Exploring Close-Rotation Microbiome Dysbiosis in Legumes

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Abstract

Food insecurity remains a significant global challenge, with millions of people lacking reliable access to sufficient and nutritious food. Moving towards more sustainable farming systems is one way to address this challenge, as these approaches aim to balance crop production with environment responsibility. Among food crops, legumes are recognised as important source of plant-based protein. However, a major problem for increasing legume production is the decline in yield caused by close rotation. Close rotation, or the repeated cultivation of the same crop on the same land, is a common practice to maximise the crop production. This practice often leads to negative consequences for soil health. However, the underlying impact of soil microbial community has not been well-studied. In this study, the impact of close rotation on the rhizosphere community was investigated. Close rotation was mimicked by replanting pea (*Pisum sativum*) using the same soil throughout three harvests. Through a combination of amplicon sequencing, genotypic profiling, and phenotypic assays, the results showed that close rotation shaped the pea rhizosphere community. Bacterial and fungal diversity decreased after close rotation, and specific taxa groups such as the Proteobacteria were recruited. The enrichment of *Rhizobium* spp. with plant growth–promoting traits were observed. *Pseudomonas* spp. populations displayed back-and-forth dynamics of selection during close rotation. These phenomena suggest adaptive responses within the rhizosphere community. In addition, the bacterial natural community obtained after close rotation was tested to determine the effect on pea health. The result showed that soil isolates can enhance plant growth and exhibited a trend of reduced disease symptoms. This finding illustrates the possibilities for microbiome training under close rotation. Overall, this study contributes to a broader understanding of how cropping practices shape the plant–microbe interactions which could help in developing microbial biocontrol approaches and support more sustainable farming practices.

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Abbreviations

ANOVA	Analysis of Variance
ANOSIM	Analysis of Similarities
ASV	Amplicon sequence variants
CER	Controlled environment room
CFC	cetrimide/fucidin/cephalosporin
CRB	Congo Red binding
DNA	Deoxyribonucleic acid
gDNA	Genomic DNA
HCN	Hydrogen cyanine
IAA	Indole-3-acetic acid
i-TOL	Interactive tree of life
ISR	Induction of systemic resistance
ITS	Internal transcribe spacer
KB	King’s medium B
LB	Lysogenic broth
NatCom	Natural community
OD	Optical density
PBS	phosphate-buffered saline
PDA	Potato Dextrose Agar
PERMANOVA	Permutational Multivariate Analysis of Variance
PGPR	Plant growth-promoting rhizobacteria
RT	Room temperature

SynCom	Synthetic community
TAE	Tris-acetate-EDTA
TY	Tryptone-yeast
YMA	Yeast mannitol agar
YMAA	Yeast mannitol antibiotics agar

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Chapter 1 –
General Introduction

Chapter 1 - Literature review

General background

The global population is steadily rising, with an annual rise of roughly 70–80 million individuals, resulting in increasing stress on agricultural outputs that already face challenges to maintain demand (United Nations Department of & Social, 2024). In 2025, ~319 million individuals in 67 countries experienced acute food insecurity (World Food, 2025). The primary factors contributing to these crises include warfare, economic instability, climate change and displacement, which are worsened by rising costs of goods and inflation (Marzi et al., 2025). Consequently, addressing these problems are essential as food availability and the nutritional quality of diets are inadequate for a significant portion of the world's population that is likely to increase without intervention. One of the largest problems confronting the global population is increasing production of nutritious food to alleviate world hunger and dietary deficiencies whilst minimising negative impacts on the environment (Willett et al., 2019). This issue particularly affects densely populated areas where suitable agricultural land and water are scarce (Food & Agriculture Organization of the United, 2021). As the world's population continues to expand, the world requires farming systems that are both environmentally friendly and productive in the near future. These farming systems need to provide significant increases in yields and improved nutrition without harming ecosystems.

Dietary change is widely recognised as a key step towards future food security, with many studies suggesting that a reduction in animal-based protein will be necessary (Ranganathan et al., 2016). Animal-derived foods generally use more land and water than plant-based ones, and their production places extra pressure on the environment (Espinosa-Marrón et al., 2022). In practice, livestock farming is especially resource intensive because it depends on forage crops and, at the same time, it is a major source of methane, one of the most potent greenhouse gases (Chen & Qi, 2025; Espinosa-Marrón et al., 2022). By contrast, plant-based proteins can provide affordable and healthy nutrition with a much lower environmental cost. This shift in perspective has helped renew interest in legumes, which are being promoted once again as valuable crops for both farmers and consumers (Ferreira et al., 2021).

