bacteria, binds to the iron in the media and creates an iron-free clear halo (B) Glycine supplemented agar plate showing HCN production by the endophytic bacteria. The brown or deep orange colouration of the Whatman filter paper indicates the HCN production by the test isolates due to their ability to utilise glycine

The ACC deaminase-producing isolates were 32% of the recovered endophytic bacteria. Compared to farm J, the endophytic bacteria producing ACC deaminase were more in Farm S, mainly from the plant roots, especially lettuce. The genus *Bacillus*, *Enterobacter*, *Pantoea*, *Pseudomonas* and *Serratia* could produce ACC deaminase. For HCN production, only *Pseudomonas agarici*, *Enterobacter bugandensis* and *Robertmurraya siralis* from farm J were positive for this trait as most endophytic bacteria could not metabolise the glycine supplemented media (Figure 5-4B).

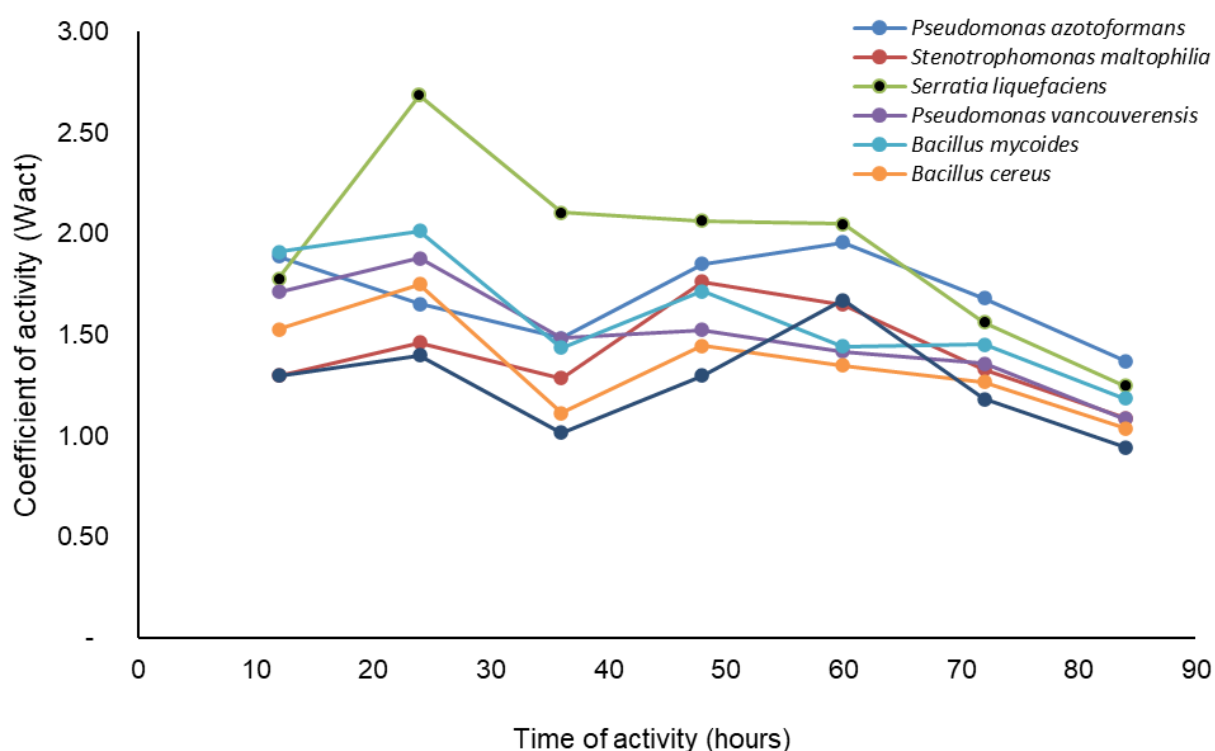


Figure 5-5 Coefficient of activity amongst siderophore-producing endophytic bacteria. The coefficient of activity was evaluated over 84 hours of incubation and was calculated from the mean of triplicate values of the halo zones on the CAS blue agar

5.3.4.4 Hydrolytic enzyme production

There were more amylolytic and lipolytic producing endophytes in vegetables from farm J compared to farm S, and most of these isolates had high enzyme activity (Supplementary Table 5-2). About 66% and 47% of the endophytic bacteria showed amylolytic and lipolytic activity, respectively. Only 21% of the isolates was positive for gelatinolytic and 44% for proteolytic activity. Most lipase and protease-positive isolates were recovered from the stem tissues and mainly from lettuce in farm J. On the contrary, the amylase and gelatinase-positive isolates were predominantly from the leaf and root tissues, respectively and were highly prevalent in cabbage and lettuce. Cellulase activity was not common among isolates; only 24% of endophytic bacteria could produce cellulase. These isolates were mostly found in cabbage from Farm J.

5.4 Discussion

Bioprospecting for beneficial microbes in diverse environments has been a key strategy for discovering novel and efficient bacterial strains to drive the production of quality microbial inoculant, a major resource in developing sustainable agroecosystems. Interestingly, several environments such as the rhizosphere, soil, and composts (Adeleke et al., 2019b; Ahkami et al., 2017; Raimi et al., 2022), have been well studied for ecologically beneficial microbes. However, niches, including plant tissues, are rarely investigated for their phytobeneficial microbes, such as endophytes. The plant growth-promoting (PGP) traits expressed by the endophytes are of great importance in selecting efficient bacterial candidates for inoculant production (Bakhtiyarifar et al., 2021). Thus, this study explores the endophytic bacterial communities of three vegetable species, primarily focusing on strains with efficient PGP traits, an excellent resource in developing bioinoculant formulations.

One of the major challenges in endophytic microbial research is the epiphytic propagules, which often associate intimately with the plant surface, penetrating superficial epithelial layers without truly becoming endophytes (Mckinnon, 2016; Sahu et al., 2022). The interference of viable and nonviable propagules or epiphytes was eliminated using surface sterilisation, allowing only the true endophytic microbes to be isolated (Muzzamal et al., 2012; Waheeda and Shyam, 2017). Various sterilisation methods have been used to recover endophytes depending on host type, organ and age (Liotti et al., 2018; Mckinnon, 2016). The efficiency of the methods is critical, as this affects the diversity and community composition of the endophytes (Sahu et al., 2022). Similar to other studies, a good sterilisation efficiency was achieved, and true endophytic bacteria were isolated (Gaiero et al., 2013; Marag and Suman, 2018). Several studies emphasized that colonisation frequency, population density and functional diversity of endophytic bacteria are influenced by the plant type, organ and location (Kandel et al., 2017; Knief et al., 2010). The highest number of endophytic bacteria was recovered from the stem tissues, contrary to other studies that reported a high number from the leaf (Yadav et al., 2016) and root tissues (Liotti

et al., 2018). However, a different trend was observed for bacterial colony count. The leaf tissues had a higher average bacterial density (8×10^6 CFU g⁻¹) compared to the stem and root tissues. Though this was inconsistent with the range 2×10^4 to 5×10^9 (CFU g⁻¹) reported for endophytes from the leaf in previous studies (Elbeltagy et al., 2001; Karthik et al., 2017), the challenges of culture-dependent techniques used in this study may have contributed to the variations and the low abundance of the recovered endophytes. Aside from being laborious and time consuming, microbial cultivation technique can only access about 1% of the total bacterial communities in the microbial world (Salmonová and Bunešová, 2017), suggesting the need for a more inclusive approach such as culture independent technique, e.g., next-generation sequencing (NGS), which can access more microbial diversity with sizeable coverage (Kulski, 2016).

Under favourable conditions, the rhizosphere bacteria may eventually transform and gain entry through the root to become true endophytes (Ahkami et al., 2017), contributing to the high cell density of endophytes within root tissues. A higher endophytic bacterial population has been reported in the root than in other organs of *Oryza sativa* L. (Hameed et al., 2015) and *Zea mays* (Marag and Suman, 2018). On the contrary, this study showed that roots generally had a low bacterial density compared to the stem and the leaf tissues, similar to other reports (Etesami et al., 2014; Moronta-barrios et al., 2018). Plant age affects microbial composition and structure, which in turn influence bacterial density and colonisation frequency (Marag and Suman, 2018; Wagner et al., 2016). Due to the same plant age and size investigated, the colonisation rate obtained was similar across the tissues. Endophytic bacterial structure are largely shaped by the anatomical and nutritional composition of the host and the specificity and adaptability of the endophytes (Gaiero et al., 2013; Liotti et al., 2018). The stem nutritional components such as starch, sugar and xylem sap influence microbial growth, suggesting the high density of endophytic bacteria reported in the stem. Unlike the roots hidden in the soil, leaves are unprotected against the direct effect of various abiotic stresses, including temperature fluctuation, irregular humidity, high ultraviolet

radiation and limited nutrient levels (Dastogeer et al., 2020). Adaptation to these variations may adversely alter microbial community composition, causing the low diversity of endophytes in the leaves (Jin et al., 2014; Liotti et al., 2018). Moreover, geographical location shapes plant endophytic bacterial composition by defining growth conditions, such as soil nutrients, climate, humidity and moisture, that considerably affect plant development (Karthik et al., 2017; Knief et al., 2010). Similarly, this study showed that farm location significantly ($P < 0.05$) affects the endophytic bacterial community population.

Plant species are defined by the variations in their genetic makeup, which affect metabolite production (Papik et al., 2020). Plant metabolites such as sugar, phenolic, and amino acids act as signalling molecules, inducing potential endophytes to colonise plants through wounds, hydathodes, and stomata (Ahkami et al., 2017; Hardoim et al., 2015). Thus, the varying endophytic bacterial diversity and composition in this study due to the different vegetable species examined is predictable. In addition, there were fewer shared species than unique species across the vegetables, suggesting the plant type affects the structure of endophytic bacteria. Studies have reported the impact of host species and genotype variations on taxonomic composition across plant species (Karthik et al., 2017), such as in cultivars of sweet potato (Marques et al., 2015), maize (Marag and Suman, 2018), banana (Karthik et al., 2017) and Guarana (Liotti et al., 2018).

The taxonomic classification reveals endophytic *Bacillus*, *Enterobacter*, *Pseudomonas*, *Pantoea*, and *Serratia* were the most prevalent genera across the vegetable tissues. These genera have been reported in various vegetables, including baby spinach, green leaf lettuce (Jackson et al., 2013), carrot, cucumber, tomato (Khalaf and Raizada, 2016; Nithya and Babu, 2017) and young radish (Seo et al., 2010). These endophytes constitute the common rhizobacteria of agronomic importance. Many rhizosphere bacteria penetrate and colonise the root tissues to become endophytes (Sturz and Nowak, 2000), suggesting a taxonomically similar root endophytic bacteria and rhizobacteria. However, our result suggests otherwise, the phyllosphere had more endophytic bacterial species compared to

the roots. PGPR and endophytic *Microbacterium* and *Pseudomonas* with high PGP ability have been reported (Ahemad and Kibret, 2014; Zinniel et al., 2002). Although endophytic bacteria could be Gram-positive or Gram-negative, belonging to Proteobacteria, Firmicutes or Actinobacteria, the Gram-negative largely contributed more to the endophytic bacteria in the vegetables (Etesami et al., 2014; Seo et al., 2010). Similar to other studies, Actinobacteria, Bacilli, Betaproteobacteria and Gammaproteobacteria were the most predominant classes (Hryniewicz et al., 2010; Jackson et al., 2013; Puri et al., 2018), which have also been reported to possess efficient PGP ability. Taxonomic classification of bacteria are widely studied using the hypervariable regions of the 16S rRNA gene (Liotti et al., 2018; Parthasarathy et al., 2018). For this study, the V3-V4 regions were employed due to their reported maximum nucleotide heterogeneity and maximum discriminatory power for bacterial classification (Graspeuntner et al., 2018).

Similar to other rhizobacteria, recovered endophytic bacteria such as *Bacillus*, *Pseudomonas* and *Serratia* participate in ecosystem functions and play key roles in plant growth and development through biogeochemical cycling, nutrient solubilisation and phytohormone synthesis (Pirhadi et al., 2018; Vardharajula et al., 2017). The production of IAA, siderophore and NH_4^+ were prevalent amongst the endophytic bacteria (Mburu et al., 2021). The phyllosphere endophytes had higher IAA and PO_4 concentration compared to the root endophytes, which had higher NH_4^+ and siderophore production. According to Haidar et al. (2018), *Bacillus* is prolific in IAA production, while *Pseudomonas* is efficient in siderophore and NH_4^+ production. A similar range of IAA (up to 33 $\mu\text{g/mL}$) produced by the endophytic bacteria in this study has been reported (Etesami et al., 2014). ALKahtani et al. (2020) also observed a high IAA production for endophytic *Bacillus* (above 40 $\mu\text{g/mL}$). IAA promotes plant root architecture and elongation and contributes to plant-microbe interaction. Patten and Glick (2002) showed that Canola seeds cultivated with IAA-producing strains had longer root lengths than the control. Interestingly, IAA production is associated with bacterial N-fixing ability, suggesting why many of the endophytic bacteria producing IAA also

produced high amounts of NH_4^+ , contributing to soil nitrogen content (Defez et al., 2017). Siderophore-producing endophytes improve iron uptake and suppress pathogen activities in their host through iron sequestration and competition (Passari et al., 2016). Studies have shown that siderophore-producing endophytes enhance seed germination, tissue development and root biomass in crops compared to the control (Etesami et al., 2014; Hameed et al., 2015). Thus, plant inoculation with siderophore-producing endophytic bacteria can improve crop yield, especially under iron-deficient soil.

Furthermore, plants mitigate stress through ethylene production; however, when in excess, it is harmful to plant, causing leaf chlorosis, abscission and senescence (Penrose and Glick, 2003). The ACC deaminase of associated bacteria can transform plant ACC —ethylene precursor— to ammonia and α -ketobutyrate instead of ethylene, thereby regulating ethylene production (Glick, 2014). Endophytic bacteria, including *Bacillus*, *Enterobacter*, *Pseudomonas* and *Serratia*, have been widely reported to produce ACC deaminase (Jasim et al., 2013; Khalaf and Raizada, 2016). Apart from PGP functions, endophytes play crucial roles in ecosystem sustainability and biocontrol of phytopathogens through hydrolytic enzymes and HCN production (Liotti et al., 2018; Seo et al., 2010). Cellulase, amylase and protease improve soil quality (Das and Varma, 2011) and are key factors in initiating endophytic colonisation (Rana et al., 2020). Lipase-producing endophytes contribute to the biodegradation of organic pollutants and plant nutrient supply (Bhandari et al., 2021; Das and Varma, 2011). HCN is a highly toxic agent and suppresses pathogens by hindering the cytochrome oxidase pathway. Endophytic *Pseudomonas* and *Enterobacter* producing HCN have been reported to show strong antagonism against root pathogens such as *Sclerotium rolfsii* and *Ralstonia solanacearum* (Aydi et al., 2016; Vardharajula et al., 2017).

5.5 Conclusion

This study revealed that vegetable crops are colonised by diverse beneficial endophytic bacteria, including *Pseudomonas*, *Bacillus*, *Enterobacter*, *Pantoea* and *Serratia*. These endophytes possess various PGP traits, including P-solubilisation, N-fixation and production

of IAA, siderophore, HCN and ACC deaminase, indicating their potential contributions to ecosystem functions and sustainable agricultural production. In addition, geographical location influences endophytic bacterial community density. The results suggest endophytic bacteria, especially *Pseudomonas azotoformans*, *Serratia liquefaciens*, and *Bacillus mycoides*, with efficient PGP ability, present an essential resource for the development of a single or a consortium bioinoculant formulations for improving crop productivity. Although *in vitro* assays provided rapid screening of efficient strains, further assessment of PGP capabilities of the bacterial endophytes in the greenhouse is necessary to at first ascertain the level of effectiveness of the organisms before the field experiment. The greenhouse experiment allows for growing the plants under controllable conditions, which supports the effectiveness of the PGP endophytes. Thus, the next chapter describes the plant growth promotion of selected recovered bacterial endophytes in a greenhouse experiment.

5.6 References

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CHAPTER 6 :

ASSESSMENT OF PLANT GROWTH-PROMOTING CAPABILITY OF BACTERIAL ENDOPHYTES

6.1 Introduction

Organic and conventional fertilisers are widely used for improving soil nutrient content with a major aim of promoting crop productivity. However, long-term application and poor management of these agro-inputs have resulted in various ecological challenges (Hernandez et al., 2021; Ngetich et al., 2012). Conventional fertilizer application is associated with soil quality damage, environmental pollution, loss of soil microbial diversity, and water body contamination (Savci, 2012; Thorat and More, 2022). The management of organic fertilisers is a major concern in agroecosystems. Studies have reported the incidences of antibiotic-resistant microbes and genes in agricultural soils through the application of animal manure, posing a major health risk to both animals and humans (Adegoke et al., 2016; Scaccia et al., 2021). Additionally, the high cost and limited availability of these agro-inputs make them inaccessible, especially to the less privileged smallholder farmers who are regarded as the backbone of the agricultural economy in many developing nations (Raimi et al., 2017). Thus, developing strategies to address challenges related to organic and conventional fertilizer application is a major goal in sustainable agriculture. A relatively new and Innovative technique that has gained much attention among agronomists is microbial technology, which involves the application of microorganisms (Compant et al., 2010; Singh et al., 2016).

Over the years, biotechnologists have extensively studied and harnessed plant-microbe interactions to improve plant productivity and nutritional quality (Berg et al., 2014b; Kumar et al., 2019). The mutualistic association between plants and their associated microbes is beneficial to both parties, with considerable impact on host plant growth and health, while the host plant provides a safe niche and chemical substances to the microbes (Hardoim et

al., 2015). Endophytic microbes are a unique group of plant microbiomes that inhabit plant tissues for at least a part of their life cycle without causing any harm to the host plant (Tadych and White, 2019). Most endophytes contribute to global element cycling, nutrient solubilization, enzyme production and stress tolerance of plants (Raimi and Adeleke, 2023; Sahoo et al., 2017). They also promote plant growth through the production of several plant hormones, such as auxin, gibberellin, ethylene, and cytokinin (Chaudhary et al., 2022) and by mobilising insoluble soil nutrients, e.g., phosphate, fixing nitrogen and synthesizing various substances that act as biocontrol agents against phytopathogens (Raimi and Adeleke, 2023). Endophytic microbes perform various physiological activities within host plant tissues, which enhance their capability to compete with pathogens inhabiting the same ecological niches, signifying their potential in the biocontrol of plant pathogens (Chaudhary et al., 2022; Hu et al., 2017; Vurukonda et al., 2018). Thus, bacterial endophytes are viable PGP agents that have huge applications in agroecosystems as an inexpensive, eco-friendly and sustainable technology to enhance crop productivity (Adeleke et al., 2019; Raimi et al., 2017).

Despite the numerous benefits of endophytes, their potential in agroecosystems has not been fully realized, suggesting the need to intensify research on endophytes to promote their use in the production of inoculants, which can enhance crop productivity. Endophytes are a better resource in inoculant technology compared to non-endophytes due to their compatibility, reinfection ability, and colonisation patterns with the host plants, as well as the complexity of their interactions, which have huge ecological benefits (Chaudhary et al., 2022; Wani et al., 2015). Previous studies have investigated the PGP ability of microbial inoculants on economically important crops such as maize, sunflower, and wheat (Forchetti et al., 2007; Mashiane et al., 2018). However, despite the rising demand and health benefits of vegetable crops, they are less investigated, consequently presenting a research gap that needs to be addressed to improve sustainable vegetable production.

Leafy vegetables are an important part of the human diet, mostly eaten raw as salad without any form of thermal processing that can alter their microbiological quality (Ülger et al., 2018). While vegetables, such as spinach, carrot, beetroot, cabbage, lettuce and onion have various human health benefits, including reduction in obesity and lowering of high blood pressure due to their high nutrient density (Kumar et al., 2020), their endosphere is regarded as an important reservoir for emerging opportunistic pathogens (Al-Kharousi et al., 2016), suggesting the assessment of their microbiological quality should be considered a priority. Interestingly, vegetables have been implicated in foodborne disease outbreaks in many parts of the world. For example, *Listeria monocytogenes*, a major threat to the food industry, has been reported in South Africa and the United States (CDCP, 2015; Tchatchouang et al., 2020). This pathogen is the causative agent of several outbreaks of listeriosis worldwide and has been reported as an endophyte of vegetable crops (Kljujev et al., 2018). According to Berg et al. (2014), bacterial endophytes, including *Pseudomonas*, *Enterobacter*, *Acinetobacter*, *Serratia*, *Staphylococcus* and *Bacillus* have bivalent associations with the host plant, contributing to plant growth promotion and biocontrol of pathogens (Adeleke et al., 2019a; Raimi and Adeleke, 2023) and causing diverse diseases when they colonise human organs (Berg et al., 2014a). Therefore, it is crucial to assess the pathogenicity and virulence potential of endophytes to gain full insight into their importance in agroecosystems and human health management (Kljujev et al., 2018).

This study aims to investigate the PGP ability of bacterial endophytes on vegetables in a pot experiment. It further assessed the antibacterial, antibiotic susceptibility and virulence potential of bacterial endophytes using selected antibiotics and cytotoxic activities on the HuTu-80 human epithelial cell line ((American Type Culture Collection (ATCC): HTB-40™). The study hypothesised that the bacterial endophytes could colonise plant tissues with differential effects on seed germination rate and plant growth. To confirm successful colonisation, confocal microscopy was used to visualize the bacterial endophytes in the plant tissues. Confocal microscope systems in combination with the fluorescence protein gene

have been used in studying plant-microbe interactions to determine the ecological niche of endophytes after inhabiting the host plants (Hu et al., 2017; Li et al., 2017). In addition, this study may provide insights into how microbial inoculum can improve vegetable productivity, thereby contributing to sustainable agriculture. The results of this study may unravel the potential of leafy vegetables to spread antimicrobial-resistant bacteria and improve the characteristics profile of the investigated endophytes, which is important for making key decisions in sustainable agriculture and human nutrition.

6.2 Materials and Methods

6.2.1 Bacterial endophytes with efficient plant growth-promoting abilities

The isolation and screening of PGP bacterial endophytes used in the present study have been performed and reported in a previous study (Raimi and Adeleke, 2023). However, due to the importance of endophytic bacteria in human nutrition through the microbiological quality of vegetables, this study further assessed the virulence potential of the endophytes using antibiotic susceptibility, cytotoxicity and hemolytic testing. Based on *in vitro* assessment of PGP attributes and 16S rRNA identification, four efficient bacterial endophytes: *Bacillus cereus*, *Pseudomonas azotoformas*, *Enterobacter bugandensis* and *Serratia liquefaciens*, which have potential to improve seed germination, plant productivity and agroecological performance through N-fixation, phosphorus solubilization and production of siderophore, hydrogen cyanide, and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (section 5.3.4) were selected and used for further study in the pot experiment. These bacterial endophytes were also confirmed not to possess any virulence ability.

6.2.2 Bacterial endophyte culture

Pure colonies of the recovered bacterial endophytes and test organisms: *Escherichia coli*, *Serratia marcescens*, *Candida krusei*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, and *Micrococcus luteus* were freshly grown on nutrient agar plates and inoculated in a Mueller

Hinton broth (Biolab, Ca, USA) overnight at 36 °C in an incubator with shaking at 100 rpm and stopped during log phase. The freshly grown culture was adjusted to 0.5 McFarland standard (1.5×10^8) or the turbidity was adjusted to an OD of 0.1 with a spectrophotometer at 600 nm using MHB. These cultures were used for the antibacterial, antibiotic-susceptibility and cytotoxicity testing.

6.2.3 Antibiotic susceptibility

The Kirby-Bauer disk diffusion method was employed for the antibiotic-susceptibility testing of the bacterial endophytes following the protocol described by Hudzicki (2012). Four classes of antibiotics were used in this study based on their mechanisms of action. These included 1) cell wall biosynthesis inhibition: ampicillin (10 µg), amoxicillin (10 µg), cefazolin 30 µg and vancomycin (30 µg). 2) bacterial protein synthesis inhibition: gentamicin (10 µg), tetracycline (30 µg) and streptomycin (10 µg) (30S rRNA subunit inhibitor) and chloramphenicol (30 µg), erythromycin (15 µg) and clindamycin (15 µg) (50S rRNA subunit inhibitor). 3) DNA synthesis inhibition: ciprofloxacin (5 µg) and 4) folic acid synthesis inhibition: trimethoprim 25 µg (Etebu and Arikekpar, 2016). Antibiotic disks (6 mm diameter) (Biolab, South Africa) were placed on Mueller-Hinton agar plates inoculated with the bacterial endophytes for 24 h at 37 °C. Inhibition zones were measured and compared with standards to classify the isolates as either susceptible, intermediate or resistant to antibiotics.

6.2.4 Haemolytic and extracellular enzyme production by bacterial endophytes

Haemolytic enzyme production was tested using 5% sheep blood agar (Biolab, MA, USA) and incubated at 37°C. A clear zone or green coloration around the colonies indicates beta and alpha haemolytic activities, respectively. The DNase agar (Merck, South Africa) containing pancreatic digest of casein 15 g, papaic digest of soybean meal 5g, sodium chloride 5g, deoxyribonucleic acid 2g and agar 15 g, pH 7.3 ± 0.2 at 25 °C was composed following manufacture's instruction. The plates were flooded with 1N HCl and a clear zone

around the colonies or streak is positive, while no colour change is a negative result. The result was read immediately within 5 minutes of adding 1N HCl.

6.2.5 Crude extract bacterial endophyte for minimum inhibitory concentration

Following the method of Anto et al. (2014), 100 ml of tryptic soy broth (TSB) (Biolab, USA) was inoculated with a single colony of the bacterial cells and incubated at 36°C in the dark for 24h. These cells were later transferred into a 400 ml sterile medium and incubated for 96h at 36°C with shaking at 200 rpm. The OD was monitored at intervals by measuring a 10-fold dilution of 10 ml at 600 nm. At a constant OD, the culture was centrifuged to separate the culture fluid from the bacterial cells at 10 000 x g for 20 min at 4°C. The supernatant was filtered through a 0.45 µm filter to obtain a cell-free culture filtrate. The mixture was neutralised with conc. hydrochloric acid and extracted with an equal volume of ethyl acetate and chloroform. The organic phase was dried in a vacuum drier. A stock solution of 0.020 g bacterial extracts was dissolved in 1 ml of dimethyl sulfoxide to make a 20 mg/ml concentration. A serial dilution was made using Mueller Hinton broth as the diluent to obtain a dilution of 128 to 0.5 mg/ml. A 96-well plate was filled with 100µl of the test organisms and 100µl of the different concentrations of the crude extracts. Streptomycin antibiotic and dimethyl sulfoxide were used as the positive and negative control, respectively. The plates were incubated at 37°C in a plate reader for 24h. After incubation, 10µl of 0.02% (w/v) of resazurin sodium salt solution was added to each well as an indicator of bacterial cell growth. The plate was further incubated for 2h at 37°C. A blue colouration indicates growth inhibition while a pinkish colour indicates bacterial growth.

6.2.6 Cytotoxicity assay

6.2.6.1 Cell maintenance

Cytotoxicity assay was performed on recovered bacterial endophytes following the methods described by Prinsloo et al. (2013). HuTu-80 human intestinal cells (HTB-40™) obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA) were first cultured

in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, Darmstadt) supplemented with 10% foetal bovine serum (FBS) (Thermo Scientific, USA) and maintained in a humidified incubator, with 5% CO₂ at 37°C. Cells were handled in a sterile laminar flow chamber disinfected with 70% ethanol and UV light for 15 min (Prinsloo et al., 2013). These cells are genetically modified to never stop growing and do not have all the constituents of primary cells. However, they are a good model for assessing the toxic potency of chemicals or organisms as a first-tier screening for cytotoxicity.