1.1 Legumes

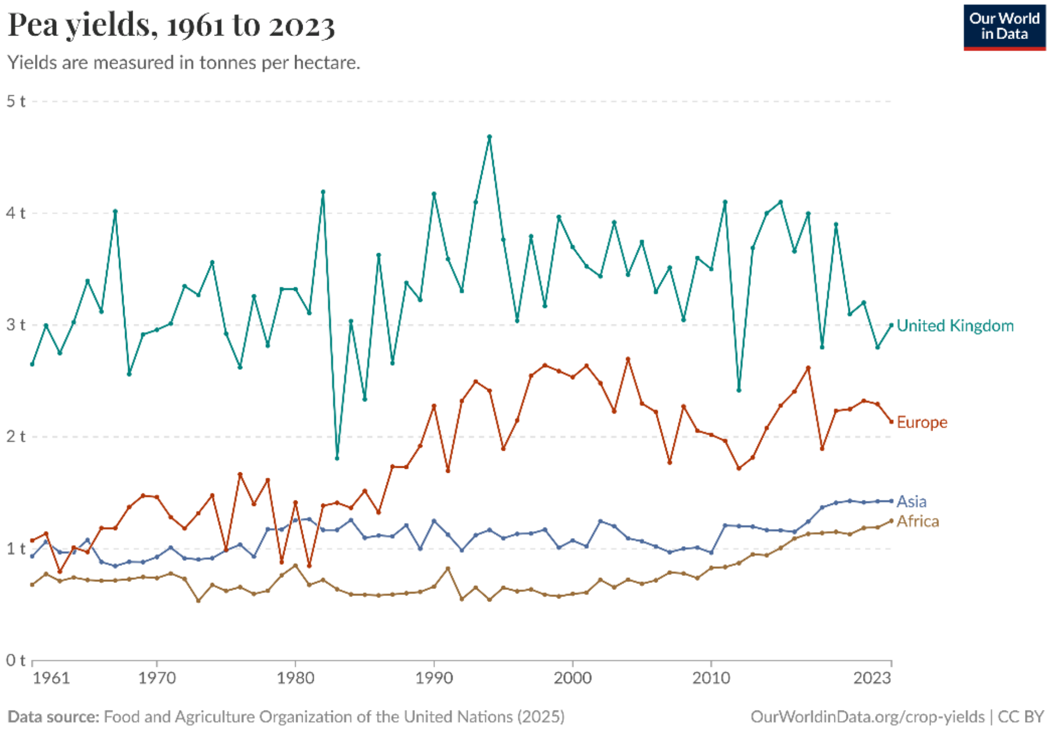
Leguminous plants characterised by their production of seeds in pod and comprises members of the *Fabaceae* family, formerly referred as *Leguminosae*. This family is composed of 670 genera with nearly 20,000 species (Lewis et al., 2005). Legumes are recognised as the third-largest family of angiosperms with the *Papilionoideae* subfamily comprising many economically important species for food, forage, and industrial crops (Kenicer, 2005). Legumes display wide variation in shape, size, colour, and include well-known crops such as chickpeas, beans, peas, lentils, and lupins (Smýkal et al., 2015). Globally, legumes account for around one-third of humanity's direct protein consumption (Smykal et al., 2012).

Pisum sativum, commonly referred as pea, is one of the major food legumes that can grow in different regions (Wu et al., 2023). Peas are a readily available source of protein for human and animal, rich in fibre, and low in sodium and fat (Dahl et al., 2012). A typical pea contains about 50% starch and 24% protein by dry weight (Tzitzikas et al., 2006). Due to their affordability, peas are widely consumed in developing and underdeveloped countries, where they play an essential role in addressing protein-energy malnutrition (Henchion et al., 2017). The United Nations (UN) have recognised peas as a sustainable and nutritious food source for the future (Foyer et al., 2016)

Despite pea's beneficial traits to human health, their production is limited by both biotic and abiotic stresses (Bagheri et al., 2023; Larmure & Munier-Jolain, 2019; Rubiales et al., 2023). Pea plants are vulnerable to numerous bacterial, viral, and fungal pathogens, insect, mite, and nematode pests (Endres & Kandel, 2021). Pea yields are significantly lower than their potential and vary dramatically by area. For example, countries in parts of Africa such as Ethiopia frequently record yields under 1,000 kg/ha, whereas in Western Europe yields may reach 4,000–5,000 kg/ha under favourable conditions (Smykal et al., 2012).

Pea yields show marked regional variation over the past six decades (Figure 1.1). The United Kingdom has regularly attained the highest pea yields relative to other locations. Nevertheless, yields exhibit significant variability, demonstrating considerable year-to-year changes. In contrast, yields throughout Europe have consistently increased from approximately 1 t/ha in the 1960s to 2–2.5 t/ha in recent decades, indicative of progress in agriculture and variety improvement. Asia has maintained yields around 1 t/ha for a significant duration, with only slight improvements post-2010, whereas Africa demonstrates the lowest and most stagnant yields, hardly surpassing 0.8 t/ha. The production rates of field peas in the UK are significantly lower than their biophysical potential. Official statistics indicate average yields of 3.0 t/ha in 2023 and 3.3 t/ha in 2024, exhibiting significant regional variability due to a wet spring that can reduce sowing windows (Department for Environment & Rural, 2024). The combination of inconsistent yields and high vulnerability to soil-borne diseases has discouraged some farmers from continuing pea cultivation.

Pea pathogens include several pathogen groups: (i) The root rot complex, produced by soilborne pathogens like *Aphanomyces euteiches* and fungi such as *Fusarium* spp*.,* *Pythium* spp., and *Rhizoctonia solani*. *Fusarium* spp., specifically *F. avenaceum* and *F. solani* f. sp. *pisi,* are the major pathogens identified in infected roots of commercial peas (Esmaeili Taheri et al., 2017; Feng et al., 2010). Infected plants have water-soaked, brown, and rotted roots, which slow growth, prematurely yellow, and reduce yields by 10–30% under moderate pressure and up to crop failure in conducive settings (Wu et al., 2022) Moreover, *A. euteiches* produces oospores that can survive in soil for over a decade (Gaulin et al., 2007). Root rot pathogens build 10–15-year resting structures in soil, and pea close rotations can increase this disease pressure (Bainard et al., 2017). (ii) The Ascochyta blight complex, caused by a collection of closely related necrotrophic fungi, is one of the most important pea foliar and stem diseases. *A. pinodes*, the most aggressive and widespread species, causes severe epidemics under humid and cool environments (Tivoli & Banniza, 2007). Chickpea, pea, faba bean, and lentil are infected by *A. rabiei* (Pass.) Labr., *A. pisi* Lib., and *A. lentis* Vassiljevsky, respectively (Peever, 2007). In vulnerable cultivars, necrotic lesions on leaves, stems, and pods can cause production and quality losses of over 60% in conducive weather (Bretag et al., 2008). (iii) Fusarium wilt, induced by *Fusarium oxysporum* f. sp. *pisi* (Fop), limits pea cultivation worldwide. Wilting, chlorosis, stunted development, and plant death result from root infection and vascular system colonisation by the pathogen (Chakrapani et al., 2023). Fusarium wilt alone can cause 30–40% yield losses, while synergistic interactions with other diseases can cause near-total crop failure under favourable conditions (Van Haeften et al., 2024). (iv) Pea bacterial blight caused by *Pseudomonas syringae* pv. *pisi* (Psp) (Richardson & Hollaway, 2011). Seed-borne and crop residue-surviving bacteria provide the primary inoculum for epidemics in successive growing seasons (Hollaway et al., 2007). Water-soaked lesions on leaves, stems, and pods grow, consolidate, and girdle tissues, causing extensive necrosis and leaf drop. (v) Pea enation mosaic virus (PEMV) is special because two viruses must work together. Pea enation mosaic virus-1 (PEMV-1) and PEMV-2 (Demler et al., 1993). Several aphid species, like the pea aphid (*Acyrthosiphon pisum*), transmit PEMV persistently, non-propagatively, allowing rapid disease progression in favourable vector settings.


Pea yields, 1961 to 2023

Yields are measured in tonnes per hectare.

Data source: Food and Agriculture Organization of the United Nations (2025)

OurWorldinData.org/crop-yields | CC BY

Figure 1.1 Pea yield from different regions of the world between 1961-2023. Graph shows pea yield in tonnes per hectare. The countries and regions represented are the United Kingdom (green), Europe (orange), Asia (blue), and Africa (brown) (Food & Agriculture Organization of the United, 2025).

1.2 Sustainable and regenerative agriculture

Conventional agriculture, which relies heavily on synthetic fertilisers, pesticides, and monocultures, has succeeded in boosting yields but has also led to environmental degradation, biodiversity loss, soil depletion, and water pollution (Tilman et al., 2002). To effectively meet future food demands, agriculture requires a transition toward practices that balance high productivity with environmental responsibility. Sustainable agriculture attempts to enhance resource-use efficiency, reduce dependence on chemical inputs, improve soil health, and integrate ecological concepts into farming systems (Figure 1.2) (Pretty, 2008). Regenerative agriculture advances beyond simple sustainability by aiming to restore and enhance agricultural systems, so strengthening resilience and supporting climate change mitigation (LaCanne & Lundgren, 2018; Lal, 2020; Newton et al., 2020).