6.2.6.2 Cytotoxicity measurement using the xCELLigence cell analyser system

The cytotoxic potential of the endophytic bacteria on the human intestinal cell line was determined using a real-time cell analyser, the xCELLigence system RTCA single plate (SP) instrument from ACEA Biosciences with RTCA software (version 1.2.1). This software records the electrical impedance that flows across micro-electrodes at the bottom of each well of the gold-plated E-plate. The growth of Hutu-80 cells is measured in real-time and converted into cell index (CI) values. Cells were seeded at a density of 8.0×10^4 cells/mL and left to adhere for 24 hours. The cell line was exposed to 10 µL of different bacterial endophyte broths (equal cell concentration 1.5×10^8) solution in triplicate. Three wells with unexposed cells received DMEM cell culture medium only and acted as the control. Impedance readings were recorded every 10 min for 186 hours. The cytotoxicity effects of bacterial endophytes were calculated based on the time taken for the HuTu-80 cells to significantly decrease. The isolates that showed significant effect with shorter periods are considered more virulent.

6.2.7 Seed germination and viability test

This study used spinach because it is easy to cultivate and grows to maturity within a short period. It is also well cultivated using the greenhouse method and requires less technicalities or expertise. Spinach seeds were procured from Die Blomwinkeltjie Florist (Pty) Ltd., Potchefstroom, South Africa and their viability was assessed as described by (Davies et al.,

2015). Briefly, distilled water and filter papers (Whatman no 1) were sterilized by autoclaving. Seeds were surface sterilized following the method of McKinnon (2016), using a combination of 5% v/v sodium hypochlorite and 0.05% v/v Tween 80 and 70% ethanol. Thirty seeds were placed on the filter papers moistened with equal volumes of distilled water in sterile Petri plates. The Petri plates were made in five replicates, placed in a container lightly closed with a plastic overlay to reduce moisture loss, and incubated at room temperature. Viability was confirmed by the radicle protruding at least 2 mm from the seed coat and germination was monitored daily to ascertain the normal growth of seedlings (Rao et al., 2006). The following equations were used for calculating the percentage of germination and viability (Kader, 2005).

$$\text{Germination (\%)} = \frac{g}{x} * 100$$

$$\text{Viability (\%)} = \frac{(g + f + a)}{x} * 100$$

Where:

g = numbers of germinated seeds,

x = numbers of sown seeds (infested and empty seeds are removed),

f = no of fresh seeds and no of abnormal seedlings

Pure colonies of the inoculum were grown overnight on nutrient agar plates before being inoculated into freshly prepared 2 litres of 15% Luria-Bertani (LB) broth (Biolab Diagnostics, South Africa) or Tryptone Soy broth and placed in an incubating shaker at 100 rpm and 37 °C. The growth was monitored to reach approximately 1.5×10^8 CFU/ml (0.5 McFarland standard). Then, the surface-sterilized seeds were soaked (submerged) in the bacterial inoculum of 1.5×10^8 CFU/ml (0.5 McFarland standard) using dilution factor on the initial density) for about 24 hours before determining the germination and growth rate. Sterilised distilled water was used as a control. The bacterial endophyte rate of germination promotion

was determined following the method of (Kader, 2005), as described by Makhaye et al. (2021). Parameters including mean germination time (MGT), germination index (GI), germination rate index (GRI), first day of germination (FDG), final germination percentage (FGP), coefficient of velocity of germination (CVG), and time spread of germination (TSG), were used to determine the germination dynamics. FGP (%) is the final number of seeds that germinated in percentage.

$$MGT = \Sigma f.x / \Sigma f$$

$$CVG = N1 + N2 + \dots + Nx / 100 \times N1T1 + \dots + NxTx \text{ GRI}$$

$$GRI = G1/1 + G2/2 + \dots + Gx/x$$

$$GI = (10 \times n1) + (9 \times n2) + \dots + (1 \times n10)$$

Where;

f = seeds germinated on day x,

N = number of seeds germinated each day,

T = number of days from seeding corresponding to N,

G1 = germination percentage on the first day after sowing,

G2 = germination percentage on the second day after sowing,

n1, n2 . . . n10 = numbers of germinated seeds on the 1st, 2nd until the 10th day; and

1 = weights given to the number of germinated seeds on the 1st, 2nd, and subsequent days.

6.2.8 Plant materials and cell transformation with plasmids.

Plasmid pLV-mCherry (36084) and pFPV-mCherry (20956) were acquired from the Addgene repository in 2022. These plasmids were gifts to Addgene from Pantells Tsoulfa (Addgene plasmid #36084) and Oliva Steele-Mortimer (Addgene plasmid # 20956.). The pLV-mCherry (36084) contains a 6782 base pair backbone and 704 base pair gene insert while the pFPV plasmid has a backbone size of 4000 base pair and an insert of 711 base pair. Both

plasmids express red fluorescence due to their gene inserts that are monomeric red fluorescence proteins with antibiotic-resistant genes for ampicillin used as selection markers. Only the plasmids that were successfully cloned into the bacterial cells were used.

6.2.9 Plasmid DNA isolation

A single colony of bacteria containing the plasmids were first grown in 15-ml tubes containing 2 ml LB broth (Biolab Diagnostics, South Africa) supplemented with ampicillin (100 µg/ml) for 24 h at 100 rpm and 37°C in a shaker incubator. Using the ZymoPURE™ Plasmid Miniprep kit (Zymo Research, CA, USA), the plasmid DNA was extracted from the bacteria and diagnostic restriction digestion was performed with *HindIII*, *NdeI*, *XbaI* and *SaI* (New England Biolabs, Germany). The plasmid's integrity and quality were verified on a 1.5% agarose gel electrophoresis and visualised in a Gel Doc (BioRad Laboratories, CA, USA).

6.2.10 Endophytic bacterial cell transformation

The bacterial endophytes were first made into competent cells following the method previously described by Chang et al. (2017). Bacterial cells were transformed with mCherry following the protocol described by (Bjornson et al., 2019; Kuduğ et al., 2019). The competent bacterial cells, cuvette, sterile microcentrifuge tubes and SOC medium were placed on ice. 40 µl of the competent cells and 2 µg of the ligation mixture (plasmids coupled with mCherry insert) were placed into the microcentrifuge tube. This mixture was pipetted into a chilled 0.2 cm electroporation cuvette and inserted into a pulser controller attached to a Bio-Rad Gene Pulser machine (Bio-Rad Laboratories, England) set to 2.5 kV/cm and 25 µF and 200 Ω. The bacterial cells were removed from the cuvette and added to SOC medium to make 1000 µl and the mixture was then incubated at 37°C on a shaker set at 250 rpm for 1 h. 100 µl of the transformants were inoculated on a pre-warmed (37°C) LB agar supplemented with 100 µg/ml ampicillin and grown at 37°C overnight. Positive transformants were picked and transferred into a new broth amended with 100 µg/ml ampicillin, incubated

overnight at 37°C with shaking at 50 rpm. The bacteria cells were stored at –80°C after adding an equal volume of 40% glycerol.

6.2.11 Screening and verification of the transformed bacterial cells

The transformed bacteria were screened using their ability to grow on freshly prepared LB agar supplemented with 100 µg/ml of ampicillin, on which the untransformed bacterial cells could not grow. The cells were screened for fluorescence expression using the Confocal imaging systems (ZEISS Microscopy, Deutschland, Germany) reconstructed with Zeiss LSM 980 with Airyscan 2 and equipped with C- PlanApochromat 63×/1.4 Oil DIC UV-Vis IR M27 objective with 32-channel detectors at the imaging facility, North-West University, Potchefstroom. Successful fluorescence gene incorporation into the bacterial cells was verified using the total genomic DNA extracted from a washed colony pellet transformants using ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research, CA, USA). Primer sequences (mCherry-F, 5'-CCCCGTAATGCAGAAGAAGA-3'; mCherry-R, 3'-TTGGTCACCTTCAGCTT GG-5') that amplify the fluorescence gene inserts of the plasmids were utilized in a polymerase chain reaction. The PCR was carried out in a 25 µl reaction consisting of 12.5 µl of one Taq 2x Master Mix with standard buffer (New England Biolabs Inc., CA, USA), 10µM each of forward and reverse primers, ~50 ng plasmid DNA template and nuclease-free PCR-grade water to final volume. The PCR conditions included initial denaturation at 95°C for 5 min, followed by 30 cycles at 95°C for 15s, 55°C for 15s and 72°C and a final extension at 72°C for 5 min.

6.2.12 Bacteria transformants and inoculum concentration

The preparation of transformed bacterial species cultures and inoculum concentrations (CFU/ml) followed the procedures already described for the untransformed bacterial cells. The bacterial cells were made to a concentration of 1.5×10^8 CFU/ml using the 0.5 McFarland standard used for the vegetable seed treatments in the pot experiment.

6.2.13 Pot trials and bacterial endophyte localization assessment

The pot experiment used two different spinach variant types (BeetFordhook Giant (SBG) and Beet Lucullus (SBL)) to study the growth-promoting ability of the bacterial endophytes. Pool sand (0.5 - 1.2 mm particle size), vermiculate and plastic pots (4 litres) were purchased from Die Blomwinkeltjie Florist (Pty) Ltd., Potchefstroom, South Africa and sterilized before use in the greenhouse. The soil was mixed with the vermiculate in a ratio of 3:1 to increase the moisture-retaining capacity of the soil. The greenhouse temperature was set at 22/18°C day/night and seeds were sown at a depth of 10 mm with approximately 7 x 30 cm spacing. Ten inoculated seeds were sown in each of the pots filled with sterile soil and watered regularly. Each of the bacterial endophytes represents a treatment (*Bacillus cereus*; treatment 1 (T1), *Pseudomonas azotoformas*; treatment 2 (T2), *Enterobacter bugandensis*; treatment 3 (T3) and *Serratia liquefaciens*; treatment 4 (T4), while uninoculated seeds treatment 0 (T0) served as the control. The experiment was made in four replicates and arranged in a randomized Complete Block Design (RCBD). The untransformed bacterial endophytes were used to compare the impact of the transformation on the PGP ability of the bacterial endophytes, while also understanding the localization of the bacterial endophytes in the infected plant to give further insight into how bacteria and host interaction promote host growth. The vigour index and disease incidences were also evaluated.

Vigour index = [(mean root length (cm) + mean shoot length (cm)) x percentage of seed germination]

Disease incidence = (No of infected plants ÷ No of total plants) x 100.

The localization of bacterial endophytes in the infested plant was assessed using red fluorescence-protein (RFP) tagged bacterial endophytes and visualisation with a confocal microscope. The plant leaves were analysed by digital imaging using a confocal microscope. To further verify the infection of the inoculum, a PCR was done using the primer specific to the fluorescence gene regions in the plasmids. DAPI staining was also performed on the

transformed bacteria. The stock solution of DAPI (4',6-diamindino-2-phenylindole) (Thermo Scientific, Ca, USA) was diluted to 300 nM and added to the transformed bacteria that had been washed in a phosphate buffer solution. The DAPI solution and transformed bacteria cells were incubated for 3-5 min, washed again about 2-3 times in PBS and fixed on imaging slides from microscopy.

6.2.13.1 Determination of chlorophyll contents

A 0.5 g fresh leaf sample was macerated in a mortar with 20 ml of 80% acetone and kept chilled over ice while processing under low luminosity. The mixture was centrifuged for 10 min at 3000 rpm in a microcentrifuge (Thermo Scientific, Ca, USA) and the supernatant was removed and made up to 50 ml using 80% acetone. The mixture was covered with aluminium foil and incubated for 30 min in a fridge in the dark. The solution was diluted appropriately and the colour intensity of the green pigment was measured with a spectrophotometer (Thermo Scientific, Ca, USA) at 645 nm for chlorophyll a and 663 nm for chlorophyll b and 652 nm for total chlorophyll. The results of a, b and total chlorophyll were expressed in mg/g.

6.2.13.2 Evaluation of seed germination, vigour index and fresh and dry weight

The inoculated seeds were sown in different pots with at least 10 seeds per pot and after 7 days, seed germination percentages and the vigour index were evaluated for each treatment following the method described by Abdul-Baki and Anderson (1973, in Sharma, 2018). The plants were harvested after two months of growth in the greenhouse and the yield and yield contributing parameters such as the plant height, root length, number of leaves, plant fresh weight, and dry weight and disease incidence were measured. To determine the dry weight, harvested plant samples were cut into pieces, placed in foil containers and oven-dried at 65°C for 2 days until a constant weight was achieved. The leaf chlorophyll a and b content was quantified using the methods of Pérez-Patricio et al. (2018). Vigor index was calculated as shown above.

6.2.13.3 Visualisation of the red fluorescence protein-tagged endophytic bacterial cells in spinach tissues

Microscopic observations of bacterial transformants and the colonised plant tissues were captured using a confocal microscope (ZEISS Microscopy, Deutschland, Germany). The plant tissues were cut into pieces (10 mm x 10 mm), fixed on a microscopic slide using Eukitt Quick Mounting Media (Thermo Scientific, USA) and allowed to stand for two hours before microscopic visualization. Bacterial cells were excited at 594 nm and emission was detected between 587–610 nm. Autofluorescence within the leaves were excited with the 405 nm diode laser and the emission was detected between 436–484 nm.

6.2.14 Data collection and statistical analysis

The cytotoxicity data obtained after the exposure period was normalised using the RTCA software. Normalisation was done by the manipulation of data at a specific time point (start of the test compound treatment) which is then set as 1.0 by the software. Vegetable yield and germination data were analysed for normality using the Shapiro-Wilk test and if data were not normally distributed and cannot be normalized using log or square root transformation, nonparametric tests such as the Wilcoxon Rank sum test and Kruskal-Wallis test were used, otherwise, parametric tests such as two-sample t-test and ANOVA with P value considered at < 0.05 . For significant values, the post hoc analysis of the means was performed with Tukey's multiple comparisons.

6.3 Results

6.3.1 Hemolysis and DNase enzyme activity

Of the total bacterial endophytes tested for virulence properties, only four and seven bacterial endophytes were beta and alpha-haemolytic, respectively (Table 6-1). Nine of the endophytes were also positive for the DNase test, an indication that the bacteria could produce DNase enzyme for deoxyribonucleic acid hydrolysis (Table 6-1).

Table 6-1: Bacterial endophyte hemolytic and DNase enzyme production

Sample code	Bacterial endophytes	Hemolysis	DNase
B34	<i>Acinetobacter johnsonii</i>	γ	–
BLIN10	<i>Arthrobacter citreus</i>	α	–
J12B	<i>Bacillus cereus</i>	γ	–
J30B	<i>Bacillus haynesii</i>	γ	–
J1	<i>Bacillus mycoides</i>	γ	+
J38	<i>Bacillus nakamurai</i>	γ	–
J34	<i>Bacillus pumilus</i>	α	+
B19	<i>Bacillus subtilis</i>	γ	–
J11B	<i>Bacillus subtilis</i>	γ	–
B55	<i>Cytobacillus solani</i>	α	–
J35	<i>Enterobacter bugandensis</i>	γ	–
B65	<i>Lysinibacillus pakistanensis</i>	γ	–
B51	<i>Lysobacter soli</i>	α	+
J33	<i>Microbacterium paraoxydans</i>	β	–
J2	<i>Microvirga calopogonii</i>	α	+
B57	<i>Neobacillus bataviensis</i>	β	+
BF66	<i>Paenibacillus amylolyticus</i>	α	+
S20	<i>Pantoea conspicua</i>	γ	–
S2	<i>Priestia megaterium</i>	β	+
J21	<i>Pseudomonas atacamensis</i>	γ	–
J20	<i>Pseudomonas atacamensis</i>	γ	+
S31	<i>Pseudomonas azotoformans</i>	γ	–
S6	<i>Pseudomonas fluorescens</i>	γ	–
T29	<i>Pseudomonas syringae</i>	α	–
B48	<i>Rhizobium zeae</i>	γ	–
S29	<i>Serratia liquefaciens</i>	γ	–
J22	<i>Staphylococcus cohnii</i>	β	+
S50	<i>Stenotrophomonas maltophilia</i>	γ	–

6.3.2 Antibiotic sensitivity test

Of the 18 bacterial endophytes tested for antibiotic susceptibility, three were resistant to at least one antibiotic from the four classes with cell wall biosynthesis, bacterial protein synthesis, DNA synthesis and folic acid metabolisms (Table 6-2) as revealed by the zone of inhibition (Figure 6-1). Eight bacterial endophytes were either susceptible or intermediate while *Pseudomonas azotoformans*, *Enterobacter bugandensis*, *Pantoea conspicua*, and *Serratia liquefaciens* were susceptible to most of the antibiotics. All the endophytes were

found to be susceptible to streptomycin. Resistance among the bacterial endophytes was higher for amoxicillin while they exhibited intermediate sensitivity to ciprofloxacin.

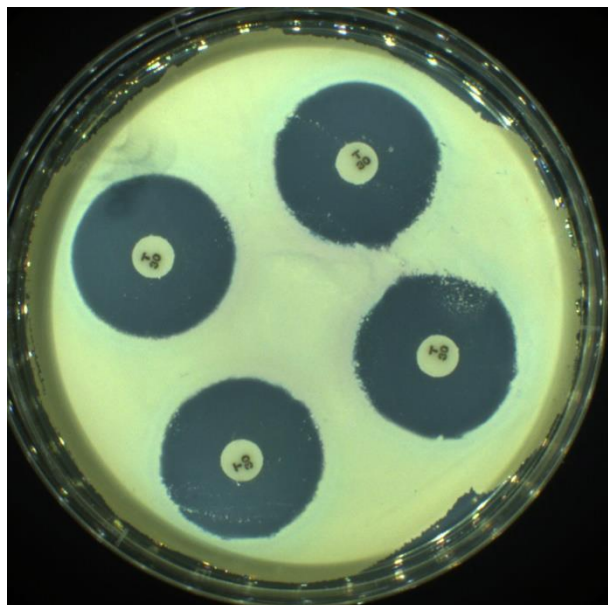


Figure 6-1: Plate showing antibiotic susceptibility with the zone of inhibition.

6.3.3 Antibacterial properties of the crude extracts

All the bacterial endophytes produced active extracts that inhibited at least one of the test organisms. The MIC of the extracts from *B. cereus* against *S. marcescens* and *E. faecalis* was 0.5 mg/mL while the extracts from *P. azotoformans* had a MIC of 2 mg/mL against *K. pneumoniae*. Both *E. bugandensis* and *P. azotoformans* showed a MIC of 0.5 mg/mL against *C. krusei*. In addition, the crude extract from *P. conspicua* and *E. bugandensis* had a MIC of 0.5 mg/mL against *M. luteus*.

Table 6-2: Antibiotic sensitivity pattern of bacterial endophytes recovered from vegetable crops

organisms	Cell wall synthesis				Protein synthesis						Folic acid	DNA synthesis	
	Amp	Van	Cef	Amo	Gen	Tet	Stre	Chlo	Ery	Cli	Tri	Cip	Class
<i>Pseudomonas azotoformans</i>	S	I	S	S	S	I	S	I	S	S	S	I	0
<i>Pantoea conspicua</i>	S	S	S	S	S	I	S	I	S	S	S	I	0
<i>Bacillus haynesii</i>	R	I	R	R	S	R	S	R	S	S	R	R	4
<i>Bacillus cereus</i>	S	S	S	S	S	I	S	I	S	S	S	I	0
<i>Bacillus mycoides</i>	R	I	R	R	S	R	S	R	I	I	R	R	4
<i>S. maltophilia</i>	R	I	S	R	S	I	S	I	S	S	S	I	1
<i>Comamonas terrigena</i>	S	I	R	R	R	I	S	I	S	I	R	R	4
<i>Paenibacillus amylolyticus</i>	S	S	S	S	S	S	S	I	S	I	S	I	0
<i>Priestia megaterium</i>	S	I	S	R	I	S	S	I	S	S	S	I	1
<i>Rhizobium zeae</i>	S	I	S	S	S	I	S	I	S	I	S	I	0
<i>Leclercia adecarboxylata</i>	S	S	S	S	S	S	S	S	S	S	S	I	0
<i>Bacillus pumilus</i>	R	I	S	R	S	I	S	S	S	R	R	S	3
<i>Bacillus subtilis</i>	S	I	S	S	S	S	S	I	S	I	R	I	1
<i>Serratia liquefaciens</i>	S	S	S	S	S	I	S	S	R	R	S	I	2
<i>Acinetobacter johnsonii</i>	R	I	R	R	S	I	S	I	S	S	R	I	2
<i>M. paraoxydans</i>	I	S	S	S	S	R	S	S	S	S	S	S	2
<i>Enterobacter bugandensis</i>	S	S	S	S	S	I	S	I	S	S	S	I	0
<i>Cytobacillus solani</i>	S	I	ND	ND	S	I	S	I	ND	ND	ND	ND	0

Degree of susceptibility: >20 mm—Sensitive; 10–19.9 mm—intermediate; 0.0–9.9 mm resistant. chlo, Chloramphenicol (30µg/disc); Gen, Gentamicin (10µg/disc); Amp, Ampicillin (10µg/disc); Van, Vancomycin (30µg/disc); Tet, Tetracycline (30µg/disc); Stre, Streptomycin (30µg/disc); Cip, Ciprofloxacin (5µg/disc); Cef, Cefazolin (30µg/disc); Tri, Trimethoprim (10µg/disc); Ery, Erythromycin (15µg/disc); Amo, Amoxicillin (20µg/disc); Cli, Clindamycin (2µg/disc). The antibiotics were classified according to their mechanisms of action.

6.3.4 Cytotoxicity of bacterial endophytes

Cell viability was monitored every 15 minutes for 76h using the exCELLigence real-time cell system. The degree of cytotoxicity was established based on the time the cell viability was significantly decreased (Mann-Whitney, $P < 0.05$). The cell viability observed for each of the test organisms was also compared with the control (unexposed cells). The endophyte that significantly reduced the viability of the HuTu-80 cells within the shortest time was considered more virulent (Table 6-1). The percentage viability of the cells before and after exposure is presented in Figure 6-2. The cell index values (CI) for the total experimental period were expressed in terms of the unexposed control cells (cells receiving DMEM only) and were expressed as percentage viability (Figure 6-2). To compensate for the effect of sterile broth, a second control was added at the same time as the bacteria.

Based on the number of hours the organisms used to inhibit growth and kill the HuTu-80 cells (Supplementary Table 6-1), the cytotoxicity of the bacterial endophytes was grouped into four categories; very high, high, medium and slow (Figure 6-2). *P. azotoformans*, *S. liquefaciens*, *B. subtilis*, *E. bugandensis*, and *R. zea* were found to have a slow cytotoxicity effect on the HuTu-80 cells compared to the control. Three of the endophytes: *Lysinibacillus pakistanensis*, *Microvirga calopogonii* and *Pantoea conspicua* had a cytotoxicity that could be described as gradual, killing the cells over a period of 20 h compared to the control (Supplementary Figure 6-1).

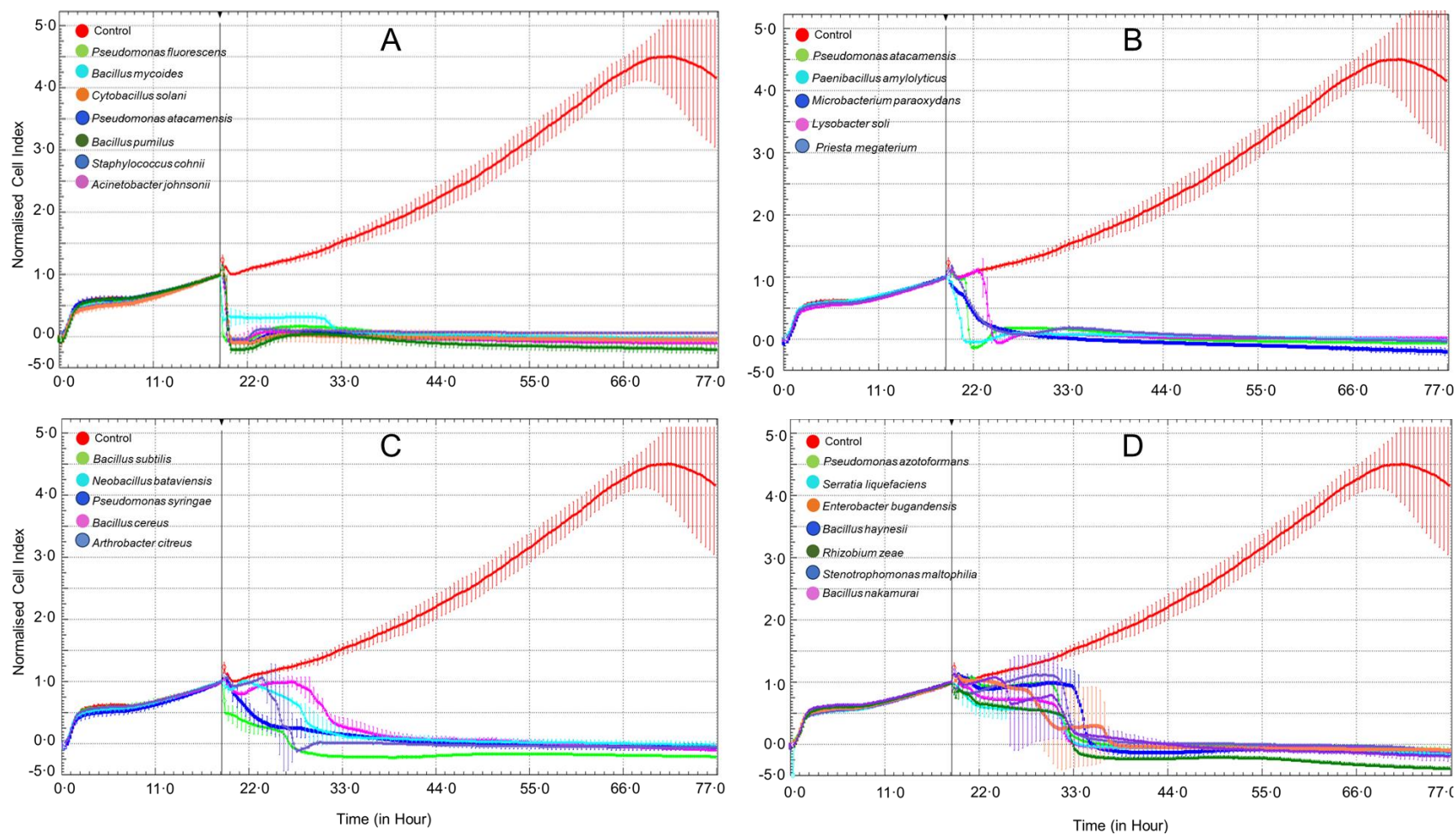


Figure 6-2:Growth curve of the human intestinal cells exposed to bacterial endophytes. The graph was drawn with the standard deviation and categorized as A) Very high, B) High, C) Medium and D) Slow cytotoxicity based on the hour inhibition and killing of HuTu-80 cells. Graphs are normalised at 19 h, i.e., the time bacterial endophytes were added to the cells. The cells died off at 76 h.

6.3.5 Bacterial endophytes improve seed germination and viability

Of the different techniques available for testing seed viability, germination testing is the most accurate and reliable (Rao et al., 2006). The results in this study reveal a high germination percentage, with over 70% mean germination percentage for the seeds of the tested plant species. In addition, the bacterial endophytes greatly influenced the plant seed germination parameters with variations among the measured parameters, including the final germination percentage, mean germination time coefficient of velocity of germination, germination index, and germination rate index (Table 6-3). *P. azotoformans*, followed by *E. bugandensis*, *S. liquefaciens* and *B. cereus*, had the highest and fastest rate of seed germination population and the highest and fastest germination rate per day. These bacteria promoted a high rate of germination and were further used in the pot experiment. For *P. azotoformans*, about 98% of seeds germinated within the first 6 days, while 95% was observed for *S. liquefaciens*. Aside from *E. bugandensis*, which germinated on the 4th day, bacteria endophytes having above 70% GI had an FDG of 3. The CVG, which shows the speed of germination did not follow the same trend, it was highest in *E. bugandensis*, while *S. liquefaciens* and *P. azotoformans* had 1.15 and 1.12, respectively (Table 6-3).

Table 6-3: Effect of bacterial endophyte inoculants on seed germination parameters

Sample	FGP (%)	MGT (day)	GI	CVG	GRI (%/day)	FDG (day)
Control	75	8.04	44.33	1.21	75	7
<i>Pseudomonas atacamensis</i>	65	6.44	59.3	0.84	65	3
<i>Acinetobacter haemolyticus</i>	55	6.64	48.0	0.73	55	4
<i>Bacillus cereus</i>	85	6.42	78.0	1.09	85	3
<i>Pantoea conspicua</i>	50	7.77	32.0	0.78	50	7
<i>Pseudomonas azotoformans</i>	98	5.68	105	1.12	98	3
<i>Enterobacter bugandensis</i>	85	6.88	70.0	1.17	85	4
<i>Serratia liquefaciens</i>	95	6.07	94.0	1.15	95	3
<i>Bacillus haynesii</i>	60	8.03	36.0	0.96	60	6
<i>Glutamicibacter mysorens</i>	55	7.39	39.67	0.81	55	5

FGP; final germination percentage, MGT; mean germination time, GI; germination index, CVG; coefficient of velocity of germination and GRI germination rate index.

6.3.6 Bacterial endophyte transformation and microscope visualisation

Bacteria transformants containing the RFP-tagged endophytes produced red fluorescence colonies and were visualized with the confocal microscope (Zeiss LSM 980 with Airyscan 2, Germany). Red fluorescence signals were detected for the RFP-tagged bacterial transformants (Figure 6-3A). Similarly, a blue and red fluorescence was observed when the RFP-tagged transformants were stained with DAPI (Figure 6-3B). The untransformed control cells did not have fluorescence. The red fluorescence expression was detected in the bacterial cells at an excitation wavelength of 587 nm and emission wavelength of 610 nm.

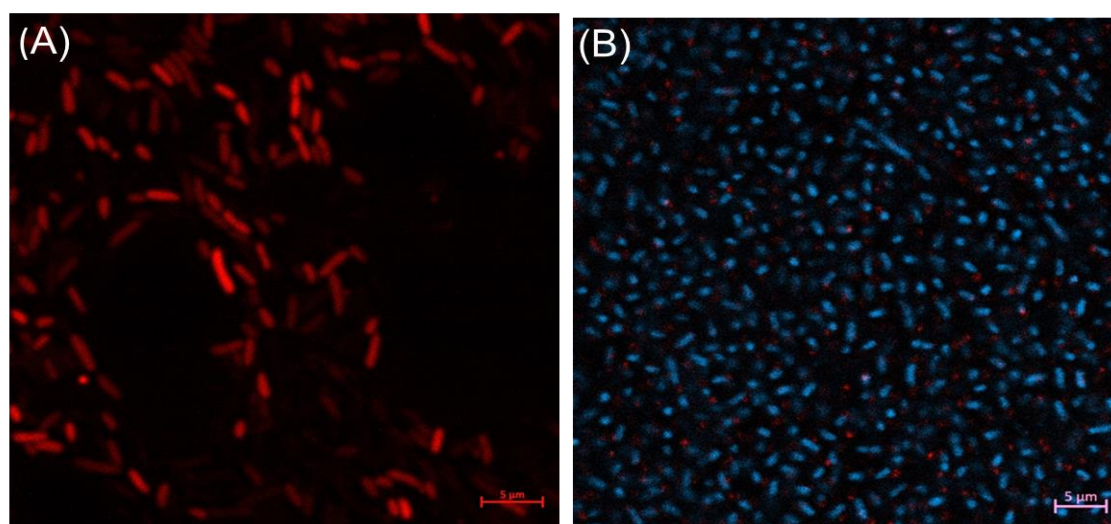


Figure 6-3: Confocal microscopy image of bacterial endophytes. (A) Fluorescence micrograph (Zeiss LSM 980) showing red fluorescent protein of bacteria with pLV-mCherry plasmids after incubation in Lauria-Bertani medium for 24 h. (B) Bacteria transformants stained with DAPI (4',6-diamindino-2-phenylindole) showing blue and red fluorescence. The scale bar is 5 µm.

The presence of the pLV-mCherry plasmids in the bacterial cells was confirmed by gel electrophoresis analysis of the RFP-mCherry gene (Figure 6-4) after PCR amplification. The transformants are resistant to ampicillin.

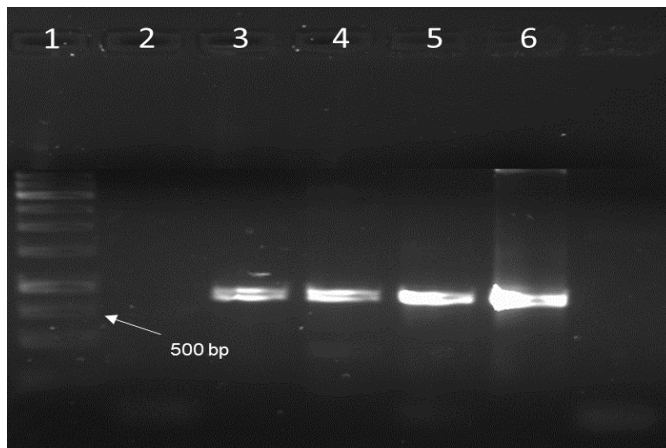


Figure 6-4: Endophytic bacterial pLV-mCherry plasmid in gel electrophoresis. Lane 1; DNA ladder (1 Kb), 2; control and 3-6; mCherry gene. **Microscopic observation of colonised plant tissues**

The microscopic assessment using a confocal laser scanning microscope revealed that the bacterial endophytes colonised the leaf tissues (Figure 6-5). The confocal microscope transmitted light allowed the visualisation of the plant tissues, while the bacterial transformants were detected by the fluorescent light. As revealed in Figure 6-5, the plant tissues had a high colonisation frequency across the two types of spinach crops (Figures 6-5 A and B), while as expected, the control experiment that had no bacterial inoculum showed no colonisation (Figure 6-5C). Additionally, the figures showed a section where stomata were clearly visible with evidence of colonisation establishment.

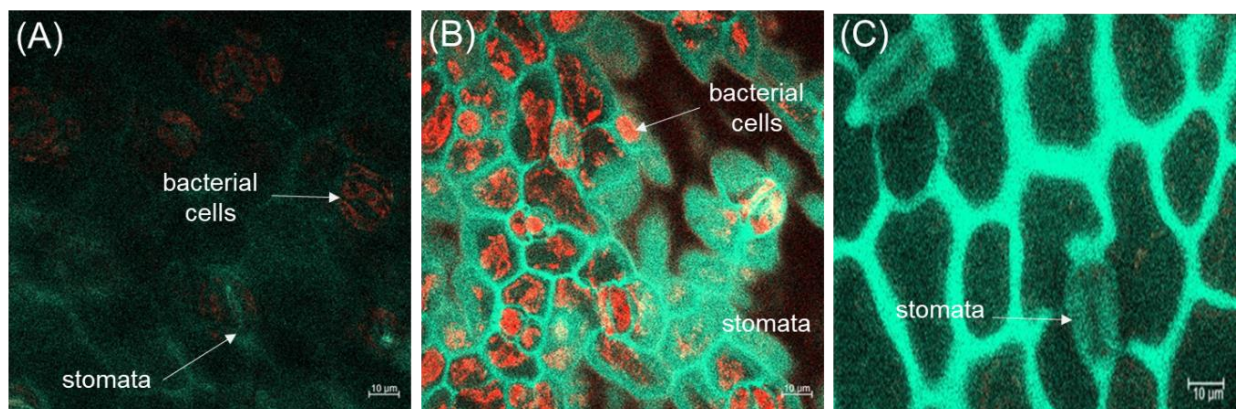


Figure 6-5: Confocal microscopy image showing the red fluorescence of the bacterial endophytes containing pLV-mCherry plasmids colonising the spinach plant tissues. (A) leaf tissue (top side) (B) leaf tissue (below side) (C) Uninoculated plant tissue.

6.3.8 Evaluation of plant growth-promotion of endophytic bacteria

All the bacterial endophytes used in the pot experiment showed a higher ability to promote the growth of spinach compared to the control (Figure 6-6A-E) with varying growth effects. Although the endophytic bacteria enhanced the growth parameters for both types of spinach, the plants treated with *P. azotoformans* showed considerable growth parameters and particularly enhanced the yield in SBG samples when compared with the control and the SBL spinach samples (Table 6-4). Root length, shoot number, number of leaves (Table 6-4), fresh weight and dry weight (Figure 6-7) were significantly ($P < 0.05$) higher in plants inoculated with *P. azotoformans* compared to the control. In the SBL samples, *B. cereus* caused a higher level of shoot length and the number of leaves compared to the control and other bacterial endophytes. Interestingly, the total chlorophyll was highest (78.97) among plants inoculated with *E. bugandensis* and *S. liquefaciens*, despite having lesser growth parameters compared to *P. azotoformans* and *B. cereus*. The dry weight was observed to be significantly highest (13.3 ± 0.89) in SBG plants (Figure 6-7) inoculated with *E. bugandensis* followed by *P. azotoformans*, while the highest level of vigour index was observed in crops inoculated with *P. azotoformans* in comparison with other inoculants and uninoculated control plants. However, a considerable level of vigour index was also observed in plants inoculated with *E. bugandensis* and *B. cereus*. Bacterial endophytes caused a significant increase in plant roots and shoots as well as enhanced the dry weight and chlorophyll contents compared to the control. Seed sterilization did not affect the performance of the inoculum as there were no noticeable differences between the plant yield parameters for sterilized and unsterilized plant seeds. No disease incidence was noticed in any of the experiments.



Figure 6-6: Plant growth-promoting ability of bacterial endophytes in pot experiment. Pot experiment set up A) the arrangements of the pots in a randomized complete block design B) plant root length between treatments 1-4 and the control 5, Fully grown spinach for treatment 1 (C) and (D) and no inoculum control (E). The plants in Figure 6-6B are arranged according to the root length.

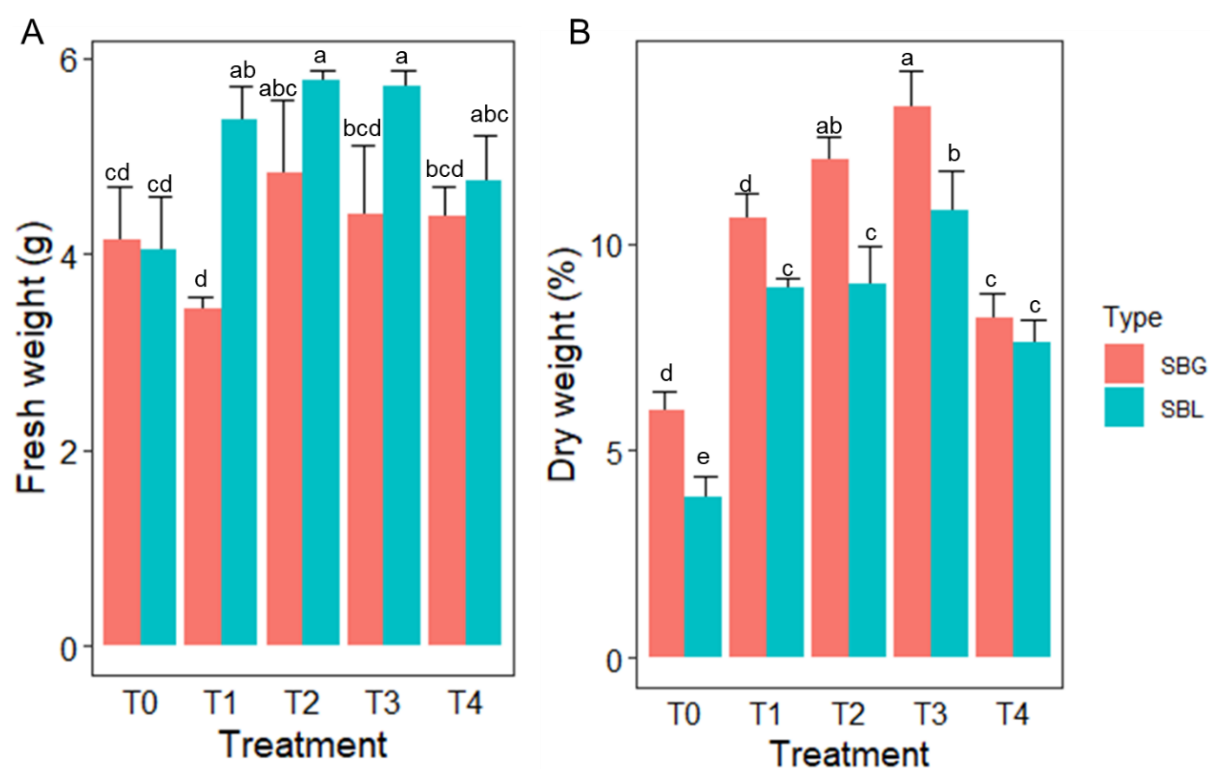


Figure 6-7: Fresh and dry weight of plant leaf tissue. Statistically significant treatments are represented with different alphabets (ANOVA, $P < 0.05$). The treatment; T0; control, T1; *Bacillus cereus*, T2; *Pseudomonas. azotoformans*, T3; *Enterobacter bugandensis* and T4; *Serratia liquefaciens*.

Table 6-4: Effect of bacterial inoculum on the growth parameters of spinach (*Spinacia oleracea*)

Treatment	Crop	Root length	Shoot length	No of leaves	Germination %	vigour index	Chlorophyll a	Chlorophyll b
Control	SBL	12.15 ± 0.62abc	7.13 ± 0.43ab	7 ± 0.82a	60 ± 7.07c	1156.8	28.77 ± 0.25a	15.11 ± 0.51d
<i>B. cereus</i>	SBL	14.03 ± 3.55ab	8.28 ± 0.99a	7 ± 1.00a	70 ± 4.08abc	1561.7	20.18 ± 1.20c	12.06 ± 2.83d
<i>P. azotoformans</i>	SBL	16.88 ± 3.40a	6.75 ± 0.99ab	6 ± 0.58a	80 ± 5.77a	1890.4	17.73 ± 1.41d	31.55 ± 1.68c
<i>E. bugandensis</i>	SBL	11.75 ± 1.23bc	7.83 ± 0.56a	6 ± 0.50a	75 ± 5.77ab	1468.5	26.86 ± 0.77a	52.11 ± 0.99a
<i>S. liquefaciens</i>	SBL	8.38 ± 1.11c	5.68 ± 0.46b	7 ± 0.58a	65 ± 7.07bc	913.9	23.65 ± 0.48b	42.97 ± 0.85b
Control	SBG	8.95 ± 0.47c	7.25 ± 0.87a	6 ± 0.58a	60 ± 7.07c	972	31.67 ± 0.42a	24.46 ± 0.32d
<i>B. cereus</i>	SBG	15.18 ± 1.58ab	6.78 ± 1.08a	6 ± 0.58a	70 ± 4.08abc	1537.2	30.51 ± 1.23ab	33.51 ± 1.84c
<i>P. azotoformans</i>	SBG	15.85 ± 3.31a	8.38 ± 1.11a	8 ± 1.5a	80 ± 5.77a	1938.4	24.37 ± 2.24d	51.89 ± 2.24a
<i>E. bugandensis</i>	SBG	11.5 ± 0.58bc	6.83 ± 1.11a	5 ± 0.5a	75 ± 5.77ab	1374.75	27.23 ± 0.41c	47.74 ± 0.41b
<i>S. liquefaciens</i>	SBG	9.3 ± 2.00c	6.7 ± 1.62a	6 ± 0.96a	65 ± 7.07bc	1040	28.52 ± 0.18bc	52.39 ± 0.18a

Data are presented as the mean ± standard deviation (n=4). Values followed by different letters are significantly (< 0.05) different with each column using a one-way analysis of variance with Tukey's multiple comparison test. Each isolate represents a treatment.

6.4 Discussion

A major goal of sustainable agriculture is to improve crop productivity while eliminating or minimizing negative impacts on the environment (Prasad et al., 2019). To achieve this goal, there must be a reduction in the use of agrochemicals causing ecological damage (Thorat and More, 2022). The rising demand for food crops, especially of vegetables, has further promoted the use of agrochemical inputs, suggesting the need for a sustainable strategy. The relatively recent application of microbial technology such as biofertilisers has been adopted as a viable alternative to agrochemical inputs such as fertilisers and pesticides (Adeleke et al., 2019b; Raimi et al., 2017). Thus, this study investigated various plant growth-promoting endophytes (PGPEs) for their ability to promote vegetable growth in a greenhouse experiment. PGPEs are considered a promising resource in the production of bioinoculants that can enhance crop productivity without any ecological footprint (Nithya and Babu, 2017). Microbial inoculants contribute to various ecological functions through biogeochemical cycling, biodegradation or decomposition and the production of bioactive compounds (Shahwar et al., 2023). Using PGPEs presents a cost-effective and eco-friendly strategy for improving crop production and alleviating ecological challenges due to human activities (Sasaki et al., 2015).

Antimicrobial and virulence potential of bacterial endophytes

The antimicrobial profile of the tested bacterial endophytes reveals that endophytes are a promising source of antimicrobials (Casella et al., 2013a; Seo et al., 2010), and suggest they produce bioactive compounds of biotechnological importance. Several bacterial endophytes synthesize antibacterial compounds such as 4-hydroxymellein, ketoconazole, polyketide, thiodiketopiperazine and organic extracts, which have antibacterial and antifungal activities against a spectrum of human pathogens (Casella et al., 2013b; Raimi and Adeleke, 2021). Similar to the observations in this study, endophytic bacteria such as

Pseudomonas sp., *Bacillus licheniformis* and *Acinetobacter calcoaceticus* showed considerable antimicrobial activity against various human pathogens including *Salmonella typhi*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Streptococcus agalactiae* and *Proteus mirabilis* (El-Deeb et al., 2013; Martinez-Klimova et al., 2017), suggesting their application in microbial inoculant formulation. The endophytes *B. cereus*, *P. conspicua*, and *E. bugandensis* with MIC 0.5 mg/ml are potential biocontrol agent. A study has reported similar endophytes with high inhibitory activity against human and plant pathogens (Seo et al., 2010), and can be harnessed for plant and human health management.

Antibiotic resistance in microbes, including bacterial endophytes' is a serious global threat to humanity. According to WHO, AMR is among the top ten global public health challenges facing human beings (WHO, 2021). Some of the endophytic bacteria are resistant to the different classes of antibiotics investigated, suggesting their potential impact on human health when consumed. The ASVs and OTUs obtained in this study are closely related to some of the genera that comprise pathogenic species such as *Salmonella*, *Enterobacter*, *Mycobacterium*, *Pantoea*, and *Serratia*. These pathogens inhabit spinach, lettuce and rocket plants (Jackson et al., 2013; Tatsika et al., 2019) and could cause diseases in the host. Pathogenic bacteria associated with fresh leafy vegetables are rarely eliminated completely before being consumed raw or after minimal processing. This is why fresh leafy vegetables are considered a major vehicle of foodborne illness outbreaks (Hölzel et al., 2018; Ölmez, 2016), suggesting it is crucial to identify the sources of contamination and transmission of the pathogens to better manage the food safety risks associated with vegetable produce (Rincón and Neelam, 2021). Agronomic practices such as soil nutrient management using pesticides and organic manure drive the increase in antibiotic resistance genes and bacteria in amended soil and crop cultivated (Anand et al., 2021;

Wang et al., 2015). Thus, practices promoting endophytic bacterial pathogenicity and antibiotic resistance should be discontinued or at least reduced, suggesting why such microbes with pathogenic ability could not be used for inoculum production.

Furthermore, while vegetables are important in human nutrition and health management, their microbiome is known to significantly impact human gut health (Berg et al., 2014a). Diet regularly consumed by humans may determine the microbial species that will colonise and be integrated into the gut microbiome (van der Merwe, 2021), suggesting such a diet should not possess species that have detrimental effects on humans. Interestingly, the cytotoxicity assay confirmed that some of the endophytes can potentially cause cell damage as they could inhibit growth and kill HuTu-80 cells (human intestinal epithelial cells). The bacterial endophytes with very high or high cytotoxicity (Figure 6-2) may present a high health risk when consumed. A study has reported endophytic *Bacillus cereus* isolated from potato tubers to show high cell toxicity in human and mammalian cells due to the production of cereulide, which causes foodborne illness in humans (Hoornstra et al., 2013). However, it is important to note that tissue or cell cultures are more susceptible to bacteria attack compared to live human intestinal cells, which have structured mechanisms of defence against bacterial pathogens (Horn et al., 2016). The *in vitro* assay may be more comparable to the conditions of individuals with compromised immune system. On the contrary, the cytotoxicity of these endophytes may be harnessed in the treatment of cancer and other related diseases (Matloub et al., 2019; Raimi and Adeleke, 2021). However, this study employed cytotoxicity, antimicrobial and antibiotic susceptibility potential as part of the criteria for selecting efficient PGP bacterial endophytes for the pot experiment to guarantee a healthy microbiome of the vegetables cultivated with the inoculant.

Impact of bacterial endophyte inoculum on seed germination

Seed germination is a crucial and delicate phase of the plant propagation cycle that ensures plant adaptation and evolution (Bentsink and Koornneef, 2008; Li et al., 2023). Thus, seed germination was investigated to ascertain their viability and quality-growth potential. Improper germination strategies can cause loss of seed vigour, reduced plant density, low seedling growth and survival rates and consequently, lower plant yield and nutritional quality (Bentsink and Koornneef, 2008; Moyo et al., 2022). It has been reported that bacterial endophytes inoculants applied to seeds and seedlings enhanced the germination of economically important crops such as maize, and wheat. Endophytic *Bacillus selenatarsenatis* and *Achromobacter xylosoxidans* significantly enhanced the germination of *Artemisia annua* L. over the control, with the germination percentage in the range of 18-31% (Husseiny et al., 2021). A similar result was observed in the present study, the bacterial endophytes significantly ($P < 0.05$) promoted the germination of spinach seeds as high as 98% compared with the control. In addition, a commercial biostimulant containing *Bacillus* sp. significantly influenced the germination of *Abelmoschus esculentus* seeds with all germination parameters having higher values over the control (Makhaye et al., 2021). This is in agreement with the present study, most of the bacterial endophytes investigated, including *B. cereus*, *P. azotoformans*, *E. bugandensis* and *S. liquefaciens* enhanced the germination of spinach seeds with all tested germination parameters including FGP, MGT, GI, CVG and GRI significantly higher and improved when compared with the control, suggesting the endophytes have a great potential to improve seed germination rate. Genetic makeup plays a key role in plant physiological structure and activities, including the germination process, which involves DNA synthesis and repairs and growth hormone production. Corroborating with other studies, variations were observed in the germination parameters for the different types of plant species (Islam

et al., 2009). On the contrary, a study has reported no differences in the germination responses of different genotypes (Babiker et al., 2017), an indication that other factors including seed dormancy, quality, treatment and the different conditions of germination may be highly identical (Bentsink and Koornneef, 2008).

Bacterial endophyte colonisation within plant tissues

Colonisation is a prerequisite activity for efficient PGPEs to initiate and successfully promote growth and ecological benefits in plants (Rani et al., 2022). This study showed that the four efficient endophytes: *B. cereus*, *P. azotoformans*, *E. bugandensis* and *S. liquefaciens* increased the plant growth parameters of the two spinach crops, which is attributed to their successful colonisation of the host tissues (Figure 6-3). Though not investigated in this study, the colonisation density of endophytes has been reported to be high along the root surfaces and rhizoplane and become low in the shoot interior as the endophytes become established (Poonguzhali et al., 2008). The biocontrol efficiency of PGPEs is highly correlated with colonisation density and the degree of competition for niches among the root endophytes. According to Hu and others, the colonisation of endophytic *Bacillus* spp. increased and stabilized in population density over time, confirming the ability of the endophyte to establish and persist on the surface or inside the root of the plant (Hu et al., 2017). Some microbial cell structures such as the flagella, fimbriae and cell surface polysaccharides contribute to the colonisation efficiency of bacterial endophytes (Kandel et al., 2017). Investigated PGPEs such as *Pseudomonas*, *Bacillus* and *Serratia* have been reported to possess flagella (Stich, 1921), which are involved in their attachment to plant surfaces (Kandel et al., 2017). The colonisation and spreading of *Herbaspirillum* sp. B501 across the shoot tissues of rice plants is related to motility, a common feature of bacterial endophytes (Elbeltagy et al., 2001).

Plant growth-promoting ability of bacterial endophytes in greenhouse experiments

The results obtained from the pot experiments provide evidence of increased growth and yield of spinach crops by the different treatments of PGPE inoculants. Consistent with other studies, aboveground biomass (wet and dry weight) and root length were considered a measure of plant growth promotion resulting from PGPE treatment in the greenhouse experiments (Emami et al., 2020; Lobo et al., 2019). Studies have also reported bacterial endophytes in the genera *Bacillus*, *Serratia*, *Pseudomonas*, *Acinetobacter*, *Klebsiella* sp. (PnB 10) and *Enterobacter* sp. (PnB 11) to promote the growth and health of different crops (Jasim et al., 2013; Padder et al., 2016; Wang et al., 2016). However, endophytic *Serratia* is poorly studied compared to *Pseudomonas* and *Bacillus*, which have been widely used for bioinoculant production (Raimi et al., 2017; Sahoo et al., 2017; Singh et al., 2016). Compared with other studies, four endophytic *Pseudomonas fluorescens* strains have been found to effectively colonise the root of Oilseed Rape after 5 weeks with a significant increase in plant biomass in greenhouse experiments (Lally et al., 2017). This is similar to the results in this study, endophytic *Pseudomonas* sp. also caused a significant increase in spinach fresh weight and dry weight biomass after 8 weeks in a greenhouse experiment. In a field experiment, the same endophytic bacterium in consortium with other PGPRs caused a significant increase in plant length and biomass (Lally et al., 2017). In another experiment, endophytic *Pseudomonas stutzeri* and *Ochrobactrum tritici* caused an increase in plant biomass and wet and dry weight. In agreement with the results in this study, the endophytic *Pseudomonas stutzeri* (K10P4) increased N uptake and chlorophyll content in rice plants under high salinity soil (Setiawati et al., 2021). Chlorophyll contributes to plant biomass formation through photosynthesis and its synthesis is influenced by some factors including light intensity, N, Mg, Fe, trace elements, temperature and water availability (Kärcher et al., 2020). Loss of leaf green pigment is

related to low levels of chlorophyll a and b, which is usually attributed to low N content in the leaf (Setiawati et al., 2021). Treatments with endophytic *Pseudomonas* and *Bacillus* had higher chlorophyll content, corroborating the observation in other studies (Beltran-Garcia et al., 2014; Krishnamoorthy et al., 2020). (Beltran-Garcia et al., 2014) in their study investigated the flow of labelled ^{15}N from bacterial endophytes to the host plant, the results revealed the presence of labelled ^{15}N in the chlorophyll, validating the transfer of ^{15}N from bacterial endophytes to plant tissues. This observation suggests that endophytes supplied N to the plant, which contributed to biosynthesis of chlorophyll (Beltran-Garcia et al., 2014). Endophytic *Bacillus* and *Pseudomonas* used in this study have been reported to possess N-fixing ability (Li et al., 2017; Raimi and Adeleke, 2023), and may have contributed to the supply of N, which stimulated the synthesis of chlorophyll content. Additionally, maize (Lobo et al., 2019), lettuce (Arkhipova et al., 2005), spinach (Tsuda et al., 2001) and tomato (Bergna et al., 2018) have been cultivated with PGPEs such as *Enterobacter*, *Bacillus*, and *Pseudomonas* that caused a significant increase in yield parameters. Despite the health benefits of spinach, it has not been well investigated, suggesting this study may present baseline information on how PGPEs could be harnessed in spinach farming.