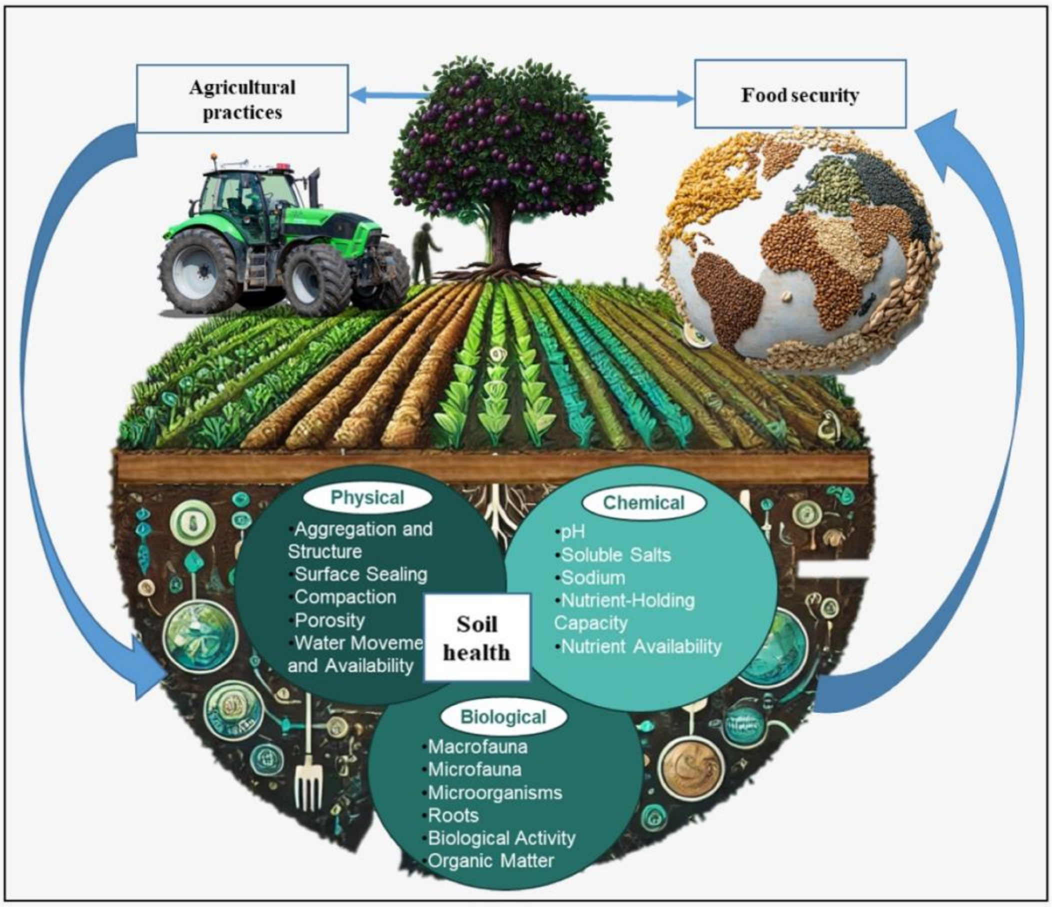


Figure 1.2 Interconnections between soil health, agricultural practices, and food security. Agricultural practices influence soil health which is determined by physical, chemical, and biological properties. The healthy soil support food security (Țopa et al., 2025).

Sustainable agriculture relies on practices that contrast with conventional farming by prioritising long-term soil health and reduced chemical inputs. Among these, crop rotation, the planned alternation of different crops on the same land, has long been recognised as a cornerstone strategy for maintaining soil fertility and ensuring effective land use (Zou et al., 2024). By disrupting pest and disease cycles and enhancing soil nutrient balance, crop rotation helps minimise dependence on synthetic inputs (Ball et al., 2005; Karlen et al., 1994). In many traditional systems, peas are alternated with cereals such as wheat or barley. Although the benefits of crop rotation are well established, close rotation or continuous cropping has still been widely adopted. This practice is characterised by the repeated cultivation of the same crop, or closely related species, over short intervals (Nafziger, 2009). A high demand of pea and farmland shortage can lead to the practice of close rotation (Chen et al., 2009). However, this strategy carries significant long-term risks (Shah et al., 2021).

1.3 Limitations of close rotation

By growing only one crop, farmers can reduce inputs, simplify mechanisation, and make harvesting easier, which may improve short-term efficiency (Durojaiye, 2024). In contrast to conventional crop rotation, which utilises diversity to maintain soil fertility and interrupt pest cycles (Karlen et al., 1994), close rotation carries significant long-term dangers. Continuous monoculture encourages the accumulation of soil-borne pathogens (Abawi & Ludwig, 2005; Bennett et al., 2012; Gossen et al., 2016), intensifies pest pressures, and accelerates nutrient depletion (Elhawat et al., 2025; Kaur et al., 2024). It also promotes microbial dysbiosis, disrupting the balance of beneficial and pathogenic taxa in the rhizosphere, which further compromises plant health and resilience (Xiong et al., 2015). These activities collectively weaken yield stability and jeopardise soil health, creating significant concerns regarding the durability of close rotation despite its immediate benefits.

It has been shown that close rotations involving multiple pulse crops like wheat, pea, lentil and pea again, led to a reduction in fungal evenness and a rise in the relative abundance of disease-causing fungi (Niu et al., 2018). Monoculture systems were found to reduce soil disease suppression capacity, for example *F. oxysporum* was able to more easily colonise the peanut rhizosphere under monocropping, which lead to a negative effect on plant health (Zhou et al., 2023). Previous research found that the highest levels of biological nitrogen fixation (BNF), a process that converts atmospheric nitrogen into plant-available form, were seen in more diverse crop rotations that included wheat and canola every 3 to 4 years. In contrast, growing field peas every year significantly reduced BNF, likely due to nutrient imbalances in the soil. This may also be linked to negative effects from frequent legume residue breakdown, such as an increase in inorganic nitrogen. Overall, pea yield and the benefits of nitrogen fixation were greatest when peas were grown less frequently in the rotation (Knight, 2012). This highlights the strong influence of crop rotation on the rhizosphere, where it shapes and enriches the microbial community (Mendes et al., 2013).