Furthermore, PGPEs employ several mechanisms to promote plant growth. Of note are the N-fixation, phosphate solubilization and phytohormone production (Adeleke et al., 2019; Singh et al., 2016). Endophytic *Pseudomonas* and *Bacillus* used in this study have been widely reported with high production of auxins and ACC deaminase (Husseiny et al., 2021; Penrose et al., 2001; Raimi and Adeleke, 2023), known to promote root hair elongation. This may have contributed to the significant ($P < 0.05$) increase in the plant root length observed for treatments involving the PGPEs, especially *Pseudomonas* and *Bacillus* (Boivin et al., 2016). Endophytic *Bacillus* usually colonise the primary root surface,

including the zone of elongation, differentiation, and lateral root junction (Hu et al., 2017). Root hairs are a major structure promoting endophyte colonisation as shown in studies that used GFP-tagged endophytes. The results revealed that the GFP-tagged bacteria were localised with high density in the intercellular spaces of epidermal cells in the roots of tomato and rice plants (Hu et al., 2017; Poonguzhali et al., 2008). Similar observations were observed for the RFP-tagged PGPEs in this study, but in the leaf tissues (Figure 6-3). Such systemic alteration in the plant after successful endophyte colonisation, resulting in growth promotion can be understood by functional genomic profiling (Kaul et al., 2016). Root elongation activated by endophyte colonisation contribute to solubilization and availability of nutrient for plant metabolism, which leads to an aboveground yield increase (Kandel et al., 2017; Verma et al., 2021). In addition, endophytes can modulate host plant metabolism and biosynthetic pathways, which impact nutrient values in the host tissues (Emami et al., 2020; Mishra et al., 2018). Similar to the results in this study, aboveground colonisation and yield increase have been reported in different crops treated with PGPEs (Lally et al., 2017; Poonguzhali et al., 2008). Inoculation of seeds was used in this study because it allows for early association of PGPEs with the host plant from the point of germination, promoting easy compatibility and establishment of the endophytes. Endophytic *Methylobacterium suomiense* CBMB120 was transmitted to the aerial part of the plant through seed inoculation irrespective of the plant species investigated (Poonguzhali et al., 2008), suggesting seed inoculation supports PGPE colonisation and growth promotion. A study did not find any significant difference in the effect of seed or foliar application of biofertiliser under waterlogging stress (Habibzadeh et al., 2012). Though seed inoculation is widely used, the use of dual inoculation (seed pre-sowing and soil after germination) combined with foliar application has been recommended for the best results (Dawa et al., 2014).

Bacterial colonisation as revealed by confocal microscopy

Results from the confocal microscopy revealed the bacterial endophytes colonised the leaf tissues and varied for the plant types (Figure 6-3). Such observations have been reported in similar studies using confocal microscope (Li et al., 2017; Wright et al., 2013). The microscopy image showed a high density of the RFP-tagged bacterial endophytes around the stomata (Figure 6-3). Stomata are known to ensure optimum conditions for photosynthesis by facilitating the intake of carbon dioxide and release of oxygen and regulating transpiration by eliminating excess water vapour (Driesen et al., 2020). Higher stomata density has been reported to improve photosynthetic induction and biomass production in *Arabidopsis* under light fluctuation (Sakoda et al., 2020), suggesting the high bacterial endophyte density around the stomata may be contributing to stomata efficiency and photosynthesis, consequently, promoting high yield of host plants. The development of a confocal laser scanning microscope combined with fluorescence protein and dye molecules has brought new insight into the study of endophytes within their hosts (Bailey et al., 2006). As observed in this study, these techniques revealed that bacterial inoculants colonise the internal tissues of crops to exhibit plant growth promotion (Li et al., 2017; Wright et al., 2013). Though the present study investigated the PGP abilities of the endophytes in a greenhouse, field experiments have been reported to present a similar observation on the PGPEs in agroecosystems (Lally et al., 2017; Lobo et al., 2019).

6.5 Conclusion

The present study explored the potential of bacterial endophytes in promoting the growth of spinach crops in a pot experiment. The results revealed that bacterial endophytes studied can promote plant growth as indicated by the significant increase in growth

parameters, including height, root length, chlorophyll content and biomass. The colonisation and localisation of the endophytes within the plant tissues were established for all the bacterial endophytes using a confocal microscope. Endophytic *Pseudomonas*, *Enterobacter* and *Bacillus*, which showed high growth parameters also had strong fluorescence colouration of the RFP-tagged bacterial endophytes, suggesting they have an excellent plant-microbe interaction that enhanced their multiplication within the host and consequently led to improved host plant growth and development. Although the potential of confocal microscopy was not fully exploited, the observed results emphasized the importance of microscopy in plant-microbe interaction. The microscopy helped to ascertain that endophytes are part of the plant's physiological development. Field experiments would be necessary to further establish the ability of the bacterial endophytes under uncontrolled environments. This will further improve the understanding of the ability of endophytes to compete and persist under natural conditions where more factors may influence their contribution to plant growth and development.

6.6 Reference

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CHAPTER 7 :

GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

7.1 General Discussion

Agroecosystems are constantly under pressure to increase crop production for the ever-rising human population, which is expected to reach over 9 billion by 2050. Unfortunately, agricultural intensification is a major practice for increasing crop productivity using agrochemicals such as chemical fertilisers and pesticides. This practice is expensive, unsustainable and harmful to the environment, suggesting the need for alternative strategies or technologies that are cost-effective and eco-friendly. A viable option in realising sustainable crop production in the present-day agroecological challenges is to harness microbial technology. Bacterial endophytes known to establish mutualistic association with all known plants present a promising alternative, as they can promote sustainable crop production through different mechanisms, including nutrient cycling, biocontrol of plant pathogens, production of growth hormones and plant tolerance to biotic and abiotic stresses without any ecological footprint. In addition, the use of synthetic substances in the production of therapeutic compounds for human health management is unsustainable, has minimal health benefits and causes various adverse effects. Interestingly, endophyte technology is an excellent approach for a safe and efficient therapeutic strategy due to the antibacterial, antifungal, antitumour and anticancer properties of many endophytes and/or their bioactive compounds. Therefore, the discovery of novel and efficient endophytes that have tremendous applications in agro-ecosystem and biotechnology is crucial.

The broad goal of this study was to isolate and characterise bacterial endophytes inhabiting vegetables and investigate their potential ecological functions and plant growth promotion in vegetable production. Increasing crop yield and quality will promote human nutrition. This study is part of a more comprehensive research, which aimed to understand how endophytes that colonise and improve plant growth can also be harnessed in human health

management. The evolution of plants is largely driven by the complex interactions of symbiotic microbes, with the development of unique mechanisms that sustain the cooperation and long-term survival of the relationships. This complex association is of great ecological importance, enhancing plant growth, development, adaptation and tolerance to ecological stress. Due to the coevolutionary selection process, endophytes have developed high compatibility, reinfection capability and a better mode of colonisation with their host. Hence, understanding the community structure and growth promoting potential of endophytes could provide a comprehensive insight for efficient inoculant production, especially for mitigating the challenges of inoculum compatibility in the field.

Several studies have exhaustively investigated plant growth-promoting microbes in the soil, the rhizosphere and other complex environments such as compost and manure (Farokh et al., 2011; Ibarra-Galeana et al., 2017; Ouertani et al., 2020; Pastor et al., 2014; Raimi et al., 2022). However, vegetables, such as beetroot, spinach, lettuce and cabbage are rarely investigated for their PGPEs, creating a research gap and limiting their application in bioinoculant technology. Vegetables such as spinach has been reported for their human health benefits, such as in cancer disease management (Chen et al., 2021). Thus, the presence of beneficial endophytes in vegetables and insight into their functional abilities may offer substantial benefits in agroecosystems for improving crop productivity and in biotechnology industries for producing efficient therapeutics for human health management.

Soil ecosystems are complex and heterogeneous, comprising different biological communities, which are essential for a number of ecological processes supporting the soil ecosystem health and services. In particular, rhizosphere microbes play a crucial role in soil ecosystem sustainability and are affected by agronomic practices such as fertiliser application and crop types, which influence the dynamics of the soil nutrient and microbial communities and consequently, the recruited bacterial endophytes. Thus, this study investigates the richness and community structure of vegetable bacterial endophytes and the

rhizosphere as they are affected by organic or conventional fertiliser application and plant species types.

The investigation was undertaken in phases. The first phase involved assessment of the vegetables cultivated under organic or conventional fertilisers to gain insight into the influence of fertiliser and plant types on diversity, community structure and ecological roles of bacterial endophytes and rhizosphere bacteria. The diversity and community composition were compared to determine the nexus between the communities. In the second phase, efficient plant growth-promoting endophytes were investigated using *in vitro* experiments. Lastly, the capabilities of the recovered efficient endophytes were assessed in a greenhouse experiment.

7.2 Conclusions

7.2.1 Diversity and community structure of bacterial endophyte of vegetables

Cabbage, lettuce, spinach and onion were sampled from four farms, two farms from each of the North West and Gauteng provinces using organic or conventional fertilisers, respectively. Using high-throughput sequencing analysis, vegetable bacterial community abundance, profile and richness as well as the nutrient content were determined. The PICRUSt2 pipeline was used to predict the functional profile of the bacterial endophytes. The results revealed a diverse collection of bacterial endophyte communities that vary across plant species and fertiliser types. Based on NMDS plot, plant type significantly influenced community structure, with onion showing distinct clustering from the other crops. There was no evidence to suggest that fertiliser type impacted the community structure, however, the soil nutrient management practices impacted the endophytic community composition and functional profile of cultivated crops. Specifically, vegetables grown under conventional fertilisers had a higher bacterial community composition compared to organic fertilisers' attributed to the increase in soil nutrient content that supported high plant nutrient content driving the recruitment, multiplication and activities of endophytes. Onion in particular had a more

diverse bacterial community. This further supports the evidence that plant species types confer specific mechanisms that allow for the recruitment or response to a unique set of beneficial microbes that can activate host plants' defence systems against abiotic and biotic stress. This information is useful for agronomists and plant breeders in identifying plant varieties or species that are best adapted to plant-microbe interactions in a particular soil nutrient management system.

Based on PICRUSt2-inferred functional profiles, conventional fertiliser-cultivated crops had a higher abundance of ecologically important pathways such as CO₂-fixation and CALVIN pathways that contribute to ecosystem functions. However, pathways directly impacting human health such as antibiotic resistance and toxin production were highly predicted in organic farms, which is attributed to the source and treatment of the organic fertilisers. Consequently, human nutrition and health may potentially be affected, especially when ready-to-eat vegetables are consumed. Based on the trends observed, functional profiles are affected by fertiliser types and as a result plant nutritional quality may be affected. Due to a lack of information on the types, source and treatment of fertilisers, seeds and irrigation systems used in the farms, it was difficult to ascertain the possible cause of these variations. However, it is highly recommended that organic manure inputs such as animal manure and compost should be well-treated to reduce or eliminate the load of antibiotic residues and antibiotic-resistant microbes before field application.

Although this study further investigates the functional structure of bacterial endophytes using predictive metagenomics profiling, which to a large extent predicts possible functional structures of bacterial communities, employing stable isotopic probing or in situ hybridization could bring about a more comprehensive insight into the functional roles of endophytes. Moreover, transcriptomics and proteomics techniques, which respectively quantify activities at the gene or protein levels are alternative methods to characterise the functional roles of bacterial endophytes (Kaul et al., 2016; Terra et al., 2020). Some of the challenges in this study can be mitigated by harnessing new and advanced molecular tools such as

metagenomics using shotgun sequencing for better profiling of bacterial endophytes in vegetables.

This study improved on the limitations of the *in vitro* study and established that soil nutrient management types, organic and conventional fertilisers, not only impact the soil and plant productivity, but also the bacterial endophyte communities of the host plants. This study is unique as it investigated four different vegetables obtained from different farm locations and relates the results to possible implications on human health management, especially when vegetables are consumed raw. Consequently, the results obtained could provide baseline information useful in agroecosystem management, especially in vegetable farming to define a unique microbial ecosystem that will promote the selection of beneficial or suppression of deleterious groups of bacterial endophytes by the potential host plants. While the study assessed the effects of location on the relative abundance and density, it could not be substantiated as this factor had no significant effects on the vegetable bacterial endophytes. This is possibly due to the small sample numbers for location datasets used in this study, suggesting a more robust samples cutting across different geographical locations should be used for further studies.

However, the accuracy of identifying bacterial communities at the species taxa level and successful classification of DNA sequences using metabarcoding is greatly limited by the completeness and availability of reference databases, which vary depending on the taxa group and genomic region investigated. Incompleteness and taxonomic mislabelling may have led to several unclassifiable group and non-detection of species that may be present in the sample (Keck et al., 2023). The short length of the output sequences may also be a limiting factor. While many of the sequences could be assigned at the genus taxa, several unclassifiable ASVs were obtained at the species levels. Third-generation sequencing technologies such as nanopore technology sequencing platform and single molecule real-time sequencing (SMRT), which can produce longer gene fragments may provide better outputs.

7.2.2 Rhizosphere bacterial communities have important ecological roles and are potential endophytes that can be recruited by plants

In this part of the study, the bacterial community abundance, diversity, structure and functional profiles were investigated across the vegetable rhizosphere. The abundance and composition of bacterial communities in the crop rhizosphere revealed plant, fertiliser and location-specific differences. The soil nutrient content was significantly different across the farms, which influenced the differences observed in the bacterial community structure. In multivariate space, bacterial community composition and structure in the rhizosphere of plants cultivated under organic differed from those of conventional fertilisers, suggesting fertiliser and plant type influence rhizosphere bacterial communities. This observation indicates fixed factors could be manipulated to obtain a unique set of bacterial communities with specific ecological roles.

The enzyme activities were significantly different across the fertiliser type except for alkaline phosphatase. The beta-glucosidase activity was highly discriminatory among the enzyme activities assessed and was higher in organic compared to conventional farm rhizosphere soil. The 16S rRNA gene predictive metagenomic profiling revealed that the potential functions of the rhizosphere bacterial communities were significantly different between the organic and conventional farm soil, suggesting nutrient addition, as well as the input of microbial communities through organic substrates could have influenced the differences and the higher functional activities in the organic farm rhizosphere. Diverse functional profiles were predicted amongst the bacterial communities, which contribute to soil ecosystem functions and plant growth promotion.

Soil bacterial community is a good indicator of ecosystem functions and soil health. However, their dynamics are influenced by the variations in soil biological and physicochemical properties, suggesting their applications in agroecosystem to determine soil health and to improve plant health may not be effectively realised until they are integrated

with related ecological factors. The results in this study showed such factors to include plant species type and soil nutrient management such as organic or conventional fertilisers.

7.2.3 Vegetables comprise diverse bacterial endophytes contributing to ecosystem functions and plant growth

Vegetables were sampled from four farms practising either organic or conventional fertiliser application. Bacterial endophytes were isolated from the plant tissues and tested for PGP abilities *in vitro*. The study revealed that vegetables, including cabbage, spinach and lettuce are colonised by diverse bacterial endophytes. Some of the bacterial endophytes in the genera *Acinetobacter*, *Bacillus*, *Enterobacter*, *Pseudomonas*, and *Serratia* significantly exhibit plant growth-promoting potential, including N-fixation, phosphate solubilisation, and production of HCN, siderophore, IAA and ACC deaminase, suggesting that they can be employed for increasing crop yield. The population of bacterial endophyte communities vary significantly across the crop types and organs, indicating that mechanisms that confer a competitive advantage for selective recruitment of endophytes with unique ecological roles differ with plant types. The variability observed in the PGP capabilities suggests endophytes might have developed unique competence for a particular function within their niche over the time of coevolution process. Some endophytes produced different hydrolytic enzymes such as lipase, protease, amylase, cellulase and gelatinase, which contribute to their ecological roles. These functionalities of bacterial endophytes are key to improving ecosystem services especially as it relates to agroecosystems.

A major limitation of this study was the inability to precisely identify some of the bacterial endophytes at the species taxa level using Sanger sequencing after successful amplification of 16S rRNA. The culture-dependent method used can only account for about 1% of microbial community in an environment. This may have caused inaccurate assessment of bacterial endophytes, as it was impossible to fully recover and identify all the isolates, consequently, phylogenetic assignment and community differentiation between species types are affected. Additionally, the assessment of plant growth-promoting traits in the

laboratory may not present a genuine picture of the field reality. In most cases, the bacterial endophyte functional ability is very different when inside the host plant and may also be influenced by different ecological factors within the plant ecosystems.

7.2.4 Plant growth-promoting capabilities of efficient bacterial endophytes

A pot experiment was carried out to assess the capabilities of selected plant growth-promoting endophytes (PGPE). The results obtained from the *in vitro* assessment were used to select three efficient PGPE found to have at least three major growth promoting properties, including phosphate solubilisation, siderophore and phytohormone (IAA and ACC deaminase) production. The efficient PGPEs must not be cytotoxic or resistant to antibiotics. The cytotoxicity test used HuTu-80 (human epithelial cells), which mimic the human intestinal cell and this is one of the unique features of this study; testing the virulence of the recovered endophytes. Germination tests also showed that the PGPE significantly promoted seed germination compared to the control. Standardised tests to assess growth promotion, including yield parameters and tissue colonisation of the PGPE were carried out. Data on plant biomass (dry and wet weight), shoot and root length, chlorophyll content, vigour index and germination parameters provided strong evidence that the PGPEs promoted vegetable growth compared to the control in a pot experiment. The presence of the mcherry gene in the plant tissues as confirmed by microscopy and gene amplification reveals the inoculated PGPEs colonised the plant tissue. Several other investigations skip this major part of inoculum testing, which is crucial to ascertain if indeed the PGPEs could colonise the plant tissues, a necessary process preceding plant growth-promotion activity. The results also showed no sign of disease infection that could have affected plant nutritional values. From the yield increase and colonisation viewpoint, the PGPEs could improve plant growth and productivity. Thus, PGPEs potentially provide a resource that is useful in the production of inoculum for agroecological development.

Human nutrition essentially influences human growth and health and is impacted by the endophytic communities of the plant food. While the human gut drives the digestive system,

they are affected by the bacterial endophytes through gut microbiome colonisation, and functioning. Consequently, plants with healthy bacterial endophyte communities will positively impact human nutrition.

7.3 Recommendations

This study provided baseline information beneficial to researchers, agriculturists and vegetable farmers, revealing the ecological roles and plant growth-promoting properties of bacterial endophytes of vegetables. Apart from agroecological functions, bacterial endophytes contribute to plant nutritional values through plant food microbiological quality. Though inoculum selection and formulation process does not always investigate the virulence potential of PGP microbes, it should be recommended as a necessary step to ascertain virulence properties of endophytes, which impact human nutrition when plant materials are consumed. Such tests include antibiotic susceptibility, presence of virulence genes (Shiga toxin, leucocidin and colicin V protein) and cytotoxicity. These tests will further guarantee the microbiological quality of vegetable crops cultivated with efficient PGPEs.

7.3.1 Future research focus

- The future focus should be a long-term study to investigate endophytic microbial community patterns within different vegetable crops cultivated under different agroecological practices and climatic conditions. Such assessment will validate the conclusions in this study and its reliability for subsequent applications. In addition, it may be required to increase the sample size to obtain statistically strong results. However, due to numerous factors that may not be easily combined in the field and other confounding factors, such investigations can be carried out under simulated conditions.
- The high-throughput sequencing method used in this study is more efficient and preferable due to its relative simplicity and ability to synthesise new materials faster and more rapidly provide a more comprehensive understanding of test samples. However, the high rate of erroneous base calls produced is a major limitation. In addition, the

relative abundance of microbial taxa could be misleading as a result of the variations in the 16S rRNA gene copy number (GCN) and PCR biases. Thus, a future study should employ normalization of GCN in the estimation of taxa abundance. Though other studies did not find any difference between normalised and non-normalised GCN, it is highly recommended to correct for the variations in GCN to reduce prediction uncertainty and improve compositional and functional profile (Gao and Wu, 2023).

- Shotgun metagenomic sequencing, which allows for a comprehensive analysis of all genetic components in all organisms existing in complex samples may provide a better understanding of the abundance, diversity and functional profile of the rhizosphere and endophytic bacterial communities of vegetables. Additionally, the long-read sequencing technologies such as the Single-Molecule real time (SMRT) technology (Pacific Bioscience, CA, USA) may present a better technique to identify microbes at species taxa rank with a high precision. However, the high cost of this technology is a limitation to its application in meta-sample analysis.
- Better taxonomic profiling of the recovered bacterial endophytes may be achieved with whole genome sequencing using Illumina Miseq, which provides a more comprehensive analysis of the entire genome and can reveal more information especially on the functional capability of endophytes (Ng and Kirkness, 2010).
- Further assessment of the performance of efficient PGPEs in the field is necessary before inoculum production processes. The efficient PGPEs have important applications for the seed industry and crop productivity by aiding in seed germination and vegetables yield increase. The compatibility, reinfection ability and ease of colonisation of the efficient PGPEs may be harnessed to produce a vegetable harbouring unique and desired endophytic microbe with biotechnological importance, a good strategy with great application in probiotics formulation. In addition, further research on the long-term use of endophytes on soil and plants should be investigated. This is crucial given the potential for endophytes in bringing about imbalances in soil microbiomes over time.

- Biotechnologists can use the results of this study to further investigate how to harness the efficient PGPEs in human health management. This can be accomplished through multidisciplinary research collaboration. The colonisation ability of endophytes can also be harnessed to improve vegetable microbiome for biotechnology importance.
- Lastly, to document and manage successful research outcomes on microbial endophytes, a dedicated library or database is necessary to warehouse information about past and present trends, which will inform any future research focus.

7.3.2 Relevance of the study

This study significantly contributes to scientific literature by providing further evidence that:

1. Vegetable crops are colonised by diverse bacterial endophytes, which vary across the different species and organ types.
2. The bacterial endophytes are key components of vegetable microbiomes and possess great potential to improve seed germination and seedling vigour.
3. Soil nutrient management practices, mainly organic and conventional fertilisers, which alter rhizosphere microbial community structure, can determine the composition and functional profile of bacterial endophytes of cultivated vegetables.
4. Bacterial endophytic communities of vegetables differ across the plant species, which may be important for plant varietal selection and breeding.
5. DNA metabarcoding enables high-throughput analysis and detection of bacterial endophytes in vegetable crops and has the potential to be harnessed by researchers for better characterisation of plant microbiomes.
6. Aside from providing insights into the potential PGP ability of bacterial endophytes, this study reveals possible bacterial candidates that are useful in bioinoculant formulations for increasing crop yield, which consequently impact human nutrition.
7. Human nutrition may be affected by the microbiological quality of the plant food. Therefore, the assessment of the bacterial endophytes' virulence factors provided

insight into the importance of bacterial endophytes in human nutrition, especially as it relates to the consumption of ready-to-eat vegetables in the form of salad.

7.4 References

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SUPPLEMENTARY MATERIALS

Supplementary Text

Supplementary Text 3-1

Demultiplexed reads were trimmed of low-quality bases using Trimmomatic software (version 0.38) (Bolger et al., 2014) by removing poor-quality trailing and leading nucleotide positions from the forward and reverse reads. Afterwards, reads with an average quality score (Phred, Q) below 20 and reads having less than 250 base pair length were expunged. Quality sequence reads were denoised and assembled using DADA2 denoiser (Callahan et al., 2016) in Quantitative Insight into Microbial Ecology (QIIME) software version 2 (Bolyen et al. 2019) to obtain amplicon sequence variants (ASVs). The ASVs taxonomic assignment was performed in QIIME2 software using a pre-trained classifier SILVA rRNA 138 database (Bokulich et al., 2023; Quast et al., 2013). Contaminants (chloroplast, plastids and mitochondria), singletons and non-bacterial sequences such as archaea and those not assigned to any domain were removed from the ASVs count tables before normalization to an even depth, which is a standard of sequence number corresponding to the sample with the least sequences. Multiple sequence alignment was performed to infer the phylogenetic relationship of each of the ASVs and assess the differences of the dominant species among different sample groups.

Supplementary Text 3-2

The absolute abundance of microbial genera (observed ASVs) and vegetable nutrient content data was uploaded to the molecular ecological network analysis (MENA) pipeline (Deng et al., 2012). Then, the pairwise Pearson correlation was employed to generate correlation coefficients. Using the absolute values of the correlation matrix and the default settings of the pipeline, a similarity matrix with threshold values was generated. Based on the RMT method, an appropriate threshold was applied to the correlation values to convert the similarity matrix into an adjacency matrix, which encodes the strength of the connection between each pair of nodes (Deng et al., 2012). A connection is assumed to have a strong

(Pearson's $r > 0.85$) and significant ($P < 0.01$) correlation. Fast greedy modularity optimisation mode was then used to identify submodules within a large module, which were visualised with the Gephi 0.92 (<https://gephi.org>).

Supplementary Text 3-3

Random networks for each of the phylogenetic molecular ecological networks (pMENS) were constructed based on the Maslov-Sneppen technique, which keeps the numbers of nodes and links constant (Maslov and Sneppen, 2002). The random networks and power-law distribution were used to ascertain whether the empirical networks were error-prone and to identify bacterial interactions that were due to non-random patterns, which depict the empirical structure of microbial communities (Pan et al., 2021). The networks were grouped into network hubs, module hubs, connectors (generalists) and peripherals (specialists). The generalists are taxa that are closely connected with others both within and among modules (network hubs), within a module (module hubs) and among different modules within a network (connectors), while the specialists are peripheral taxa with less interaction with other taxa (Guimera and Amaral, 2005). The global network properties, individual node centrality, module separation and modularity were evaluated. The modularity of the networks was calculated using MENA and other evaluated topological parameters, including average degree, average path length, clustering coefficient and graph density (Pan et al., 2021).

Supplementary Tables

Supplementary Table 3-1: Geographical location and climate parameters for the farms

Farm	Latitude	Longitude	Altitude	Temperature	Precipitation
BF1	-26.79578	27.03854	1,337	17.5	40.005
BF2	-26.79668	27.03971	1,335	17.5	40.005
BF3	-26.79724	27.03862	1,331	17.5	40.005
BF4	-26.79821	27.03851	1,332	17.5	40.005
BF5	-26.79468	27.03897	1,329	17.5	40.005
SF1	-26.71509	27.08743	1,349	17.5	40.005
SF2	-26.71299	27.08323	1,350	17.5	40.005
SF3	-26.70109	27.08363	1,349	17.5	40.005
SF4	-26.71624	27.09323	1,344	17.5	40.005
SF5	-26.72739	27.08824	1,347	17.5	40.005
JF1	-26.19619	28.06614	1,717	15.65	46.355
JF2	-26.19419	28.07211	1,714	15.65	46.355
JF3	-26.19619	28.07953	1,733	15.65	46.355
JF4	-26.19475	28.06418	1,704	15.65	46.355
JF5	-26.19497	28.06914	1,706	15.65	46.355
TF1	-26.05436	27.67032	1,563	16.9	46.355
TF2	-26.02465	27.79547	1,528	16.9	46.355
TF3	-26.02458	27.69547	1,493	16.9	46.355
TF4	-26.02465	27.59347	1,534	16.9	46.355
TF5	-26.03465	27.67587	1,514	16.9	46.355

Altitude (m), precipitation (mm) and temperature (°C) are annual averages.

Supplementary Table 3-2: Result of Wilcoxon rank sum test with continuity correction testing the difference in ranks between alpha diversity indices and plant organ.

Alpha diversity	Effect	Test statistic W and sig. P value	r (rank biserial) and 95% CI
Chao 1	negative	very large; W = 1382.00, P < 0.001	r = -0.40, 95% CI [-0.55, -0.22])
Pielou's evenness	positive	tiny; W = 2400.00, p = 0.678	r = 0.04, 95% CI [-0.15, 0.23])
Shannon-Weiner	negative	medium; W = 1659.00, p = 0.005	r = -0.28, 95% CI [-0.45, -0.09])
Inverse Simpson	negative	small (diff = -0.79, 95% CI [-1.38, -0.19], t ((133.64) = -2.63	Cohen's d = -0.45, 95% CI [-0.79, -0.11])
Core abundance	negative	very small; W = 2167.00, p = 0.534	r = -0.06, 95% CI [-0.25, 0.13])
PD	positive	very small; W = 2429.00, p = 0.588	r = 0.05, 95% CI [-0.14, 0.24])

Welch's t-test is for testing the group differences mean between the organic fertilised farms and conventional fertilised farms while the Wilcoxon rank sum exact test is for testing the difference in ranks between the OF and CF farms. The effect sizes were labelled following Funder's (2019) recommendations. Sig. P value; significant P value. PD; phylogenetic diversity, CI; confidence interval. p < 0.05 is statistically significant. Shannon-Weiner was normally distributed and was tested with the Welch t-tests.

Supplementary Table 3-3: Fixed factor interaction effects on the Shannon index

Variables	Estimate	Std. Error	t value	Pr (> t)
(Intercept)	1.32872	0.09119	14.571	< 2e-16 ***
Plant lettuce	-0.40107	0.14400	-2.785	0.00622 **
Plant onion	0.04252	0.16077	0.264	0.79185
Plant spinach	-0.29742	0.13969	-2.129	0.03528 *
Organ root	-0.04876	0.13387	-0.364	0.71635
Practice organic	-0.24765	0.14607	-1.695	0.09258.
Plant lettuce: Organ root	0.47669	0.19810	2.406	0.01764 *
Plant onion: Organ root	0.30688	0.22887	1.341	0.18251
Plant spinach: Organ root	0.33947	0.19362	1.753	0.08211.
Plant lettuce: Practice organic	0.19999	0.26377	0.758	0.44981
Plant onion: Practice organic	0.21646	0.21889	0.989	0.32469
Plant spinach: Practice organic	0.27378	0.20842	1.314	0.19148
Organ root: Practice organic	0.06620	0.20913	0.317	0.75213
Plant lettuce: Organ root: Practice organic	-0.79251	0.38620	-2.052	0.04234 *
Plant onion: Organ root: Practice organic	-0.10202	0.31031	-0.329	0.74291
Plant spinach: Organ root: Practice organic	-0.19957	0.29500	-0.676	0.50003

Degree of freedom – Df, (*) statistical significance code: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘.’ 1. The Poisson model (estimated using ML) was fitted to predict Shannon-Weiner diversity with Plant, Organ, and Practice factors. The model’s explanatory power is substantial (Nagelkerke’s R² = 0.35) and the model’s intercept, corresponding to Plant = cabbage, Organ = leaf and Practice = conventional, is at 1.33 (95% CI [1.14, 1.50], t (120) = 14.57, p < .001).

Supplementary Table 3-4: PerMANOVA results based on weighted and unweighted Unifrac distances using the bacterial community structure abundance data.

Fixed factors	Weighted Unifrac (Composition)				Unweighted Unifrac (Composition)			
	PERMANOVA		PERMDISP		PERMANOVA		PERMDISP	
	Df	R ² (%)	P	P	Df	R ² (%)	P	P
Plant	3	5.65	0.002*	0.069	3	4.76	0.001*	0.519
Organ	1	1.35	0.025*	0.022	1	1.11	0.005*	0.634
Fertiliser	1	0.32	0.990	0.007	1	0.78	0.168	0.972
Plant: organ	3	1.75	0.800	0.021	3	2.19	0.036*	0.454
Plant: Fertiliser	3	2.34	0.321	0.063	3	2.33	0.137	0.013
Organ: Fertiliser	1	0.55	0.694	0.002	1	0.69	0.504	0.827
Plant: organ: Fertiliser	3	2.06	0.512	0.048	3	2.28	0.186	0.005
Residue	120	85.98			120	85.86		

Df- degree of freedom, (*) shows statistical significance with P < 0.05, P-value is based on 999 permutations using the adonis () function of the vegan package in R.

Supplementary Table 3-5: Linear discriminant analysis of Significant features between vegetable species

Features	LDA	P. unadj	P.adj	Significance	
Gammaproteobacteria	lettuce	5.18857	0.00092	0.02973	*
Pseudomonadaceae	lettuce	5.09971	0.00048	0.02012	*
Pseudomonas	lettuce	5.09971	0.00048	0.02012	*
Pseudomonadales	lettuce	5.09205	0.00061	0.02372	*
Thermoleophilia	onion	4.49856	0.00000	0.00090	***
Enterobacteriaceae	spinach	4.32607	0.00149	0.04521	*
Acidimicrobiia	cabbage	4.22590	0.00028	0.01420	*
Gaiellales	cabbage	4.21624	0.00000	0.00011	***
Solirubrobacterales	cabbage	4.21546	0.00019	0.01064	*
Chloroflexi	onion	4.12015	0.00029	0.01443	*
Gaiellales	cabbage	4.11846	0.00000	0.00011	***
Acidobacteriota	onion	4.09665	0.00068	0.02527	*
Solirubrobacter	cabbage	4.05029	0.00009	0.00755	**
Solirubrobacteraceae	cabbage	4.04708	0.00140	0.04373	*
Vicinamibacteria	cabbage	4.02487	0.00004	0.00641	**
Vicinamibacterales	cabbage	3.98905	0.00024	0.01262	*
Beijerinckiaceae	cabbage	3.95371	0.00088	0.02944	*
Xanthobacteraceae	cabbage	3.91966	0.00045	0.02012	*
Nocardioides	cabbage	3.90861	0.00042	0.01980	*
Chloroflexia	cabbage	3.88187	0.00001	0.00190	**
Solirubrobacter	cabbage	3.87913	0.00010	0.00793	**
Microvirga	cabbage	3.85965	0.00001	0.00279	**
Sphingomonas	cabbage	3.81703	0.00008	0.00755	**
Ilumatobacteraceae	cabbage	3.79741	0.00070	0.02527	*
Thermomicrobiales	cabbage	3.78934	0.00006	0.00641	**
Sphingomonas	cabbage	3.77170	0.00085	0.02944	*
Vicinamibacteraceae	cabbage	3.76228	0.00014	0.00981	**
JG30-KF-CM45	cabbage	3.76107	0.00006	0.00641	**
Microvirga	cabbage	3.74349	0.00002	0.00291	**
Xanthobacteraceae	cabbage	3.73937	0.00013	0.00981	**
IMCC26256	cabbage	3.62420	0.00018	0.01047	*
Desulfobacterota	cabbage	3.60551	0.00061	0.02372	*
Ilumatobacteraceae	cabbage	3.54840	0.00089	0.02944	*

P.adj; P value adjusted, P. unadj; P value unadjusted * -- 0.05, ** -- 0.01.

Supplementary Table 4-1: Permutational multivariate analysis of variance (PERMANOVA) based on Bray-Curtis dissimilarities using the bacterial community abundance data.

Fixed factors	PERMANOVA			PERMDISP
	Df	R ² (%)	P	P
Farms	2	27.27	0.001	0.122
Fertiliser type	1	11.74	0.003	0.537
Plant species	3	12.75	0.227	0.964
Fertiliser type: Plant species	3	15.82	0.055	0.588

Df- degree of freedom, $P < 0.05$, P-value is based on 999 permutations using the `adonis()` function of the `vegan` package in R. ND; not determined.

Supplementary Table 4-2: Differentially abundant features between the farms

Genus	LDA	P unadjusted	P adjusted	Significance
<i>Rubrobacterales</i>	Farm_B	4.350949	0.001959	0.03330
<i>Rubrobacter</i>		4.350949	0.001959	0.03330
<i>Elsterales</i>		3.568718	0.002101	0.03330
<i>Propionibacteriaceae</i>		3.392654	0.002702	0.03330
<i>Virgisporangium</i>		2.909674	0.002548	0.03330
<i>Entothaeonellaceae</i>		2.845636	0.002864	0.03330
<i>Segetibacter</i>		2.412468	0.002835	0.03330
<i>Nordella</i>		2.348284	0.002835	0.03330
<i>Acidobacteria</i>		2.180584	0.002864	0.03330
<i>Frankiales</i>	Farm_S	3.879269	0.001959	0.03330
<i>Alicyclobacillaceae</i>		3.327737	0.002521	0.03330
<i>Tumebacillus</i>		3.327737	0.002521	0.03330
<i>Rubrobacter</i>		3.257831	0.001886	0.03330
<i>Geminicoccaceae</i>		3.100697	0.001157	0.03330
<i>Bacillus</i>		2.782937	0.002864	0.03330
<i>Terribacillus</i>		2.759578	0.002422	0.03330
<i>Bdellovibrio</i>		2.218778	0.002864	0.03330
<i>Microtrichales</i>	Farm_J	4.181742	0.002103	0.03330
<i>Thermoactinomyces</i>		3.571520	0.001649	0.03330
<i>Marmoricola</i>		3.342096	0.002751	0.03330
<i>CL500-29 marine group</i>		2.999481	0.002284	0.03330
<i>Subgroup 6</i>		2.915509	0.001697	0.03330
<i>Kouleothrix</i>	Farm_T	2.590037	0.000346	0.02171
<i>Subgroup 10</i>		2.552849	0.000342	0.02171
<i>metagenome</i>		2.493913	0.000342	0.02171
<i>Rhodopirellula</i>		2.475376	0.000342	0.02171
<i>Terrimonas</i>		2.976678	0.002259	0.03330
<i>Pontibacter</i>		2.914398	0.001839	0.03330
<i>UTCFX1</i>		2.906586	0.000849	0.03330
<i>Nocardia</i>		2.905558	0.002605	0.03330
<i>Bauldia</i>		2.869715	0.002864	0.03330
<i>Blastocatella</i>		2.811599	0.000849	0.03330
<i>Pseudogracilibacillus</i>		2.751798	0.002057	0.03330
<i>Pedomicrobium</i>		2.731728	0.002057	0.03330
<i>Pir4 lineage</i>		2.728715	0.001071	0.03330
<i>Cerasibacillus</i>		2.709531	0.002864	0.03330
<i>Kouleothrix</i>		2.695504	0.001439	0.03330
<i>Pir4 lineage</i>		2.683881	0.002864	0.03330
<i>Chthoniobacter</i>		2.648163	0.000842	0.03330
<i>Propionibacterium</i>		2.440266	0.002864	0.03330
<i>Herpetosiphon</i>		2.302259	0.002864	0.03330
<i>Oceanobacillus</i>		2.111164	0.002383	0.03330

Some of the significant features (significance $p \leq 0.0333$) from the 205 significant features

Supplementary Table 4-3: Differentially abundant features between organic and conventional farms rhizosphere soil

Group (genus taxa)	LDA	P unadjusted	P adjusted	Significance
<i>Rubrobacteria</i>	Organic	4.218127	0.000212183	0.046020136
Rubrobacterales		4.218127	0.000212183	0.046020136
<i>Rubrobacteriaceae</i>		4.218127	0.000212183	0.046020136
<i>Rubrobacter</i>		4.218127	0.000212183	0.046020136
<i>Elsterales</i>		3.316274	0.000381058	0.046020136
<i>Propionibacteriaceae</i>		3.277556	0.000285118	0.046020136
<i>Virgisporangium</i>		2.955924	0.000212183	0.046020136
<i>Geminicoccaceae</i>		2.901468	0.000509639	0.049014667
<i>Micrococcales</i>	Conventional	4.329750	0.000381058	0.046020136
<i>Marmoricola</i>		3.118914	0.000526387	0.049014667
CL500-29 marine group		2.850692	0.000282117	0.046020136
<i>Hymenobacteraceae</i>		2.833395	0.000201679	0.046020136
CL500-29 marine group		2.746453	0.000504076	0.049014667
<i>Luteitalea</i>		2.712036	0.000341952	0.046020136
uncultured actinobacterium		2.704708	0.000327429	0.046020136
<i>Nocardia</i>		2.701467	0.000780488	0.049014667
uncultured Chloroflexi bacterium		2.549217	0.00074968	0.049014667
uncultivated soil bacterium C031		2.447038	0.000639038	0.049014667

Supplementary Table 4-4: Liner discriminant effect size analysis of abundant and unique pathways across the organic and conventional farms.

Pathway comparison	LDA	P.unadj	P.adj	Sign.
Metabolism Energy metabolism Sulfur metabolism		2.04953	0.00250	0.0474
Metabolism Metabolism of other amino acids Phosphonate and phosphinate metabolism		1.69128	0.00115	0.0311
Human Diseases Cancers: Overview	Conventional farm	1.48840	0.00194	0.0419
Human Diseases Endocrine and metabolic diseases		1.11586	0.00088	0.0311
Cellular Processes Transport and catabolism Endocytosis		0.39965	0.00016	0.0170
Organismal Systems Endocrine system GnRH signalling pathway		0.39393	0.00016	0.0170
Organismal Systems Immune system Fc gamma R-mediated phagocytosis		0.37266	0.00016	0.0170
Organismal Systems Digestive system Fat digestion and absorption		0.23430	0.00115	0.0311
Metabolism Amino acid metabolism		3.13844	0.00088	0.0311
Metabolism Amino acid metabolism Arginine and proline metabolism	Organic farm	2.67648	0.00088	0.0311
Metabolism Amino acid metabolism Phenylalanine metabolism		2.40703	0.00115	0.0311
Metabolism Energy metabolism Carbon fixation pathways in prokaryotes		2.34942	0.00115	0.0311
Metabolism Carbohydrate metabolism Citrate cycle (TCA cycle)		1.84869	0.00194	0.0420
Metabolism Metabolism of other amino acids Selenocompound metabolism		1.82821	0.00115	0.0311
Metabolism Xenobiotics biodegradation and metabolism Styrene degradation		1.73386	0.00150	0.0374
Human Diseases Infectious diseases: Parasitic Amoebiasis		1.44485	0.00250	0.0476
Metabolism Glycan biosynthesis and metabolism Glycosaminoglycan degradation		1.23946	0.00029	0.0231

Conv; conventional farm, organic; organic farm, Sign.; significance ($P < 0.05$), Linear discriminant analysis; LDA P.adj; P value adjusted, P.unadj; P value unadjusted. The pathway taxonomic ranking was at level 3.

Supplementary Table 4-5: Differentially abundant functional profile of rhizosphere bacterial community across the farms

Functional profile group	LDA	P.unadj	P.adj	Sig.
Metabolism Amino acid metabolism Phenylalanine, tyrosine and tryptophan biosynthesis	Farm_T	2.3707	0.0018	0.0271
Genetic Information Processing Replication and repair Mismatch repair	Farm_T	2.3074	0.0021	0.0271
Genetic Information Processing Replication and repair DNA replication	Farm_T	2.0163	0.0025	0.0271
Metabolism Energy metabolism Photosynthesis	Farm_T	1.7818	0.0024	0.0271
Metabolism Biosynthesis of other secondary metabolites Novobiocin biosynthesis	Farm_T	1.3965	0.0020	0.0271
Cellular Processes Transport and catabolism Endocytosis	Farm_T	0.5468	0.0011	0.0271
Organismal Systems Endocrine system GnRH signaling pathway	Farm_T	0.5419	0.0011	0.0271
Organismal Systems Immune system Fc gamma R-mediated phagocytosis	Farm_T	0.5410	0.0011	0.0271
Genetic Information Processing	Farm_T	3.3329	0.0038	0.0277
Environmental Information Processing Membrane transport Bacterial secretion system	Farm_T	3.0245	0.0036	0.0277
Genetic Information Processing Translation	Farm_T	3.0002	0.0034	0.0277
Metabolism Nucleotide metabolism	Farm_T	2.8262	0.0036	0.0277
Genetic Information Processing Folding, sorting and degradation RNA degradation	Farm_T	2.5553	0.0034	0.0277
Genetic Information Processing Transcription RNA polymerase	Farm_T	1.7499	0.0043	0.0277
Metabolism Glycan biosynthesis and metabolism Lipopolysaccharide biosynthesis	Farm_T	2.7663	0.0024	0.0271
Genetic Information Processing Translation AminoACYI-tRNA biosynthesis	Farm_T	2.7050	0.0027	0.0271
Metabolism Xenobiotics biodegradation and metabolism	Farm_J	3.4191	0.0017	0.0271
Metabolism Carbohydrate metabolism Butanoate metabolism	Farm_J	2.5531	0.0040	0.0277
Metabolism Biosynthesis of other secondary metabolites Stilbenoid, diarylheptanoid and gingerol biosynthesis	Farm_J	1.8368	0.0042	0.0277
Metabolism Metabolism of terpenoids and polyketides Geraniol degradation	Farm_J	2.6789	0.0015	0.0271
Metabolism Xenobiotics biodegradation and metabolism Benzoate degradation	Farm_J	2.6467	0.0019	0.0271
Metabolism Xenobiotics biodegradation and metabolism Steroid degradation	Farm_J	2.5530	0.0024	0.0271

Metabolism Lipid metabolism Fatty acid degradation	Farm_J	2.5123	0.0023	0.0271
Metabolism Xenobiotics biodegradation and metabolism Caprolactam degradation	Farm_J	2.3068	0.0022	0.0271
Metabolism Lipid metabolism Steroid hormone biosynthesis	Farm_J	2.1687	0.0015	0.0271
Metabolism Xenobiotics biodegradation and metabolism Naphthalene degradation	Farm_J	2.1331	0.0019	0.0271
Cellular Processes Cell growth and death Meiosis - yeast	Farm_J	2.1200	0.0020	0.0271
Organismal Systems Endocrine system PPAR signaling pathway	Farm_J	2.1195	0.0022	0.0271
Metabolism Metabolism of other amino acids beta-Alanine metabolism	Farm_J	2.0362	0.0017	0.0271
Metabolism Xenobiotics biodegradation and metabolism Chloroalkane and chloroalkene degradation	Farm_J	2.0059	0.0024	0.0271
Metabolism Lipid metabolism Sphingolipid metabolism	Farm_J	1.9786	0.0021	0.0271
Metabolism Xenobiotics biodegradation and metabolism Xylene degradation	Farm_J	1.9268	0.0017	0.0271
Organismal Systems Endocrine system Adipocytokine signaling pathway	Farm_J	1.8407	0.0027	0.0271
Metabolism Xenobiotics biodegradation and metabolism Dioxin degradation	Farm_J	1.8331	0.0024	0.0271
Metabolism Metabolism of cofactors and vitamins Retinol metabolism	Farm_J	1.6107	0.0025	0.0271
Metabolism Lipid metabolism Steroid biosynthesis	Farm_J	1.4713	0.0026	0.0271
Metabolism Lipid metabolism Primary bile acid biosynthesis	Farm_J	1.3221	0.0017	0.0271
Metabolism Biosynthesis of other secondary metabolites Flavone and flavonol biosynthesis	Farm_J	0.3949	0.0020	0.0271
Genetic Information Processing Replication and repair Non-homologous end-joining	Farm_J	1.8527	0.0029	0.0275
Metabolism Xenobiotics biodegradation and metabolism Polycyclic aromatic hydrocarbon degradation	Farm_J	2.3340	0.0038	0.0277
Organismal Systems Endocrine system	Farm_J	2.2982	0.0038	0.0277
Metabolism Lipid metabolism Synthesis and degradation of ketone bodies	Farm_J	1.7649	0.0031	0.0277
Organismal Systems Digestive system Bile secretion	Farm_J	1.4792	0.0038	0.0277
Metabolism Metabolism of cofactors and vitamins One carbon pool by folate	Farm_B	2.0535	0.0030	0.0277
Metabolism Glycan biosynthesis and metabolism	Farm_B	2.8341	0.0028	0.0271
Metabolism Metabolism of cofactors and vitamins Vitamin B6 metabolism	Farm_B	1.6735	0.0019	0.0271
Metabolism Glycan biosynthesis and metabolism Glycosaminoglycan degradation	Farm_B	1.3610	0.0018	0.0271

Metabolism Amino acid metabolism Valine, leucine and isoleucine degradation	Farm_S	2.7572	0.0043	0.0277
Metabolism Metabolism of other amino acids Selenocompound metabolism	Farm_S	2.0014	0.0036	0.0277
Metabolism Metabolism of terpenoids and polyketides Biosynthesis of type II polyketide products	Farm_S	1.7453	0.0042	0.0277
Metabolism Energy metabolism Methane metabolism	Farm_S	2.7154	0.0061	0.0281

Linear discriminant analysis; LDA, P value unadjusted; P.unadj, P value adjusted; P.adj, Significant P value; sig.,

Supplementary Table 4-6: Comparison of the relative abundance of selected enzymes of agroecological importance across vegetable species

KO Terms	Enzyme and commission number	Cabbage	Lettuce	Onion	Spinach
K01505	ACC deaminase [EC:3.5.99.7]	5.13121	5.04508	4.99341	5.079096
K02585	nitrogen fixation protein NifB	1.55937	1.56163	1.42423	1.335757
K00368	nitrite reductase [EC:1.7.2.1]	5.28574	5.37568	5.19877	5.373010
K01078	acid phosphatase [EC:3.1.3.2]	6.65048	6.84074	6.84352	6.953965
K01077	alkaline phosphatase [EC:3.1.3.1]	4.87082	5.13687	5.40619	5.317880
K01093	4-phytase [EC:3.1.3.26]	0.40239	0.43370	0.39744	0.434442
K01130	arylsulfatase [EC:3.1.6.1]	0.00667	0.00757	0.00768	0.007705
K01133	choline-sulfatase [EC:3.1.6.6]	0.00456	0.00539	0.00538	0.005476
K16090	catecholate siderophore rec	2.22708	2.14491	2.47484	2.213386
K02217	ferritin [EC:1.16.3.1]	7.67035	7.42860	7.43135	7.249544
K02013	ICTS ATP-binding protein [EC:3.6.3.34]	41.2667	42.0071	41.6423	41.99524
K01183	chitinase [EC:3.2.1.14]	1.62722	1.87736	1.82461	1.819459
K07405	alpha-amylase [EC:3.2.1.1]	5.28059	5.12611	4.62818	4.895040
K07406	alpha-galactosidase [EC:3.2.1.22]	0.20130	0.23736	0.20505	0.210376
K01179	endoglucanase [EC:3.2.1.4]	9.04409	8.67790	9.57702	8.969161
K01187	alpha-glucosidase [EC:3.2.1.20]	5.27219	5.00210	4.96465	5.201447
K03332	beta-fructosidase [EC:3.2.1.80]	3.49920	3.09207	2.97534	2.939153

The relative abundance was calculated for each enzyme across the vegetable rhizosphere soil investigated. ACC; 1-aminocyclopropane-1-carboxylate deaminase, ICTS; iron complex transport system

Supplementary Table 4-7: Correlation between soil enzymes and physicochemical properties

Parameter	Dehyd	Beta gluc	Alk.phos	acid phos	Urease
TOC	0.413	0.442	0.028*	0.034*	0.771
pH	0.005*	0.001*	0.966	0.042*	0.790
Moisture	0.283	0.060	0.823	0.360	0.139
OM	0.056	0.015*	0.236	0.438	0.456
EC	0.203	0.344	0.091	0.023*	0.433
CEC	0.874	0.378	0.275	0.435	0.004*
NO ₃	0.760	0.776	0.007*	0.306	0.843
PO ₄	0.018*	0.045	0.336	0.003*	0.626
NH ₄	0.026*	0.005*	0.217	0.000*	0.708
SO ₄	0.269	0.939	0.921	0.476	0.003*
P	0.792	0.475	0.885	0.947	0.003*
K	0.029*	0.019*	0.214	0.000*	0.960
Na	0.140	0.170	0.160	0.017*	0.748
Ca	0.360	0.569	0.071	0.060	0.365
Mg	0.771	0.380	0.183	0.714	0.135
Cl	0.194	0.175	0.049*	0.005*	0.923
Particle size (mm)	0.879	0.409	0.108	0.304	0.018*
Clay	0.527	0.098	0.905	0.491	0.020*
Sand	0.625	0.251	0.295	0.972	0.108
Silt	0.056	0.418	0.817	0.278	0.032*

NH₄; ammonia, CEC; cation exchange capacity, EC; electrical conductivity, K; potassium, NO₃; nitrate, OM; organic matter, P; phosphorus, PO₄; phosphate, SO₄; sulphate, and TOC; total organic carbon, “*” Significant (P < 0.05) value. Dehydrogenase; Dehyd., Alkaline phosphatase; Alk.phos, acid phosphatase; acid.phos, beta glucosidase; Beta.gluc.

Supplementary Table 4-8: Significant environmental variables correlating with the bacterial communities (genus taxa) within the redundancy analysis triplot.

Variables	Variance	F	Pr(>F)	Mantel test		
				P value	adj. P	Sig.
TOC (total organic carbon)	2648870	3.657678	0.001	0.2901	0.009	0.0210
pH	671366.8	0.927053	0.495	-0.106	0.938	0.9380
Moisture content	873237.3	1.205805	0.280	-0.020	0.528	0.5686
OM (organic matter)	3980192	5.496026	0.001	0.1665	0.041	0.0718
EC (electrical conductivity)	2023697	2.794411	0.015	0.3151	0.006	0.0210
CEC (cation exchange capacity)	1945933	2.687031	0.020	0.0786	0.114	0.1330
NH ₄ (ammonia ion)	444958.9	0.614419	0.780	0.1240	0.078	0.1092
SO ₄ (sulphate ion)	599286.3	0.827521	0.574	0.3118	0.008	0.0210
PO ₄ (phosphate ion)	313507.4	0.432905	0.925	0.1177	0.092	0.1171
Na (sodium)	655668.9	0.905377	0.511	0.1933	0.030	0.0600
Mg (magnesium)	926024	1.278695	0.268	0.3023	0.004	0.0210
Clay	876681.4	1.210561	0.303	0.2721	0.005	0.0210
Sand	1124022	1.552099	0.149	0.3145	0.008	0.0210
Silt	296185.7	0.408986	0.936	0.1214	0.073	0.1092

Permutation test for RDA under the reduced model. The terms are added sequentially (first to last) and the number of permutations is 999. Degree of freedom = 1. The $R^2 = 0.8275777$ and adjusted $R^2 = 0.3447952$. Mantel check was used to check for significant correlations between environmental variables and distance matrix, adj. P; adjusted P values, Sig; significant value.