The pea's deep root system is beneficial for crop rotation. Pea symbiotic association with rhizobia leaves residual nitrogen in the soil, which can support the growth of subsequent cereal crops (Goyal et al., 2021). Legumes enrich nitrogen while also improving soil water retention and increasing overall productivity in agricultural systems (Gan et al., 2015; Stagnari et al., 2017). Overall, peas support crop productivity without excessive chemical inputs (Singh et al., 2020). This aligns with the broader vision of sustainable agriculture that emphasises advanced plant breeding, precision farming, and harnessing the plant microbiome to sustain crop performance even under environmental stress (Berg et al., 2014; Singh et al., 2018).

1.4 Plant-associated microbiomes

The microbiome is the collection of microorganisms and their genetic material inside a specific environment (Blaser, 2014). It contains both beneficial bacteria and pathogens, whose interactions affect their hosts in several ways (Aggarwal et al., 2023). Microbial communities function as essential elements of ecosystems. They involve every aspect of life and are intimately linked with humans, animals, and plants (Turnbaugh et al., 2007). Advances in high-throughput sequencing and other molecular technologies have accelerated microbiome research in recent decades (McDaniel et al., 2021). Plant microbiomes are important due to their significance in agriculture, food security and ecological sustainability (Sawant et al., 2025).

Plants serve as complex ecosystems that harbour a vast diversity of microorganisms. These include bacteria, fungi, protists, nematodes, and viruses, collectively known as the plant microbiota (Trivedi et al., 2020). The plant microbiota is organised into specific compartments, each shaping which microbes are present and what functions they perform (Trivedi et al., 2021). Within plant-associated microbiomes, three principal compartments have been identified, each representing a distinct ecological niche that influences microbial composition and function (Dastogeer et al., 2020). (i) The phyllosphere defines the aerial surfaces of plants, including leaves, stems, flowers, and fruits (Vorholt, 2012). (ii) The rhizosphere is the soil region directly next to plant roots, significantly affected by root exudates. This establishes a nutrient-dense environment encouraging to microbial activity (Hartmann et al., 2009; Pierret et al., 2007). Microorganisms in the rhizosphere can boost plant growth by improving nutrient recycling, decomposing organic materials, and suppressing soil diseases (Mendes et al., 2013). The rhizosphere has drawn much interest and is considered as one of the most complex ecosystems on earth (Liu et al., 2023; Philippot et al., 2013). (iii) The endosphere consists of the internal plant tissues, encompassing roots, stems, and leaves (Dastogeer et al., 2020). These microorganisms enhance plant growth by synthesising phytohormones or providing protection against biotic and abiotic stressors (Hardoim et al., 2015).

A two-step model of root microbiome assembly has been widely proposed to explain how plants structure their associated microbial communities (Bulgarelli et al., 2013). In the first step, root exudates including sugars, amino acids, and secondary metabolites reshape microbial composition in the rhizosphere. These compounds act as both nutrient sources and signalling molecules, selectively enriching microbial taxa adapted to utilise them while excluding others, thereby creating a distinct rhizosphere microbiome compared to bulk soil (Hu et al., 2018). The second step involves host-mediated selection at the root surface and within internal root tissues. Plant genetic factors, such as immune signalling pathways and root surface receptors, function as filters that determine which microbial taxa can colonise the root endosphere (Edwards et al., 2015). Cross-species comparisons, where different plant species are grown in the same soil, and soil-transfer experiments, where plants are cultivated in soils of different origins, provide strong support for this model. These studies show that rhizosphere microbial diversity is shaped predominantly by soil type and root exudates, whereas the endosphere community is more strongly governed by host plant genotype (Brown et al., 2020). This selection process emphasises the interplay between environmental and host factors in microbiome assembly. The root–shoot microbiome is often compared to the gut–brain axis in animals because of their systemic effect on plant (Stassen et al., 2021).

Plant growth–promoting rhizobacteria (PGPR) play key roles in plant–soil interactions by facilitating nutrient acquisition, improving soil structure, and regulating extracellular molecules (Backer et al., 2018). Based on their relationship with plants, they are commonly divided into two groups: symbiotic bacteria, such as *Rhizobium*, *Bradyrhizobium*, and *Mesorhizobium*, which establish highly specialised nodulating associations and free-living rhizobacteria, such as *Pseudomonas*, *Bacillus*, and *Azospirillum*, which colonise the rhizosphere without forming nodules (Khan, 2005; Oldroyd, 2013). In addition, many PGPR contribute to disease suppression by competing with pathogens for nutrients and root attachment sites (Yang et al., 2024). Also, they produce a variety of antimicrobial compounds, including antibiotics, siderophores, and lytic enzymes (Compant et al., 2005). Some strains also trigger induced systemic resistance (ISR), a defence response mediated by jasmonic acid and ethylene signalling (Pieterse et al., 2014). Notably, suppressive soil for diseases such as Fusarium wilt or take-all of cereals often harbours higher populations of these beneficial microbes, underscoring their ecological and agronomic importance (Weller et al., 2002).

The composition of plant-associated microbiomes is influenced by both abiotic and biotic factors (Dastogeer et al., 2020). Abiotic factors include growth-limiting nutrients, soil salinity, pH, climate, land use, and crop diversity (Buée et al., 2009; Yan et al., 2021). For example, variations in cover cropping strategies have been demonstrated to influence soil microbial communities (Cappelli et al., 2022; C. H. Wang et al., 2020). Biotic factors include a complex interactions among bacteria, fungi, and protists, are equally important and play critical roles in structuring soil microbiomes and ecological functions (Dastogeer et al., 2020).

In addition to external factors, plants actively influence their associated microbiomes through genetic characteristics and physiological mechanisms. According to many studies, plants release chemicals and signals that affect microbial recruitment. For example, Pacheco-Moreno et al. investigated the influence of barley genotype on microbial community recruitment by comparing Chevallier with a modern cultivar Tipple. It was discovered that Tipple attracted a greater abundance of *Pseudomonas* spp. in the rhizosphere than Chevallier. This recruitment was linked with differences in root exudate composition (Pacheco-Moreno et al., 2024)**.** In *Arabidopsis thaliana*, a key metabolite to shape the root microbiome called triterpenes have been identified (Huang et al., 2019). For pea, several studies indicated specific microbiomes in this plant. Metatranscriptomic comparisons of wheat, oat, and pea grown in the same soil revealed that pea exerted a stronger influence on the rhizosphere and was particularly enriched in fungi (Turner et al., 2013). Molecular hydrogen (H_2) released during nitrogen fixation in legumes can be taken up by soil microbes, leading to an increase in H_2-oxidising bacteria, including some PGPR. This enrichment enhances microbial activity and beneficial traits such as ACC deaminase production, which improves root development and soil health and ultimately supports overall plant growth. (Dong et al., 2003).

Recent advances in soil microbiome research have greatly enhanced our understanding of the complexity and functional diversity of microbial communities (Mauchline & Malone, 2017). High-throughput sequencing techniques, such as amplicon sequencing of the 16S rRNA gene, are widely used to identify and characterise bacterial taxa within soil microbiomes. Tkacz *et al* developed a multi-cropping system and demonstrated, through metatranscriptomic analysis, that both plant type and soil composition interact to shape the recruitment of soil microbial communities (Tkacz et al., 2015). Not only the descriptive studies, but modern molecular tools also provide opportunities for functional insights. For example, CRISPR–Cas genome engineering holds great potential for identifying key plant and microbial genes that regulate rhizosphere interactions (Singh & Ramakrishna, 2021). Microbiome research can be applied to engineer root-associated communities for enhancing plant growth, providing biocontrol against pathogens, and supporting environmental remediation. Hence, a comprehensive study of this topic is both necessary and advantageous for sustainable agriculture.

PGPR comprise a wide range of genera, including *Bacillus*, *Azospirillum*, *Rhizobium*, and *Pseudomonas* (Jeyanthi & Kanimozhi, 2018; Sun et al., 2024). Among these, *Pseudomonas* spp. has emerged as particularly influential in the rhizosphere. Their metabolic versatility and ability to produce antimicrobial compounds make them effective contributors to plant health and resilience (Anuroopa et al., 2022; Mehmood et al., 2023).

1.5 *Pseudomonas* spp.

Pseudomonas is an aerobic, Gram negative, γ-proteobacterial genus that colonises a wide range of plant species. With more than 220 species characterised to date, *Pseudomonas* represents one of the largest and most diverse genera among Gram-negative bacteria (Lalucat et al., 2020). Among plant-associated pseudomonads, the *Pseudomonas fluorescens* complex is particularly important. *P. fluorescens* complex is one of the most diverse groups within the *Pseudomonas* genus and can be classified into eight subgroups (Garrido-Sanz et al., 2017) including *P. fluorescens*, *P. gessardii, P. fragi, P. mandelii, P. jessenii, P.*

koreensis, P. corrugata, P. chlororaphis and *P. protegens*. The name of the group derives from its ability to produce the fluorescent siderophore pyoverdine (also known as pseudobactin), which serves as a key iron-chelating molecule and plays a critical role in both microbial competition and plant–microbe interaction (Cornelis, 2010).

Pseudomonas spp. usually switches from a motile, planktonic lifestyle to a stationary condition during plant colonisation through biofilm formation (Danhorn & Fuqua, 2007). They produce a diverse array of secondary metabolites, including phenazines, phloroglucinols, pyoluteorin, pyrrolnitrin, cyclic lipopeptides, and hydrogen cyanide (HCN), which collectively suppress soil-borne pathogens (Blumer & Haas, 2000; Haas & Défago, 2005). In addition, strains of the *P. fluorescens* complex can stimulate plant growth and induce systemic resistance by production of plant growth-promoting substances such as indole-3-acetic acid (IAA), gibberellins, cytokinins, and volatile organic compounds (VOCs) like 2,3-butanediol and acetoin (Santoyo et al., 2012).