Supplementary Table 5-1: Sterilisation procedure for vegetable tissues

Sample	85% Ethanol (sec)	Sterile distilled H ₂ O (sec)	2.5% NaClO (min)	Sterile distilled H ₂ O (min)
Leaf	60	30	4	2 - twice
Stem/root	90	40	5	2 - twice

Supplementary Table 5-2: Plant growth-promoting ability of endophytic bacteria recovered from vegetable crops

ID	Organ	Endophytic bacteria	% S	N-f	NH4	PS	Soluble PO4	Siderophore	IAA	ACC	HCN	Lp	Am	Pr	Ge	Ce
S19	CL1	<i>Pseudomonas azotoformans</i>	100	+	26.43 ± 2.9	-	22.98 ± 1.00	27.33 ± 2.52	0.97 ± 0.07	+	-	+++	+++	-	-	-
S20	CL2	<i>Pantoea conspicua</i>	100	+	35.38 ± 1.82	+	30.05 ± 2.00	-	21.84 ± 2.01	+	-	-	+++	-	-	-
S21	CL3	<i>Pseudomonas gessardii</i>	100	+	2.58 ± 1.04	+	36.25 ± 1.50	-	0.14 ± 0.05	-	-	-	+++	-	++	-
S22	CL3A	Nd	100	-	-	-	12.26 ± 1.80	-	-	-	-	-	++	-	-	-
S23	CL3B	Nd	100	+	-	-	15.58 ± 2.10	-	-	-	-	-	++	-	-	-
S24	CL4	<i>Pseudomonas reinekei</i>	100	+	60.93 ± 3.25	-	19.80 ± 2.58	24.33 ± 1.53	2.15 ± 1.00	+	-	++	+++	+	-	-
S25	CR1	<i>Pantoea conspicua</i>	100	+	6.10 ± 1.37	-	16.65 ± 1.89	-	11.59 ± 2.82	-	-	-	-	-	-	-
S26	CR2	<i>Pseudomonas azotoformans</i>	100	-	8.85 ± 1.84	-	11.24 ± 0.95	25.33 ± 0.58	2.85 ± 1.50	+	-	+++	-	-	++	-
S27	CR3	<i>Microbacterium liquefaciens</i>	100	-	1.21 ± 0.46	+	25.66 ± 1.86	-	12.66 ± 2.40	-	-	-	+++	-	-	-
S28	CR4	<i>Pseudomonas azotoformans</i>	100	+	64.89 ± 2.83	-	24.59 ± 1.24	25.33 ± 0.58	18.29 ± 3.02	+	-	++	-	+	++	+++
S29	CS1A	<i>Serratia liquefaciens</i>	100	+	45.16 ± 2.49	-	3.35 ± 1.13	24.33 ± 0.58	-	+	-	++	+++	+	+	-
S30	CS1B	<i>Pseudomonas azotoformans</i>	100	+	1.26 ± 0.52	-	4.76 ± 2.01	29.67 ± 0.58	1.59 ± 1.00	-	-	++	-	-	++	+
S31	CS2	<i>Pseudomonas azotoformans</i>	100	+	36.92 ± 2.74	+	27.47 ± 1.06	30.33 ± 0.58	2.13 ± 0.77	-	-	++	+++	-	-	+
S32	CS3	<i>Priestia megaterium</i>	100	-	1.21 ± 0.54	-	21.29 ± 1.34	26.33 ± 1.15	7.79 ± 2.02	-	-	+	+	+	-	-
S33	CS3A	<i>Serratia liquefaciens</i>	100	+	-	+	38.78 ± 2.61	-	-	-	-	+	-	-	-	+
S34	CS3B	<i>Pseudomonas azotoformans</i>	100	+	-	+	33.60 ± 1.33	33.33 ± 2.08	6.04 ± 1.43	+	-	+	+++	-	-	-
S35	LL1	<i>Pseudomonas kairouanensis</i>	99.8	-	55.71 ± 2.87	-	16.20 ± 2.75	-	0.57 ± 0.07	+	-	+	+++	+	++	Nd
S36	LL2	<i>Pantoea conspicua</i>	100	+	52.80 ± 1.04	+	25.29 ± 3.12	15.67 ± 0.58	2.18 ± 1.00	+	-	-	+++	-	-	-
S37	LL3	<i>Pseudomonas vancouverensis</i>	100	+	33.57 ± 2.61	-	17.59 ± 1.90	-	4.00 ± 0.79	-	-	-	+++	-	-	-
S38	LL4	<i>Pseudomonas vancouverensis</i>	100	+	1.98 ± 0.29	-	25.56 ± 2.00	-	3.25 ± 1.57	-	-	-	-	-	-	-
S39	LL5	<i>Pseudomonas azotoformans</i>	100	-	10.88 ± 2.11	+	23.10 ± 1.62	34.33 ± 5.52	7.98 ± 2.99	-	-	-	-	+	-	-
S40	LL6	Nd	-	+	-	-	11.96 ± 1.42	-	-	-	-	Nd	Nd	-	-	-
S41	LR1	<i>Serratia liquefaciens</i>	100	+	41.87 ± 2.89	+	21.41 ± 2.69	38.33 ± 7.23	2.50 ± 1.00	+	-	+	+++	+	++	-
S42	LR2	<i>Pantoea conspicua</i>	100	-	23.13 ± 2.13	-	12.63 ± 1.71	24.67 ± 0.58	2.85 ± 1.00	-	-	-	+++	-	-	-
S43	LR3	<i>Pseudomonas azotoformans</i>	100	-	68.52 ± 2.82	-	16.45 ± 1.01	-	4.35 ± 1.09	+	-	-	++	-	-	-
S44	LR4	<i>Pseudomonas vancouverensis</i>	100	+	46.76 ± 3.49	+	25.91 ± 1.53	-	6.68 ± 1.66	-	-	-	-	-	-	-
S45	LR5	<i>Pseudomonas azotoformans</i>	100	+	9.07 ± 2.31	+	22.98 ± 1.61	37.00 ± 1.00	2.18 ± 1.24	+	-	+	+++	+	-	-
S46	LR6	Nd	-	-	4.29 ± 1.03	-	24.96 ± 2.04	10.67 ± 1.53	11.12 ± 1.00	+	-	-	+++	-	-	-
S47	LR7	Nd	-	+	-	-	9.65 ± 1.77	-	-	-	-	-	Nd	-	-	-
S48	LS1A	<i>Acinetobacter haemolyticus</i>	100	+	51.92 ± 2.88	-	18.19 ± 2.39	-	0.44 ± 0.01	+	-	-	+++	+++	-	-
S49	LS1B	<i>Stenotrophomonas maltophilia</i>	98.1	-	4.89 ± 0.91	+	20.35 ± 0.98	-	2.10 ± 1.00	-	-	-	+++	+++	-	-
S50	LS2A	<i>Stenotrophomonas maltophilia</i>	100	-	2.75 ± 0.53	+	20.69 ± 1.31	39.67 ± 1.53	5.85 ± 1.90	-	-	+	++	+	-	+
S51	LS2B	<i>Xanthomonas fragariae</i>	100	+	43.41 ± 1.82	-	15.96 ± 1.20	-	0.89 ± 0.38	-	-	+	+++	+	-	-
S62	SL1	<i>Pseudomonas vancouverensis</i>	100	-	29.12 ± 2.35	-	18.66 ± 1.31	-	6.28 ± 1.78	+	-	-	+++	-	+	-
S63	SL2	<i>Pantoea conspicua</i>	99.6	-	5.05 ± 1.04	-	15.96 ± 1.00	-	22.62 ± 2.37	+	-	Nd	-	+++	-	-
S64	SL3	Nd	-	+	24.07 ± 2.79	-	13.50 ± 2.60	-	44.48 ± 2.87	-	-	-	-	-	-	-
S65	SR1A	Nd	-	+	-	-	15.41 ± 1.91	-	4.46 ± 1.36	-	-	-	+	-	-	-
S66	SR1B	Nd	-	+	-	+	20.62 ± 1.28	-	-	-	-	-	-	-	Nd	-
S67	SR2	<i>Glutamicibacter uratoxydans</i>	100	-	44.18 ± 2.03	-	18.11 ± 1.23	18.33 ± 1.53	16.14 ± 3.00	+	-	-	+++	-	-	-
S68	SR3A	<i>Pseudomonas azotoformans</i>	100	+	11.26 ± 2.14	-	26.35 ± 1.87	39.67 ± 1.53	4.92 ± 1.00	-	-	-	++	+	-	-

S69	SR3AN	<i>Pseudomonas azotoformans</i>	100	+	6.10 ± 1.52	+	22.18 ± 1.94	17.33 ± 0.58	-	-	-	-	+++	+	++	-
S70	SR3B	<i>Pantoea conspicua</i>	99.4	-	70.55 ± 3.09	-	18.39 ± 1.88	24.00 ± 1.00	17.85 ± 3.10	-	-	-	-	-	-	-
S71	SR4A	Nd	-	+	-	-	16.92 ± 2.12	32.00 ± 1.00	12.82 ± 1.00	+	-	+	+	-	-	-
S72	SR4B	Nd	-	-	-	-	12.13 ± 2.17	-	-	-	-	-	Nd	Nd	-	-
S73	SS1	<i>Pseudomonas azotoformans</i>	100	-	-	-	14.62 ± 1.63	-	1.11 ± 0.58	+	-	-	-	-	-	-
S74	SS1N	<i>Pseudomonas azotoformans</i>	100	+	11.48 ± 1.08	+	22.43 ± 2.37	38.33 ± 3.51	16.81 ± 3.78	-	-	+	+	+	-	-
S75	SS2	<i>Pseudomonas azotoformans</i>	100	+	35.00 ± 2.07	+	22.63 ± 1.58	-	0.51 ± 0.00	-	-	-	-	+	-	-
S76	SS2N	Nd	-	+	-	-	7.30 ± 1.03	-	-	-	-	-	-	-	-	-
S77	SS3N	<i>Pseudomonas vancouverensis</i>	100	-	-	-	17.47 ± 1.95	29.67 ± 1.53	4.81 ± 1.00	+	-	-	-	+	++	-
J40	CL1	<i>Comamonas terrigena</i>	99.7	-	5.88 ± 1.02	-	19.90 ± 1.33	7.33 ± 0.58	4.50 ± 1.03	-	-	+	+	-	-	-
J41	CL2	<i>Bacillus safensis</i> subsp. <i>safensis</i>	100	-	3.85 ± 0.93	+	29.80 ± 1.25	6.00 ± 0.00	2.66 ± 1.00	-	-	+++	+++	-	-	+++
J41B	CL2B	<i>Bacillus safensis</i> subsp. <i>safensis</i>	99.1	+	67.47 ± 2.68	+	21.09 ± 1.31	-	1.53 ± 0.00	-	-	+++	+++	+	-	+
J50	CLX1	Nd	-	+	2.53 ± 0.45	+	32.66 ± 1.25	-	3.14 ± 1.00	-	-	-	+++	-	-	-
J36	CR1	Nd	-	+	0.77 ± 0.34	+	32.16 ± 0.39	6.00 ± 1.00	3.61 ± 1.43	-	-	++	+++	-	-	-
J37	CR2	<i>Bacillus safensis</i> subsp. <i>safensis</i>	100	+	9.56 ± 1.89	+	24.00 ± 1.28	20.67 ± 0.58	10.53 ± 2.02	+	-	-	+++	-	+	++
J38	CR3	<i>Bacillus nakamurai</i>	100	+	10.44 ± 2.06	-	10.65 ± 2.39	17.67 ± 0.58	6.18 ± 1.82	-	-	-	+++	+	+	+++
J39	CR4	<i>Bacillus mycoides</i>	89.9	+	17.25 ± 1.97	+	21.22 ± 2.04	-	9.35 ± 1.73	-	-	++	+++	+	-	++
J31	CS1	<i>Enterobacter bugandensis</i>	99.8	+	-	-	9.26 ± 1.19	16.67 ± 0.58	12.60 ± 3.01	-	-	+++	-	+++	-	-
J32	CS2	<i>Microbacterium paraoxydans</i>	100	+	9.45 ± 1.72	+	31.64 ± 0.82	9.33 ± 0.58	2.01 ± 1.00	+	-	-	+++	-	-	-
J33	CS3	<i>Microbacterium paraoxydans</i>	100	+	10.33 ± 2.33	+	27.64 ± 2.42	5.67 ± 1.15	8.85 ± 2.06	-	-	-	+++	-	-	-
J34	CS4	<i>Bacillus pumilus</i>	98.5	+	19.45 ± 2.48	+	27.79 ± 2.96	1.00 ± 0.00	11.89 ± 1.83	-	-	+++	+++	-	-	+++
J35	CS5	<i>Enterobacter bugandensis</i>	100	-	63.68 ± 2.97	+	31.64 ± 1.22	20.33 ± 0.58	7.34 ± 2.00	-	-	+++	+++	-	-	-
J35B	CS5B	<i>Enterobacter bugandensis</i>	99.4	-	-	-	34.79 ± 1.55	15.67 ± 0.58	9.11 ± 2.11	-	-	-	+++	-	-	-
J27	LL1	Nd	-	-	14.95 ± 1.79	+	34.99 ± 2.15	-	5.03 ± 1.56	+	-	++	+++	+	-	+
J27B	LL1B	<i>Pseudomonas agarici</i>	99.7	+	42.36 ± 2.63	+	23.92 ± 1.72	13.67 ± 0.58	10.24 ± 1.00	+	-	+++	+++	+++	-	++
J28	LL2	Nd	-	+	60.22 ± 1.89	+	23.33 ± 1.83	10.67 ± 0.58	10.83 ± 1.94	-	-	++	+++	+	++	-
J29	LL3	<i>Pseudomonas syringe</i>	99.6	+	3.30 ± 1.01	-	14.02 ± 1.94	11.33 ± 0.58	2.19 ± 0.58	-	-	-	+++	+	-	-
J30	LL4	Nd	-	-	27.36 ± 2.10	+	21.49 ± 1.92	20.67 ± 0.58	6.04 ± 1.00	-	-	+++	+++	+	-	+++
J30B	LL4B	<i>Bacillus haynesii</i>	100	+	22.53 ± 2.98	+	28.46 ± 1.95	20.33 ± 2.08	4.26 ± 1.65	+	-	+++	+++	+	-	-
J22	LR1	<i>Staphylococcus cohnii</i>	100	-	12.09 ± 1.45	-	7.17 ± 0.82	5.33 ± 0.58	7.99 ± 2.13	-	-	++	+++	-	++	++
J23	LR2	Nd	-	-	5.93 ± 0.37	-	22.18 ± 0.74	23.67 ± 3.06	4.15 ± 1.21	-	-	+++	-	+	-	++
J23B	LR2B	Nd	-	-	63.30 ± 3.30	+	33.4 ± 0.59	14.67 ± 1.15	26.27 ± 3.90	-	-	-	-	-	-	-
J24	LR3	<i>Bacillus mycoides</i>	95.9	+	59.45 ± 2.84	+	27.79 ± 1.73	27.00 ± 1.00	5.39 ± 1.80	-	-	-	-	+	-	+
J25	LR4	Nd	-	+	74.29 ± 1.78	+	27.44 ± 2.12	24.00 ± 1.73	6.40 ± 0.99	-	-	-	-	+	++	-
J26	LR5	<i>Robertmurraya siralis</i>	99.1	-	-	+	30.30 ± 1.37	-	14.91 ± 2.65	-	-	+++	-	+++	-	++
J26B	LR5B	<i>Bacillus mycoides</i>	94.6	-	88.57 ± 2.30	-	10.02 ± 1.43	16.67 ± 0.58	0.53 ± 0.00	+	-	-	+++	+	++	-
J15	LS1	<i>Pantoea agglomerans</i>	99.6	-	73.08 ± 2.64	+	14.00 ± 2.53	11.33 ± 0.58	8.28 ± 2.00	+	-	-	+++	-	-	+
J16	LS2	Nd	-	-	20.99 ± 1.88	+	26.50 ± 2.37	15.67 ± 0.58	5.80 ± 1.83	-	-	+++	+++	+	-	-
J17	LS3	<i>Raoultella terrigena</i>	93.2	-	1.92 ± 0.00	+	31.09 ± 1.73	-	7.16 ± 1.67	-	-	-	-	+	-	-
J18	LS4	Nd	-	+	4.45 ± 1.34	-	15.71 ± 1.96	17.67 ± 0.58	1.78 ± 1.00	-	-	+++	-	-	-	-
J19	LS5	Nd	-	+	-	-	18.68 ± 1.04	12.33 ± 0.58	12.01 ± 2.07	-	-	++	-	-	-	-
J19B	LS5B	Nd	-	-	1.04 ± 0.00	+	32.88 ± 1.56	21.33 ± 0.58	7.99 ± 1.00	-	-	+++	-	++	-	+
J20	LS6	<i>Pseudomonas atacamensis</i>	99.4	-	93.24 ± 2.08	-	13.92 ± 1.67	12.67 ± 1.15	11.42 ± 3.0	+	-	++	+++	+	-	-
J21	LS7	<i>Pseudomonas atacamensis</i>	100	-	8.52 ± 1.39	+	22.08 ± 2.06	21.00 ± 1.00	3.96 ± 1.00	+	-	+++	-	++	-	-

J7	SL1	<i>Pantoea eucalypti</i>	99.1	-	51.76 ± 2.17	-	19.26 ± 1.79	-	2.07 ± 0.57	-	-	-	-	-	-	-
J8	SL2	<i>Bacillus cereus</i>	100	+	3.13 ± 1.04	+	31.17 ± 2.32	-	16.27 ± 2.69	-	-	++	+++	-	-	+
J9	SL3	<i>Bacillus cereus</i>	100	+	3.24 ± 1.01	+	22.03 ± 0.97	25 ± 1.73	33.14 ± 1.00	-	-	+++	-	+	-	++
J9B	SL3B	<i>Pantoea vagans</i>	99.8	+	3.68 ± 0.05	-	17.74 ± 1.33	9.67 ± 0.58	4.62 ± 0.94	+	-	-	+++	-	-	-
J10	SL4	<i>Bacillus velezensis</i>	99.8	+	6.26 ± 1.63	+	17.92 ± 1.26	14.00 ± 1.73	9.29 ± 2.56	-	-	-	+++	+	-	-
J10B	SL4B	<i>Bacillus velezensis</i>	100	+	5.66 ± 1.62	-	9.93 ± 1.39	14.33 ± 1.53	11.48 ± 2.06	-	-	-	+++	+	-	-
J11	SL5	<i>Bacillus cereus</i>	100	+	51.81 ± 2.91	-	7.27 ± 1.11	25.67 ± 0.58	3.85 ± 1.00	-	-	+++	+++	+	-	-
J11B	SL5B	<i>Bacillus subtilis</i>	100	+	12.86 ± 1.53	+	13.90 ± 2.60	14.00 ± 1.73	8.88 ± 1.22	-	-	+++	+++	+	-	+
J12	SR1	<i>Enterobacter roggenkampii</i>	100	+	13.02 ± 1.67	+	33.03 ± 1.99	16.33 ± 0.58	6.30 ± 1.06	-	-	-	+++	-	++	-
J12B	SR1B	<i>Bacillus cereus</i>	100	+	61.04 ± 2.46	+	31.07 ± 1.28	25.33 ± 0.58	5.15 ± 1.89	-	-	++	+++	++	++	+
J13	SR2	<i>Pantoea eucrina</i>	100	+	-	+	32.56 ± 2.57	15.00 ± 0.00	7.45 ± 1.22	+	-	-	-	-	-	-
J13B	SR2B	<i>Bacillus cereus</i>	100	+	10.55 ± 1.05	+	30.89 ± 1.83	26.33 ± 0.58	1.71 ± 0.00	-	-	++	-	+	++	+
J14	SR3	<i>Bacillus cereus</i>	100	-	20.11 ± 1.59	+	9.58 ± 1.36	-	5.74 ± 1.00	+	-	+++	+++	+	++	-
J14B	SR3B	<i>Bacillus safensis subsp. safensis</i>	100	+	19.01 ± 2.17	+	31.66 ± 1.24	1.00 ± 1.00	5.33 ± 1.22	+	-	-	-	-	-	-
J1	SS1	<i>Bacillus mycoides</i>	98.5	+	19.67 ± 1.72	+	27.72 ± 1.57	12.67 ± 1.15	2.85 ± 1.03	-	-	+++	+++	+	-	-
J2	SS2	<i>Microvirga calopogonii</i>	99.8	-	13.13 ± 1.04	+	23.75 ± 1.87	-	3.31 ± 1.00	-	-	-	-	-	-	-
J3	SS3	<i>Glutamicibacter mysorens</i>	99.4	-	20.27 ± 2.38	+	21.89 ± 2.36	1.00 ± 0.00	1.63 ± 0.58	-	-	-	+++	-	-	-
J4	SS4	<i>Leclercia adecarboxylata</i>	100	+	-	+	24.84 ± 1.79	1.00 ± 0.00	0.59 ± 0.00	-	-	+++	-	-	-	-
J5	SS5	<i>Bacillus cereus</i>	100.	-	12.09 ± 1.36	-	31.96 ± 3.10	24.67 ± 1.53	7.52 ± 1.39	-	-	++	+++	+	-	+
J6	SS6	<i>Stenotrophomonas maltophilia</i>	99.6	+	5.44 ± 1.03	+	32.41 ± 2.07	10.67 ± 0.58	2.25 ± 1.07	+	-	+++	-	+	-	+++
J6B	SS6B	<i>Enterobacter sichuanensis</i>	98.9	+	2.64 ± 1.10	+	31.29 ± 1.73	14.67 ± 0.58	9.41 ± 1.19	-	-	++	+++	-	-	-

%S- percentage similarity with the sequences in the NCBI GenBank database, Nitrogen fixation (N-f), phosphorus solubilisation (PS), and indole acetic acid (IAA). 1- aminocyclopropane-1-carboxylate deaminase (ACC), hydrocyanic acid (HCN), lipase (Lp), amylase (Am), protease (Pr), gelatinase (Ge) and cellulase (Ce). The N concentration can be obtained by multiplying the concentration of NH₄⁺ (mg N/L) by 0.778. Negative (-), Positive (+), Low (+), Medium (++), High (+++). The organ column is coded as vegetable species (cabbage C, lettuce L and spinach S) followed by organ type (stem S, leaf L or root R) and their numeric/alphabet serial number.

Supplementary Table 5-3: Jaccard similarities index values showing percentage similarity of species in vegetables from two farm locations

Vegetables	Spinach farm J	Lettuce farm J	Cabbage farm J	Spinach farm S	Lettuce farm S	Cabbage farm S
Spinach farm J	100					
Lettuce farm J	4.5	100				
Cabbage farm J	10.5	6.7	100			
Spinach farm S	0	0	0	100		
Lettuce farm S	4.8	0	0	33.3	100	
Cabbage farm S	0	0	0	25.0	27.3	100

Supplementary Table 5-4: Sørensen index values showing percentage similarity of species in vegetables from two farm locations

Vegetables	Spinach farm J	Lettuce farm J	Cabbage farm J	Spinach farm S	Lettuce farm S	Cabbage farm S
Spinach farm J	100					
Lettuce farm J	8.7	100				
Cabbage farm J	19.0	12.5	100			
Spinach farm S	0	0	0	100		
Lettuce farm S	9.1	0	0	50.0	100	
Cabbage farm S	0	0	0	40.0	42.9	100

Supplementary Table 5-5: Significant effect of farm location, vegetable species and organs on endophytic bacterial functional properties

Ammonia (NH ₄)	df	Sum Sq	Mean Sq	F value	Pr(>F)
Farm	2	1.017	0.5084	1.767	0.177
Residuals	83	23.881	0.2877		
Plant	2	0.459	0.2293	0.779	0.462
Residuals	83	24.439	0.2944		
Organ	1	0.077	0.07749	0.262	0.610
Residuals	84	24.82	0.29548		

Soluble PO ₄	df	Sum Sq	Mean Sq	F value	Pr(>F)
Farm	1	492	492.3	8.229	0.005**
Residuals	103	6162	59.8		
Plant	2	90	44.96	0.699	0.500
Residuals	102	6565	64.36		
Organ	2	57	28.38	0.439	0.646
Residuals	102	6598	64.68		

Siderophore	df	Sum Sq	Mean Sq	F value	Pr(>F)
Farm	1	2576	2576.4	41.33	1.57e-08 ***
Residuals	67	4176	62.3		
Plant	2	14	7.19	0.07	0.932
Residuals	66	6738	102.09		
Organ	2	151	75.53	0.755	0.474
Residuals	66	6601	100.02		

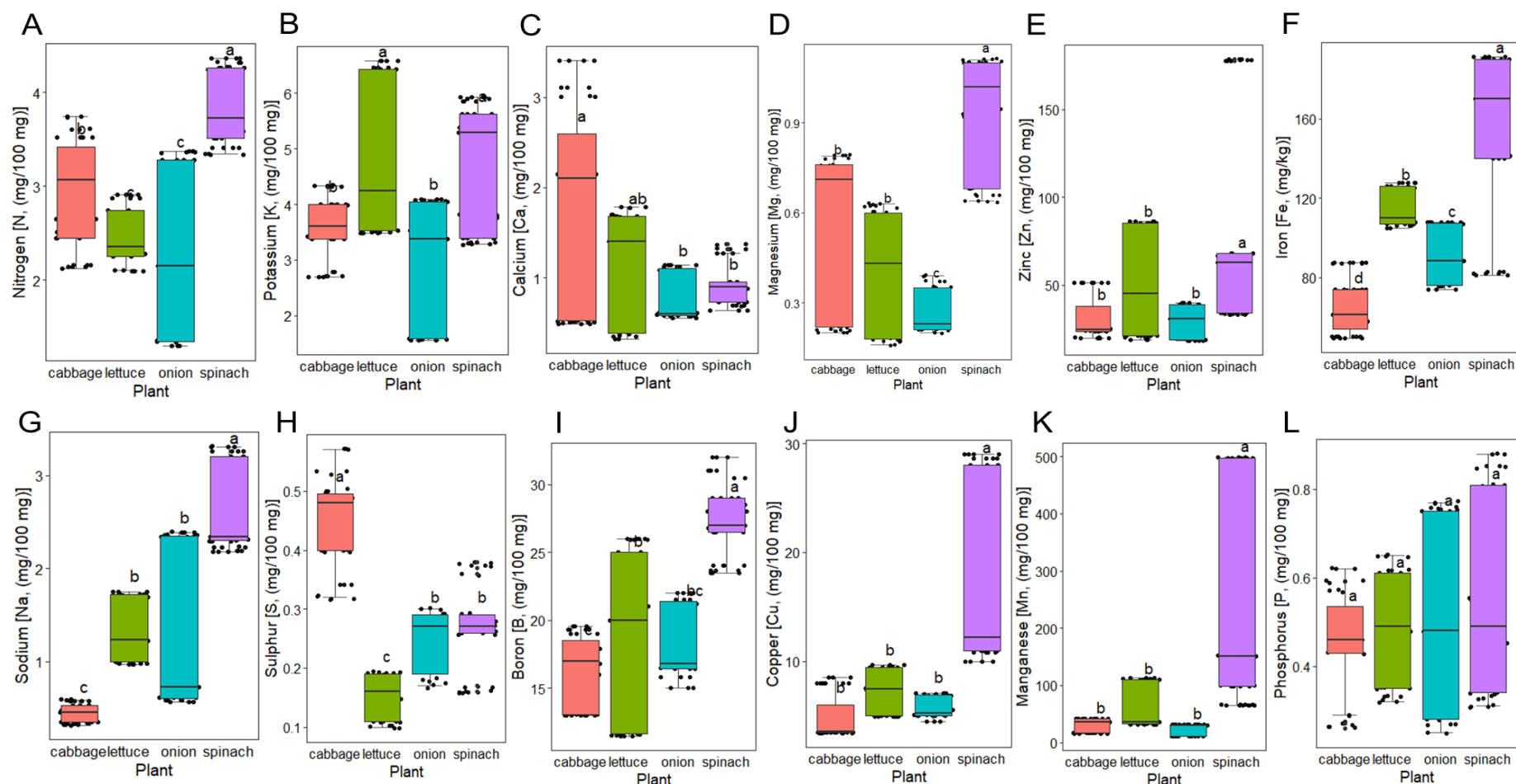
Indole acetic acid	df	Sum Sq	Mean Sq	F value	Pr(>F)
Farm	1	2	1.95	0.038	0.845
Residuals	94	4776	50.81		
Plant	2	197	98.43	1.998	0.141
Residuals	93	4581	49.26		
Organ	2	3	1.45	0.028	0.972
Residuals	93	4775	51.35		

Degree of freedom, df; Sum of squares, sum sq; mean of the sum of squares, mean sq; test statistic; F-value; P-value, Pr(>F). Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