From an applied perspective, *Pseudomonas* spp. is increasingly recognised as promising agents for sustainable agriculture due to their wide-ranging functional capabilities (Alattas et al., 2024). As biocontrol agents, *Pseudomonas* spp. suppresses phytopathogens through the production of antibiotics, siderophores, volatile organic compounds, and lytic enzymes, while also inducing systemic resistance in plants (Hernández-León et al., 2015; Leontidou et al., 2020). Genetic comparison of strains that controlled *Streptomyces scabies* growth highlighted that cyclic lipopeptides are key compounds that mediate this phenotype. Irrigation of the field was also shown to influence the population of *Pseudomonas*, with changes in water availability significantly affecting its abundance and distribution in the soil (Pacheco-Moreno et al., 2021).

Moreover, *P. fluorescens* is also known to interact with other beneficial microbes, including nitrogen-fixing *Rhizobium* spp., potentially enhancing nodulation efficiency and plant nutrient acquisition (Andrade et al., 1998; Yu et al., 2025). The interactions between *Rhizobium* spp. and *Pseudomonas* spp. are not independent but can be synergistic with both contributing to improved plant growth (Egamberdieva et al., 2010). Understanding the coexistence and interactions of these microbial partners within the pea microbiome will provide essential insights into the formation of advantageous root communities.

1.6 *Rhizobium* spp.

Among symbiotic diazotrophs, the rhizobia represent a highly diverse group within the class *Alphaproteobacteria*. Taxonomically, rhizobia span several families, including Rhizobiaceae (*Rhizobium, Sinorhizobium, Allorhizobium, Pararhizobium, Neorhizobium, Shinella*), Phyllobacteriaceae (*Mesorhizobium, Aminobacter, Phyllobacterium*), Brucellaceae (*Ochrobactrum*), Methylobacteriaceae (*Methylobacterium, Microvirga*), Bradyrhizobiaceae (*Bradyrhizobium*), Xanthobacteraceae (*Azorhizobium*), and Hyphomicrobiaceae (*Devosia)* (Lindström & Mousavi, 2020).

Rhizobium is a genus of Gram-negative, motile, rod-shaped bacteria renowned for their ability to form nitrogen-fixing symbioses with legumes *(Young et al., 2001)* . *R. leguminosarum* is one of the well-known species (Terpolilli et al., 2012). *R. leguminosarum* is linked with various legume species, including pea, field bean (*Vicia faba*), and hairy vetch (*Vicia hirsuta*) (Macdonald et al., 2011). Research on the root microbiome of red clover (*Trifolium pratense*) indicates that rhizobia can predominate within the microbial community, constituting approximately 70% of the root-associated bacterial population. These findings emphasise the ecological dominance of rhizobia in legume root niches (Hartman et al., 2017).

The process of nodule formation is a complex and highly regulated interaction between the plant host and rhizobia (Jones et al., 2007). It begins when the plant secretes specific flavonoids into the rhizosphere, which are recognised by rhizobia *nod* genes. This induction triggers the synthesis of lipochitooligosaccharides (LCOs), also known as Nod factors (Checcucci & Marchetti, 2020). Nod factors act as key signalling molecules, stimulating cortical cell division in the root and leading to the differentiation of specialised nodule tissues (Poole et al., 2018). Each nodule can contain up to 10^9 nitrogen-fixing bacterial cells. Nodules are usually red in colour because they contain leghaemoglobin, a plant-derived haemoprotein that helps regulate oxygen levels and allows oxygen to diffuse in a controlled way to the symbiosomes, where nitrogen fixation occurs (Ledermann et al., 2021). The biochemical basis of nitrogen fixation is carried out by the nitrogenase enzyme complex, which is composed of six protein subunits, two each of NifH, NifD, and NifK. This enzyme catalyses the energetically demanding reduction of atmospheric nitrogen (N_2) to ammonia (NH_3), which plants can assimilate into organic forms (Poole et al., 2018). Many of the symbiosis genes required for nodulation and nitrogen fixation are located on mobile genetic elements such as plasmids or symbiosis islands (Galibert et al., 2001). Thus, they can move horizontally within and between rhizobia populations, contributing to both ecological adaptation and evolutionary process (Wardell et al., 2021). The specificity of rhizobia interactions is mediated by the NodD transcriptional regulators, which respond to sets of plant flavonoids (Yu & Zhu, 2025). Taken together, the study of *Rhizobium* spp. reveals their ecological importance in legume root systems.

1.7 Objectives

To achieve sustainable agriculture and meet the rising demand for plant-based protein and food in general, it is essential to optimise the role of legumes such as pea within cropping systems. While traditional crop rotation is well recognised for maintaining soil fertility and crop health, the impact of close rotation on pea-associated bacterial and fungal communities remains poorly understood. Elucidating the dynamics of the rhizosphere microbiome has therefore become a key research objective. The investigation was structured around these main objectives.

1. Investigate pea plant health under close rotation and observe the dynamic changes in the pea rhizosphere community, including bacterial and fungal communities, together with soil nutrient shifts.
2. Evaluate the impact of close rotation on pea rhizosphere microbiome structure and composition using both culture-dependent and culture-independent approaches.
3. Investigate the colonisation of *Pseudomonas* spp. and *Rhizobium* spp. strains across harvests under close rotation, focusing on both phenotypic and species-level selection.
4. Explore the influences of close rotation-derived microbial community on pea plant health.