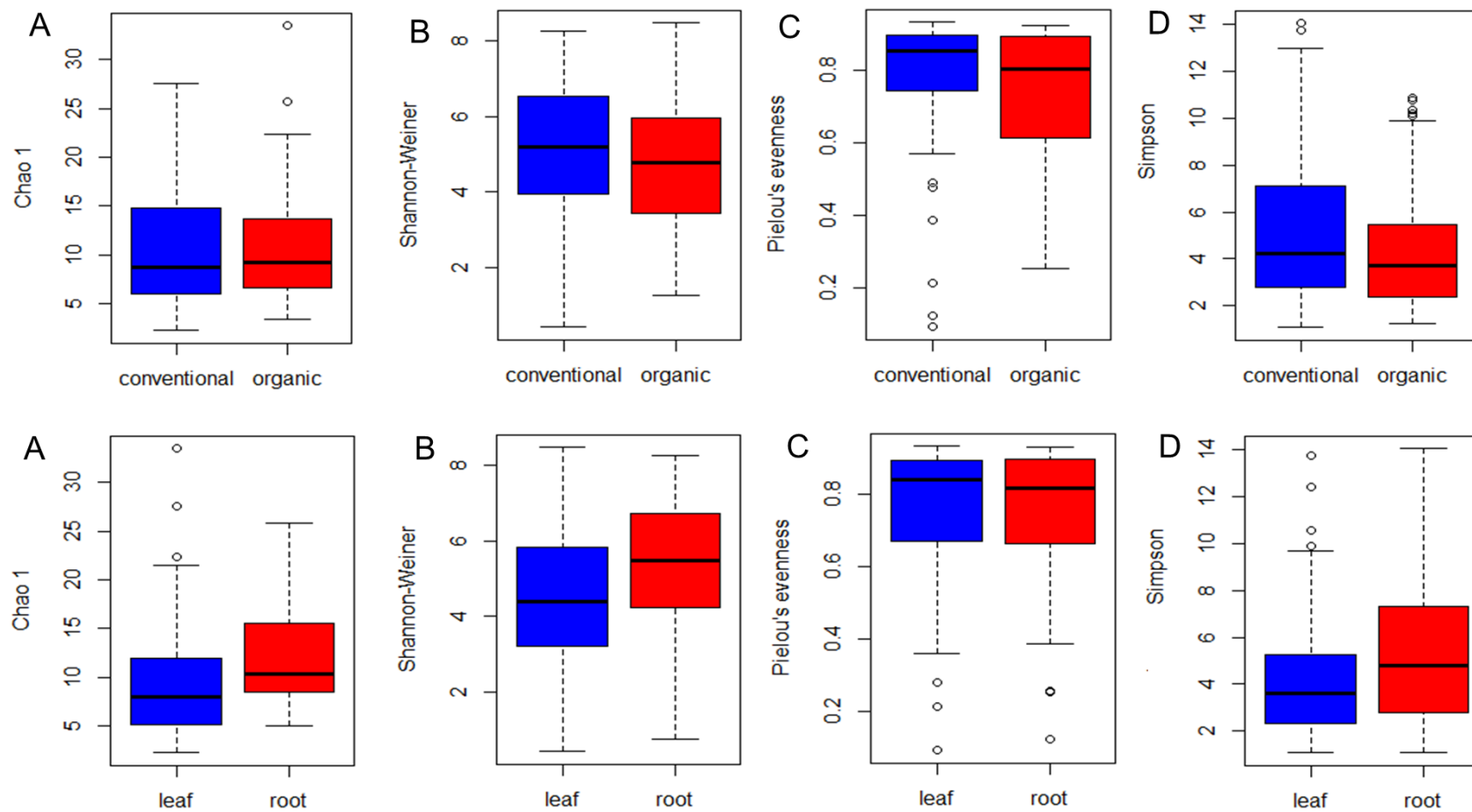
Supplementary Table 6-1: Cytotoxicity of bacterial endophytes

organism	killing Time h	Category
<i>Pseudomonas atacamensis</i>	<1	Very High
<i>Acinetobacter johnsonii</i>	<1	
<i>Staphylococcus cohnii</i>	<1	
<i>Cytobacillus solani</i>	<1	
<i>Bacillus pumilus</i>	<1	
<i>Arthrobacter citreus</i>	2	High
<i>Pseudomonas atacamensis</i>	3	
<i>Lysobacter soli</i>	4	
<i>Microbacterium paraoxydans</i>	5	
<i>Paenibacillus amylolyticus</i>	5	
<i>Pseudomonas syringae</i>	7	medium
<i>Arthrobacter citreu</i>	7	
<i>Neobacillus bataviensis</i>	9	
<i>Bacillus subtilis</i>	10	
<i>Bacillus cereus</i>	12	
<i>Microvirga calopogonii</i>	12	slow
<i>Pseudomonas fluorescens</i>	13	
<i>Bacillus nakamurai</i>	13	
<i>Stenotrophomonas maltophilia</i>	13	
<i>Bacillus subtilis</i>	13	
<i>Rhizobium zeae</i>	14	
<i>Bacillus haynesii</i>	15	
<i>Enterobacter bugandensis</i>	16	
<i>Pseudomonas azotoformans</i>	17	
<i>Serratia liquefaciens</i>	17	
<i>Pantoea conspicua</i>	20 h	
Control	20 h	
<i>Bacillus mycoides</i>	stimulated growth	
<i>Lysinibacillus pakistanensis</i>	stimulated growth	

Supplementary Figures

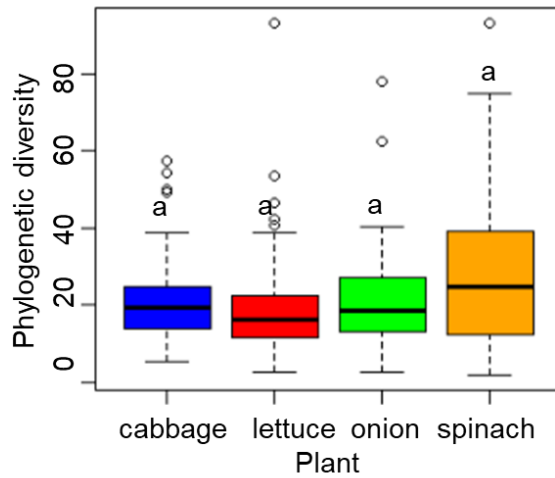


Supplementary Figure 3-1: Selected vegetable nutrient content across the vegetable crops and the statistical significance of their mean values represented with either a, b, c or d to show the main effects across the crop species.

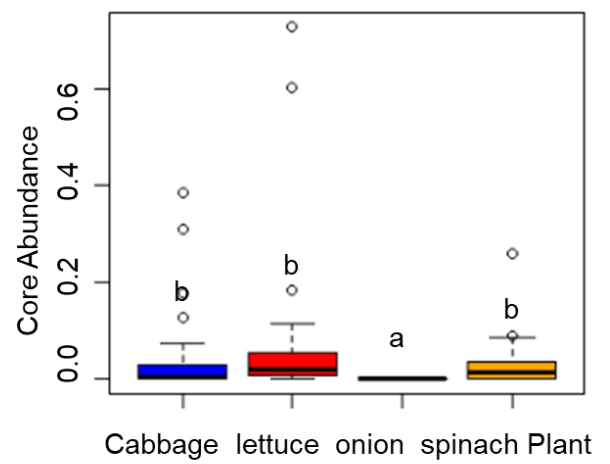


Supplementary Figure 3-2: Alpha diversity measures across fertiliser types (conventional and organic) and organ type (leaf and root) for A) Chao1, B) Shannon-Weiner index, C) Pielou's evenness, and D) Simpson index.

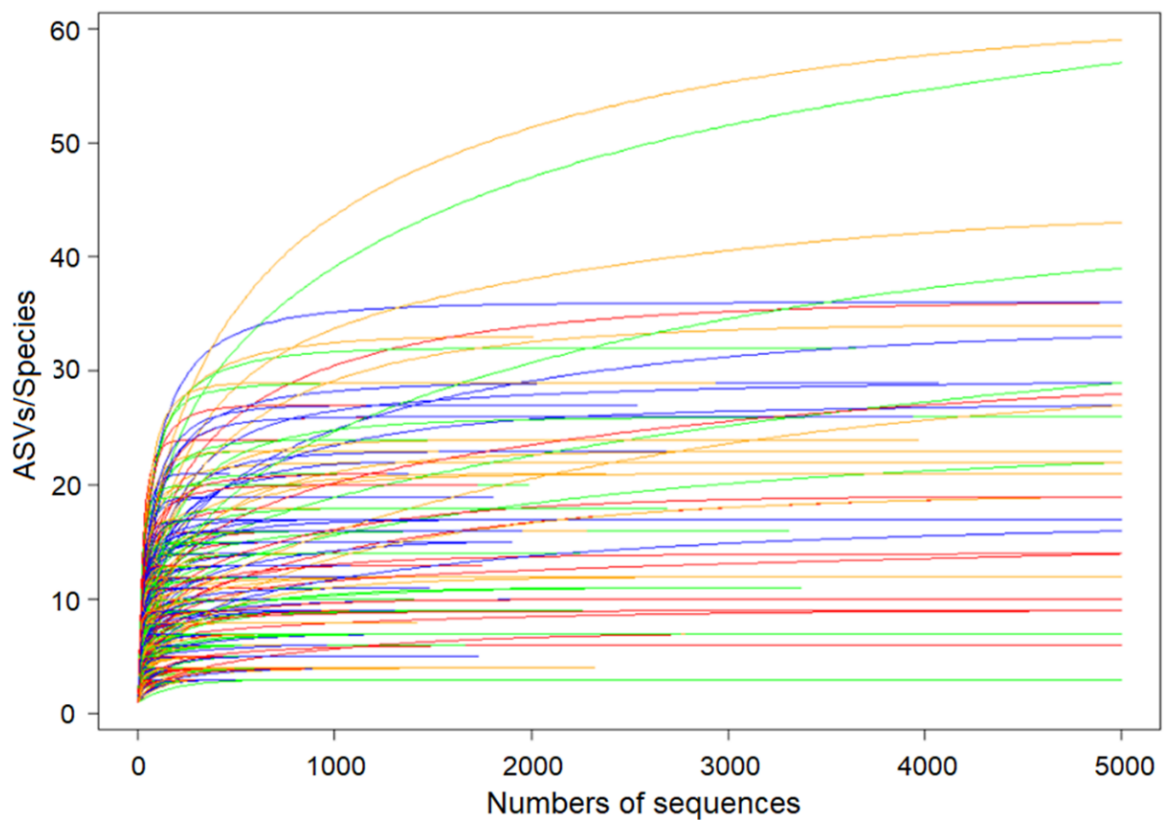
A



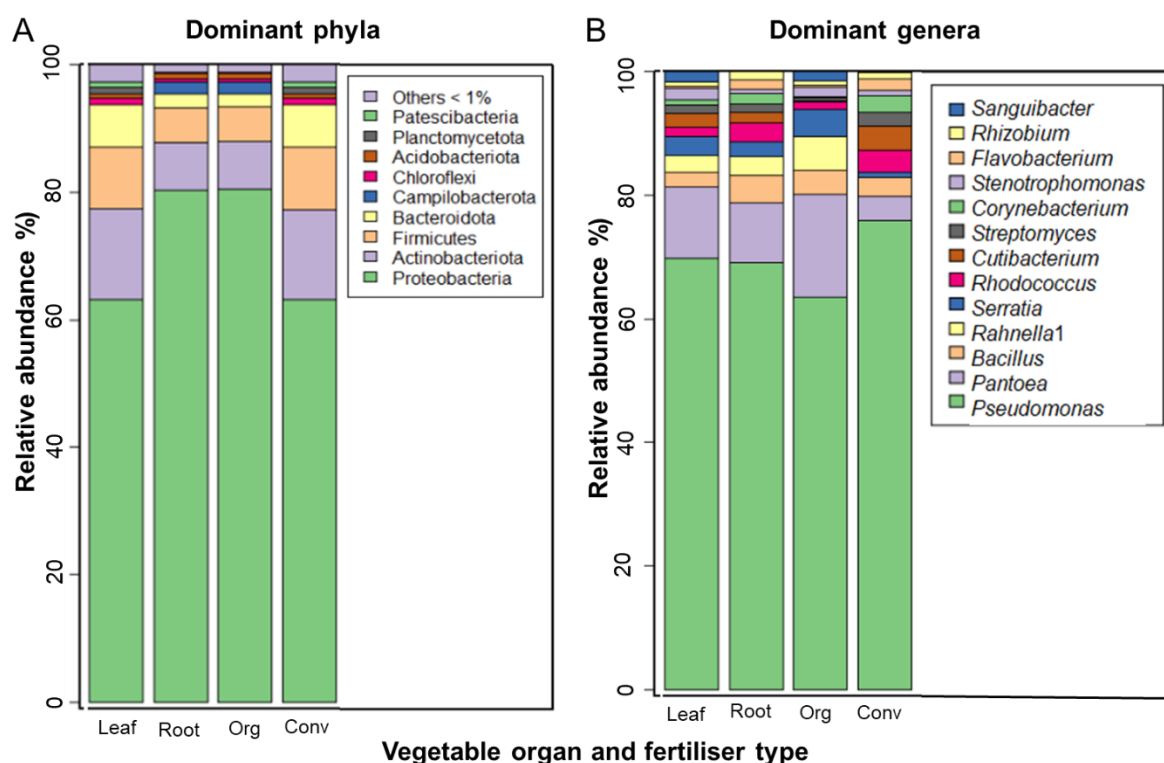
B



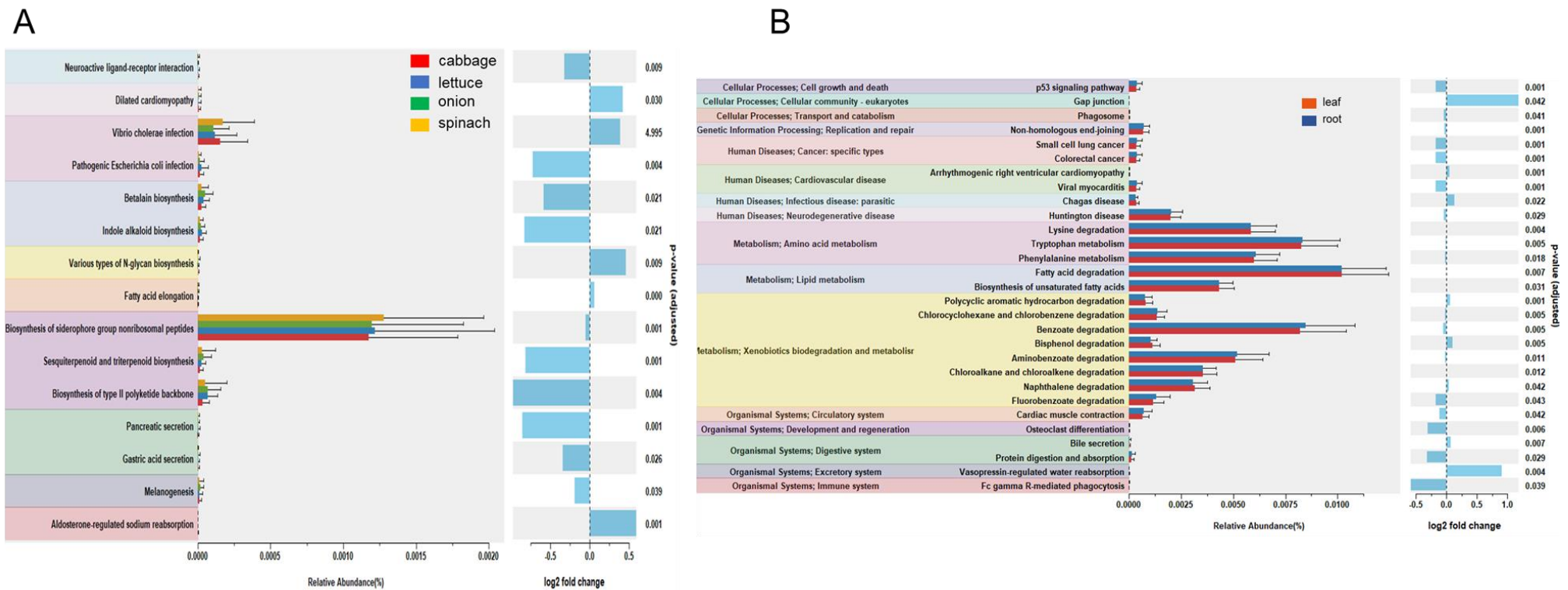
Supplementary Figure 3-3: Alpha diversity measure across the plant species showing A) Phylogenetic diversity B) Core abundance

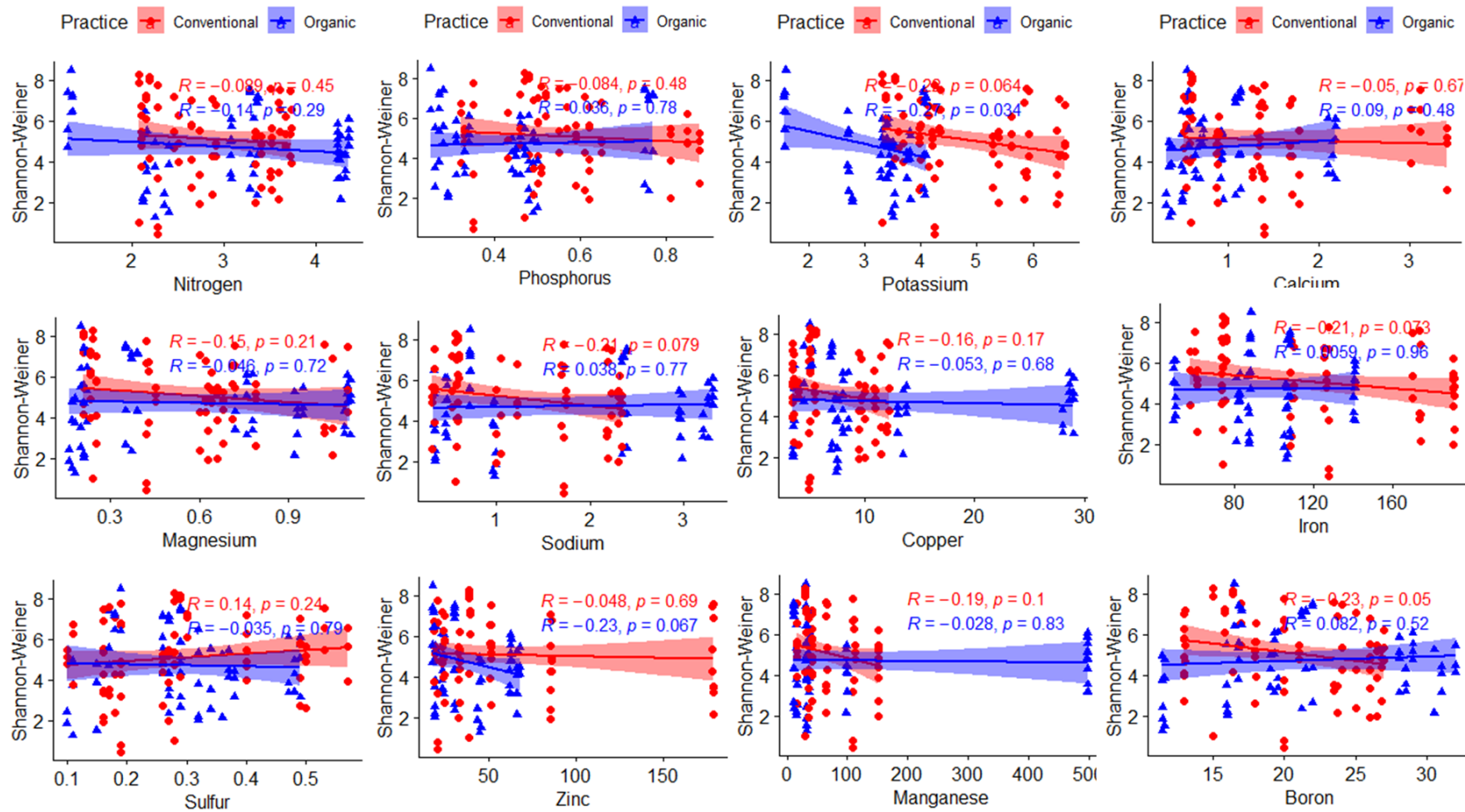


Supplementary Figure 3-4: Rarefaction curve plot for amplicon sequence variants (ASVs) of plant species from organically and conventionally fertilised farms. The rarefaction was subsampled at a depth of 5000.

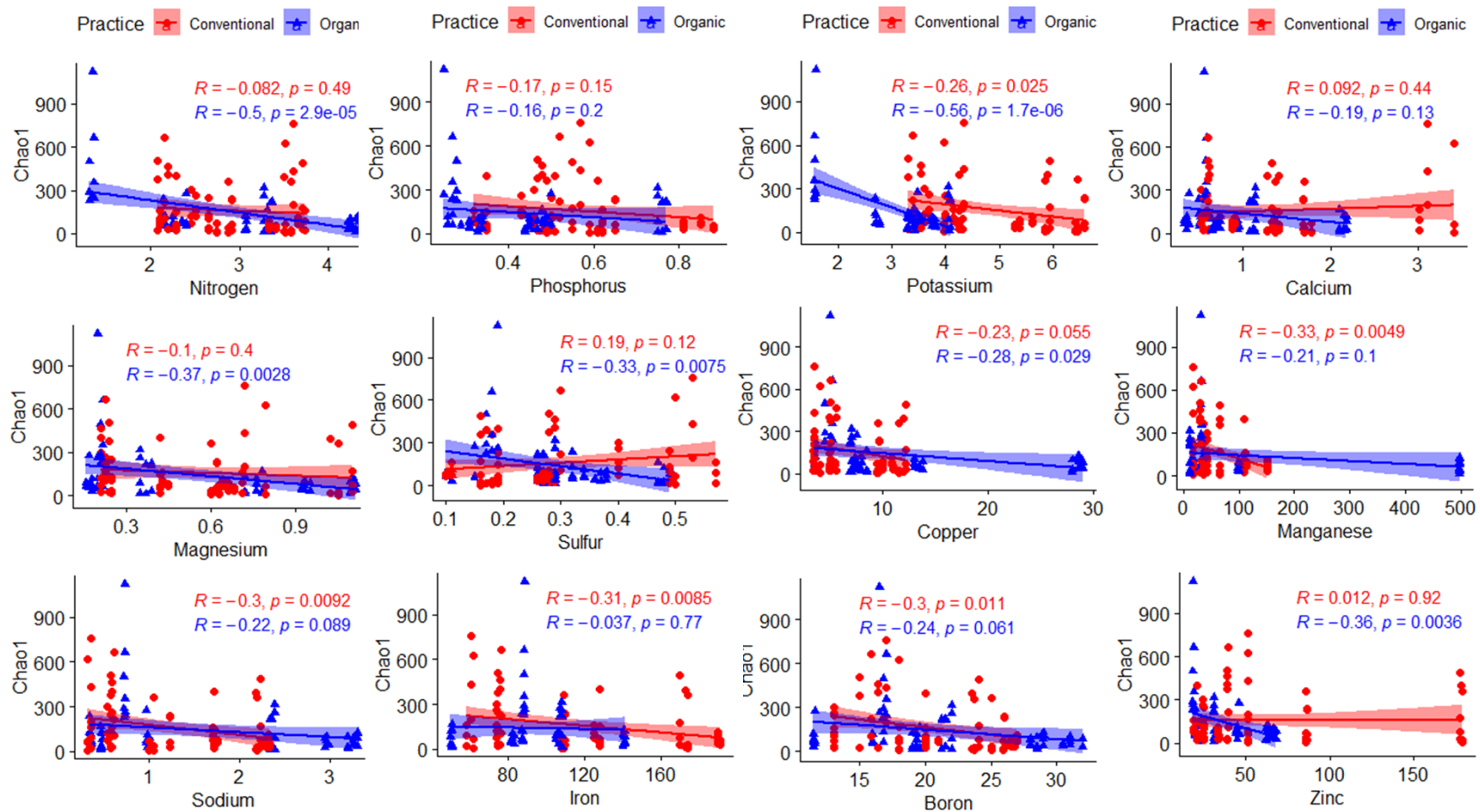


Supplementary Figure 3-5: Relative abundance of endophytic bacterial (> 1% relative abundance) at the a) Dominant phylum and b) Dominant genus taxa. Org; organic fertiliser farm and Conv; conventional fertiliser farm. The relative abundance at the genus level was computed amongst the bacterial communities with above 1% relative abundance after removing the unclassifiable and non-culturable bacterial communities from the data. Each taxon is represented by a different colour while the height represents the relative abundance of the taxon in each sample.

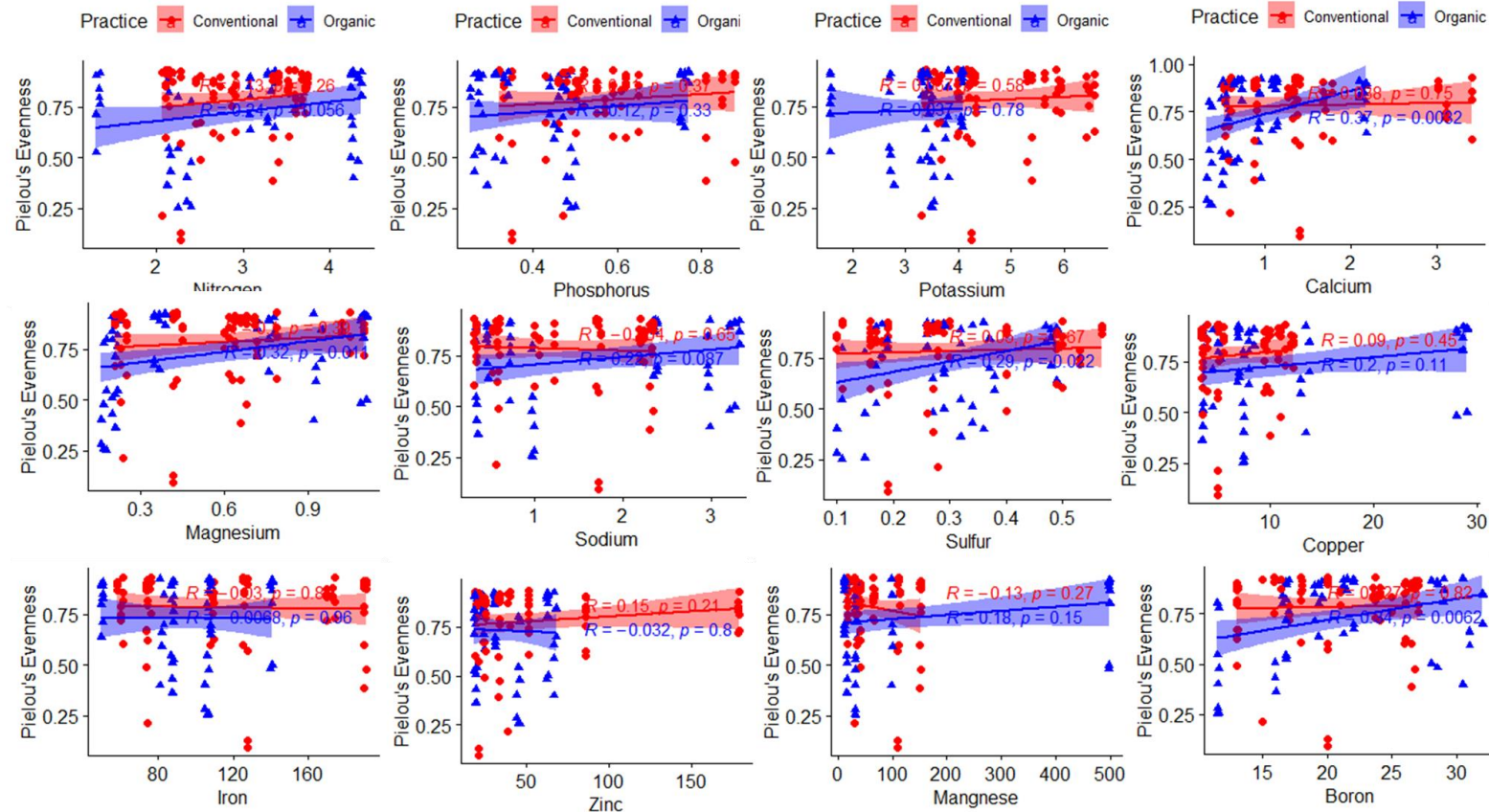




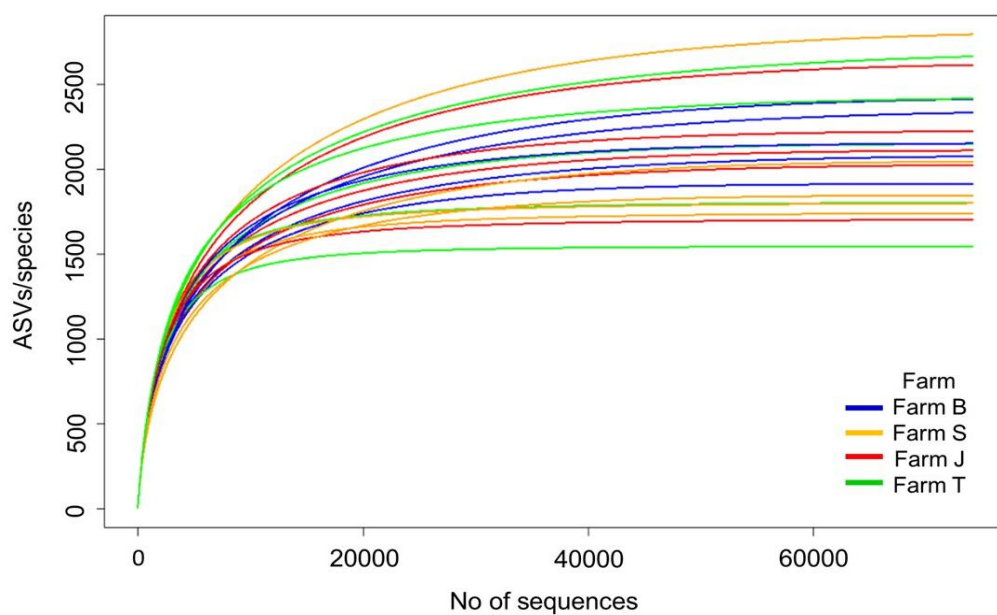
Supplementary Figure 3-7: Relationship between Shannon-Weiner diversity index and vegetable nutrient content



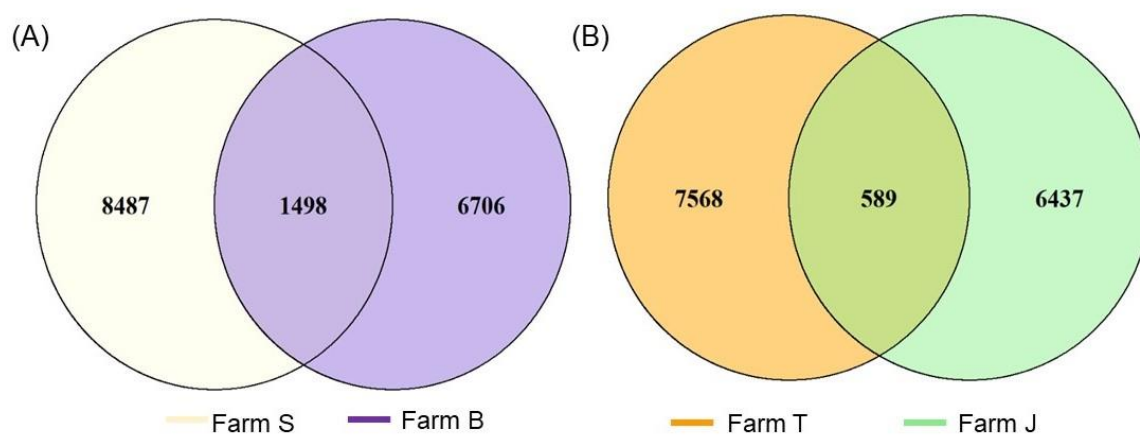
Supplementary Figure 3-8: Relationship between Chao 1 index and vegetable nutrient content



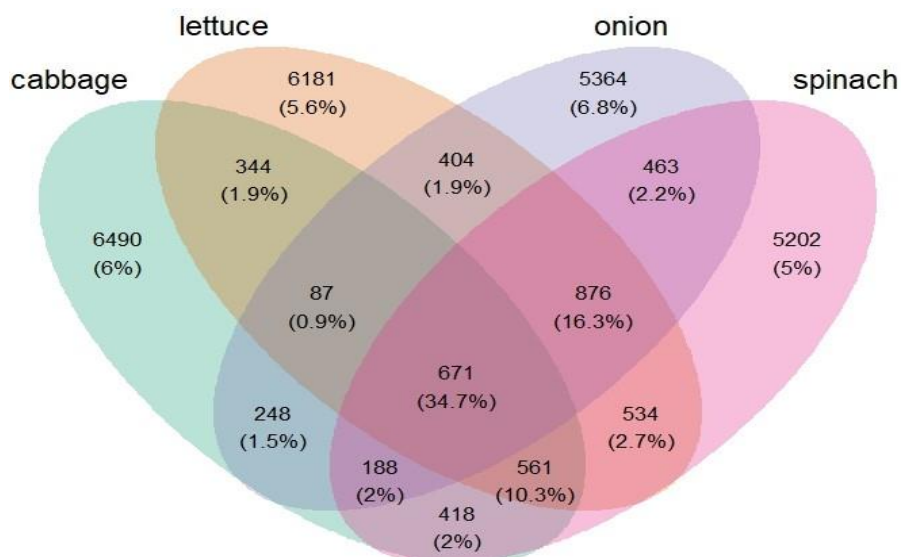
Supplementary Figure 3-9: Relationship between Pielou's evenness and vegetable nutrient content



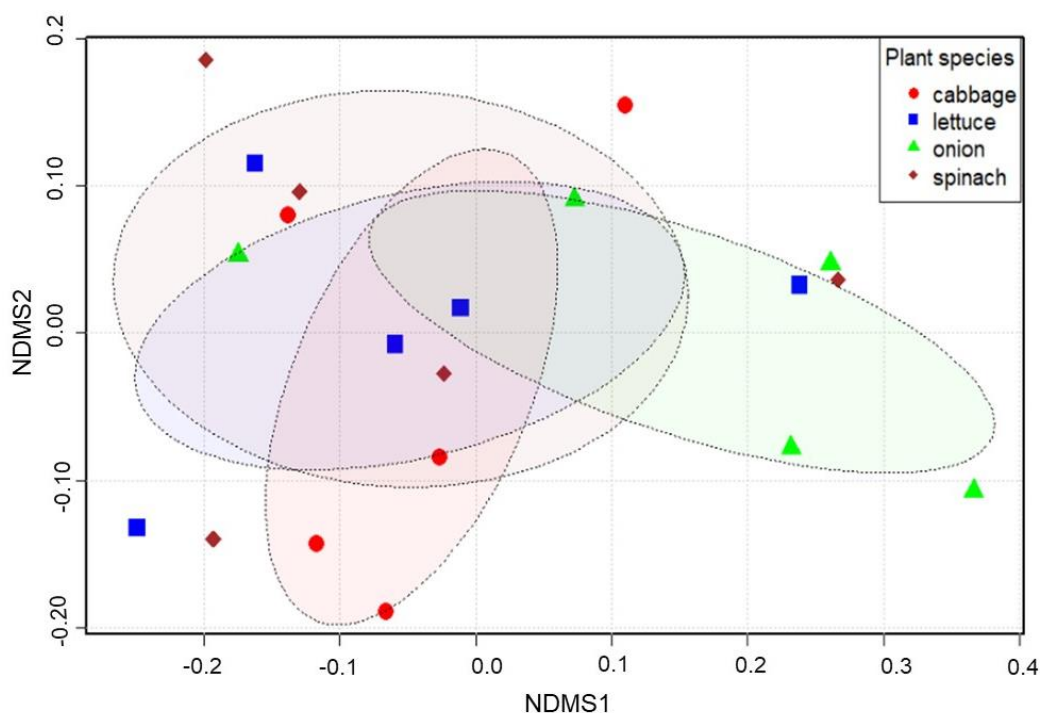
Supplementary Figure 4-1: Rarefaction curve for rhizosphere bacterial communities from different farms. Subsampling of the community was done at an even depth of 74170 sequences per sample. The plateau in the sequence graph indicates that the richness of the community was adequately represented.



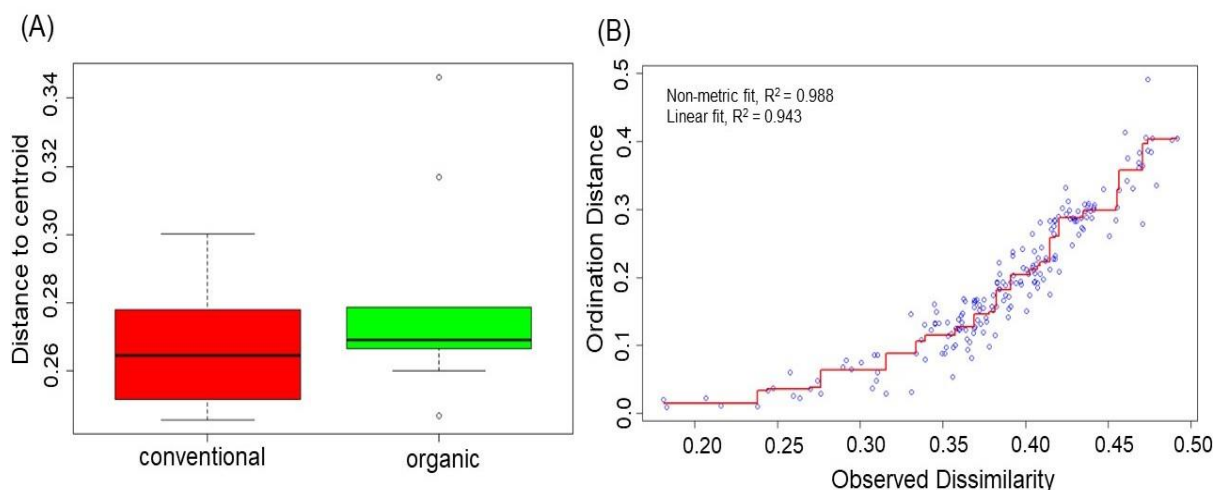
Supplementary Figure 4-2: Comparison of shared and unique amplicon sequence variants (ASVs) in (A) organic farms and (B) conventional farms



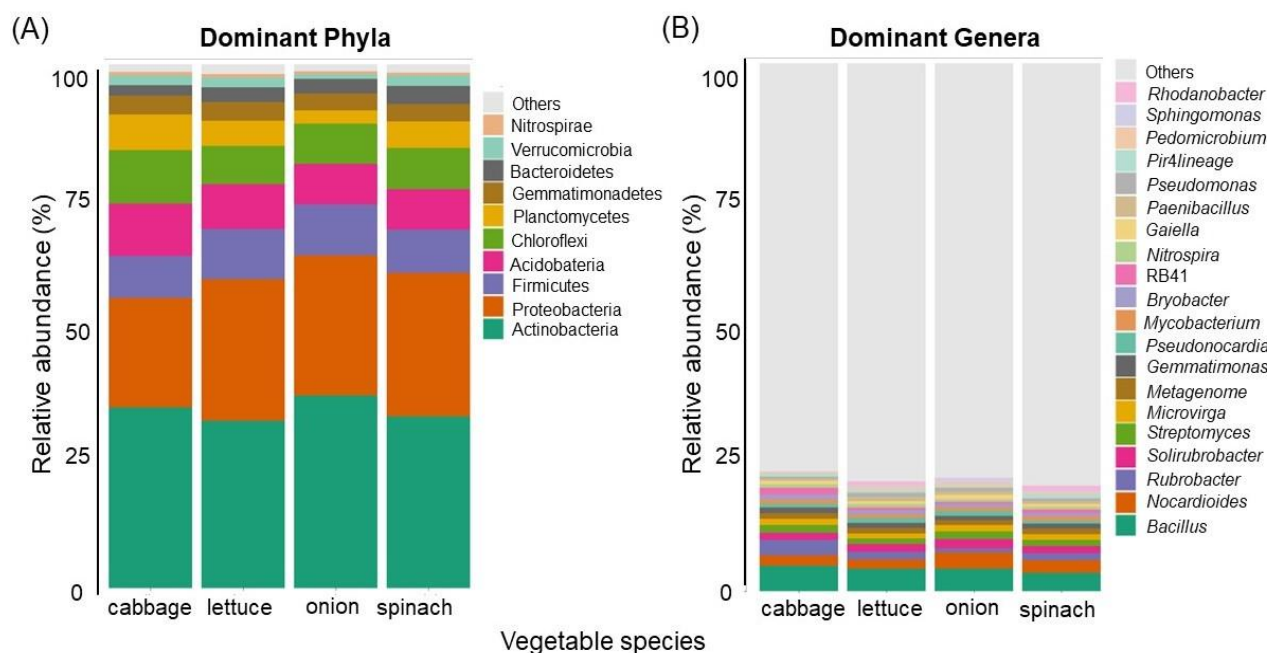
Supplementary Figure 4-3: Unique and shared amplicon sequence variants (ASVs) across the rhizosphere soil of vegetable crops



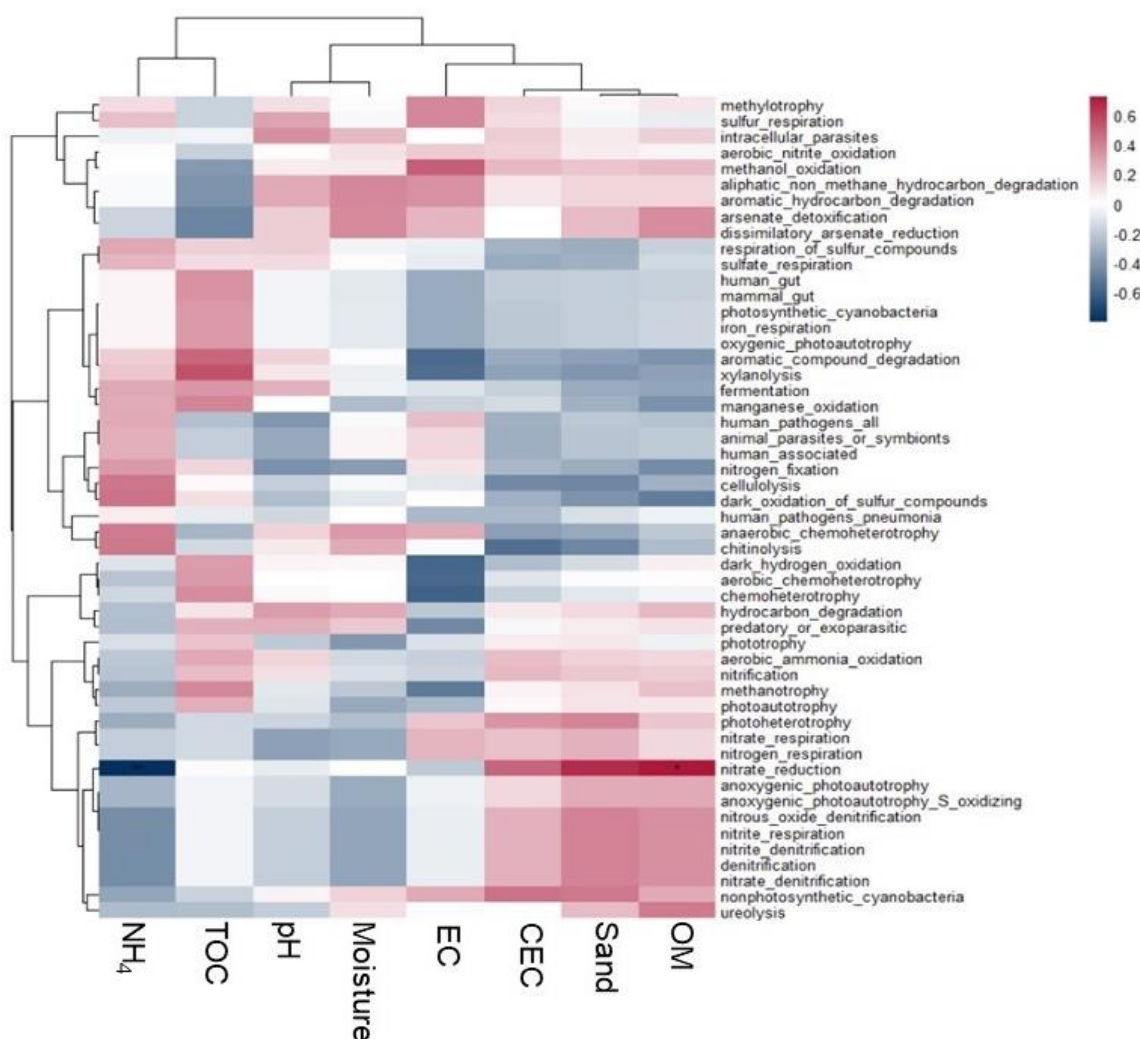
Supplementary Figure 4-4: Bray-Curtis dissimilarity of bacterial communities of rhizosphere soil from different plant species based on non-metric multidimensional scaling (NMDS). The eclipses in the NMDS plot shows 95% confidence intervals (standard error) in multivariate space within the group centroids, The stress plot (Supplementary Figure 4-5B) of the plot showed that the original dissimilarities are well preserved (stress = 0.165047) in the reduced number of dimensions



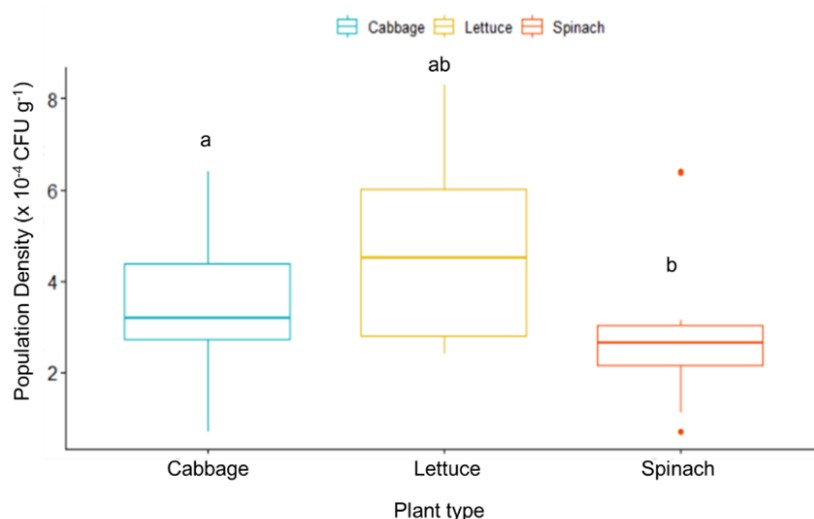
Supplementary Figure 4-5: Rhizosphere bacterial community structure variability in conventional and organic farm rhizosphere soil (A) Average distance of the community dispersion to the median and (B) stress plot. The higher median indicates a high probability of higher community dispersion in organic farms compared to conventional farms. The stress plot was generated from the non-metric multidimensional scaling plot and was constructed with the stress plot () function of the vegan package in R software. The stress was completed at 0.1650471.



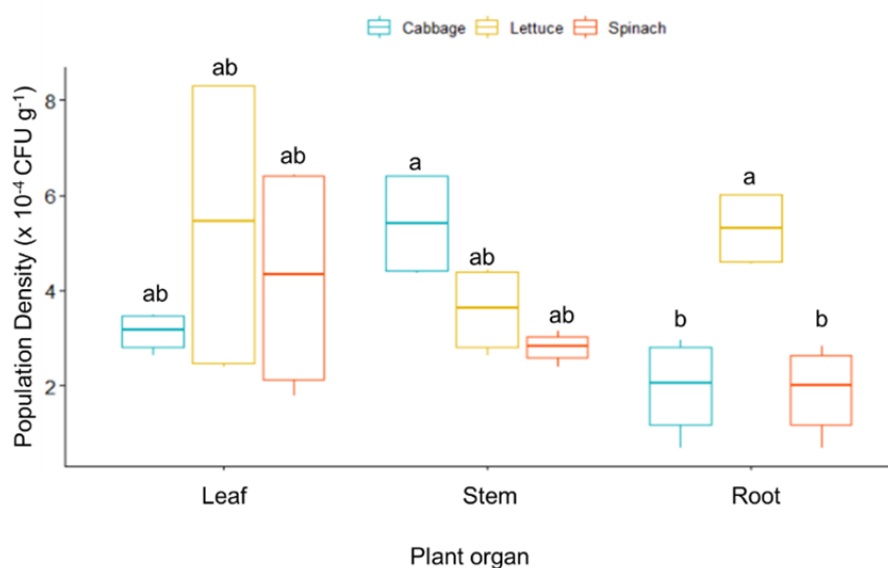
Supplementary Figure 4-6: Average relative abundance of the dominant bacterial community in the rhizosphere soil of different vegetables (A) Dominant phyla (B) Dominant genera taxa level. The phylotypes with average relative abundance below 1% and the unculturable and unclassified at the genus taxa level constitute the others. The bar plots were constructed based on average relative abundance per plant type.



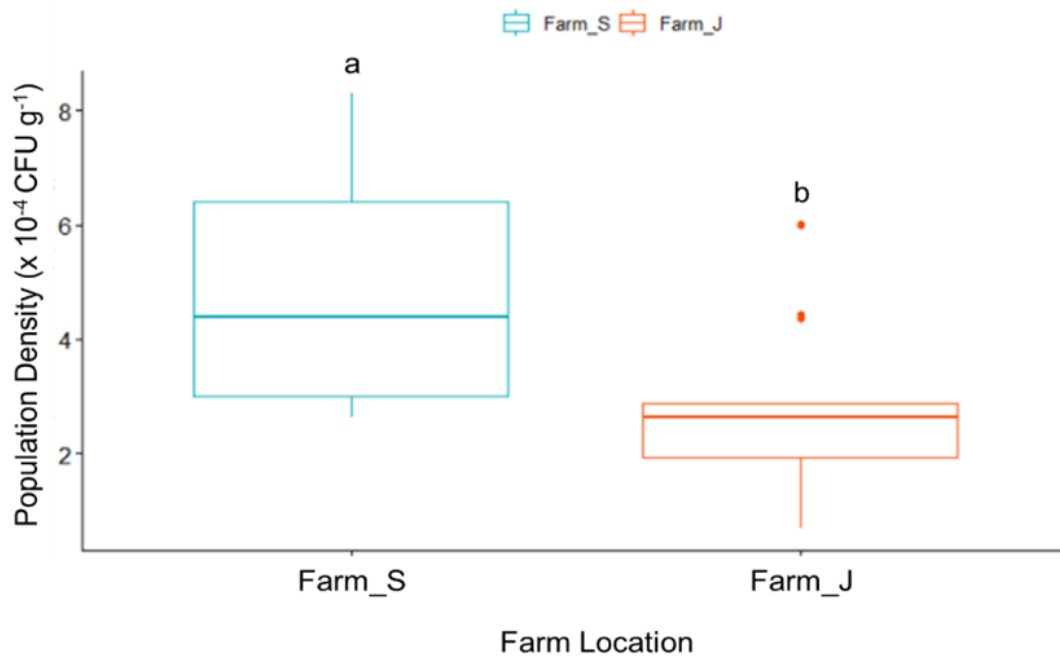
Supplementary Figure 4-7: Correlation between species function percentage of bacterial community with soil properties. NH₄; ammonia, TOC; total organic carbon, EC; electrical conductivity, CEC; cation exchange capacity, OM; organic matter



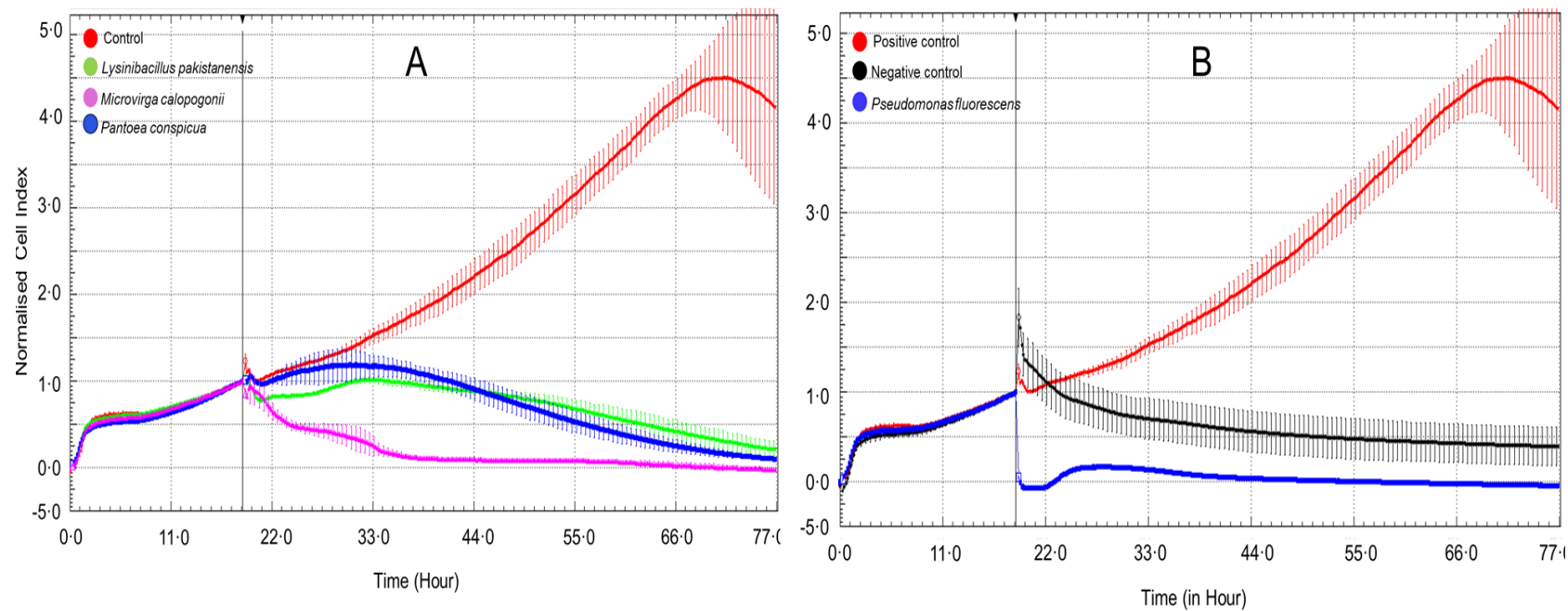
Supplementary Figure 5-1: Box plot showing endophytic bacterial population density across the plant species. The colony count (CFU g⁻¹) was significantly ($P < 0.05$) different across the plant species (Kruskal-Wallis chi-squared = 7.5112, df = 2, P -value = 0.0234). Plants with the same letter are not significantly different. The data were log-transformed using the log () in R.



Supplementary Figure 5-2: Box plot showing endophytic bacterial population density across farm location. The colony count (CFU g⁻¹) was significantly ($P < 0.05$) different across the farm location (Kruskal-Wallis chi-squared = 18.654, df = 1, P -value = 1.57e-05). Farms with similar letter are not significantly different. Data were log-transformed with log () in R.



Supplementary Figure 5-3: Box plot showing endophytic bacterial population density across plant organs. The average colony count (CFU g^{-1}) was not significantly ($P > 0.05$) different across the organs (Kruskal-Wallis chi-squared = 3.0103, $df = 2$, P -value = 0.222). However, when the combined effect of plant species and organ types were considered, the average colony count (CFU g^{-1}) was significantly (Kruskal-Wallis chi-squared = 23.60309, $df = 8$, P -value = 0.0026) different. Plant organs with the same letter are not significantly different. The data were log-transformed using the log () in R.



Supplementary Figure 6-1: Cytotoxicity of bacterial endophytes with gradual activity