Chapter 2 -

Materials and Methods

Chapter 2 – Materials and Methods

2.1 Growth Media

2.1.1 *Pseudomonas* selective agar (CFC agar)

CFC agar was prepared using 24.5 g of ready-made *Pseudomonas* agar base (CM0559, Oxoid, UK) per 500 mL deionised water, followed by 5 mL glycerol. The media was sterilised via autoclaving at 15 psi and 121°C for 15 minutes. SR0103 CFC solution was prepared by aseptically adding 2 mL 50% ethanol to SR0103 CFC (cetrimide-fucidin-cephalosporin) supplement vial (Oxoid, UK) and added to 500 mL molten agar cooled down to 50°C for final concentrations of 10 mg/L cetrimide, 10 mg/L fucidin, and 50 mg/L cephalosporin.

2.1.2 *Rhizobium* semi-selective media

A semi-selective medium was prepared using deionised water with the following composition: 1 g/L mannitol, 178.5 mg/L anhydrous Na_2PO_4, 100 mg/L $MgSO_4 \cdot 7H_2O$, 33.25 mg/L $FeCl_3 \cdot 6H_2O$, 53 mg/L $CaCl_2 \cdot 2H_2O$, and 500 mg/L NH_4NO_3, with the pH adjusted to 6.8. After pH adjustment, 15 g/L agar (Formedium, UK) was added. The media was sterilised via autoclaving at 15 psi and 121°C for 15 minutes.

The vitamin solution was prepared in deionised water with following composition: 50 mg/L biotin, 50 mg/L thiamin, and 50 mg/L calcium pantothenate. The vitamin solution was sterilised by membrane filtering (pore size 0.22 μm), and 1 mL added to per 100 mL molten agar cooled down to 50°C.

To inhibit unwanted microbial growth, antimicrobials for final concentrations 100 mg/L cycloheximide, 3 mg/L penicillin G, 2.5 mg/L neomycin, 0.003% Congo red, and 0.003% Malachite green (Louvrier et al., 1995; Soenens & Imperial, 2018).

2.1.3 Yeast mannitol broth (YMB)

YMB medium was prepared by dissolving 12.8 g of ready-made YMB powder (NutriSelect®Plus) per litre of deionised water. Heat was applied as necessary to ensure complete dissolution. Component concentration was 10 g/L mannitol, 0.2 g/L K_2HPO_4, 0.1 g/L of NaCl, 0.2 g/L $MgSO_4$, 1.0 g/L yeast extract, and 1.0 g/L $CaCO_3$. Yeast mannitol antibiotics agar (YMAA) was prepared for *Rhizobium* isolation by adding 15 g/L agar

(Formedium, UK). The media was sterilised via autoclaving at 15 psi and 121°C for 15 minutes.

2.1.4 Potato dextrose agar (PDA)

PDA medium was prepared by dissolving 39 g of ready-made PDA powder (NutriSelect$^{®}$Plus) per litre of deionised water. The media was sterilised via autoclaving at 15 psi and 121°C for 15 minutes.

2.1.5 Tryptone yeast (TY)

TY medium was prepared using deionised water with the following composition: 5.0 g/L Tryptone (Merck Millipore), 3.0 g/L Yeast extract (Merck Millipore), 0.9 g/L $CaCl_2 \cdot 2H_2O$, with the pH adjusted to pH 6.8. The media was sterilised via autoclaving at 15 psi and 121°C for 15 minutes.

2.1.6 Lysogeny broth (LB)

LB Miller was prepared by dissolving 25 g of ready-made powder (Formedium, UK) per litre of deionised water. Component concentration were 10 g/L peptone from casein (tryptone), 5 g/L yeast extract, and 10 g/L NaCl (Miller, 1972). For solid media, 11 g Agar (Formedium, UK) was added per litre of deionised water. The media was sterilised via autoclaving at 15 psi and 121°C for 15 minutes.

2.1.7 King's medium B (KB)

KB media was prepared using deionised water with the following composition: 10 g/L of protease peptone, 1.5 g/L $MgSO_4$, 1 g/L K_2HPO_4, and 10% glycerol (King et al., 1954). For solid media, 16 g Agar (Formedium, UK) was added per litre of deionised water. The media was sterilised via autoclaving at 15 psi and 121°C for 15 minutes.

2.2 Fungus strain

Fusarium solani f. sp. *pisi* was obtained from National Institute of Agricultural Botany (NIAB) by isolation from pea roots in 2021.

2.3 Primers

Table 2.1 Primer pairs used in this study

Primer Name	Target (reference)	Primer sequences
F27	Universal 16sRNA bacterial (Busti et al., 2002)	AGAGTTTGATCMTGGCTCAG
R1492		TACGGYTACCTTGTTACGACTT
F979	*Rhizobium* genus (Macdonald et al., 2011)	CCCGGCTACYTGCAGAGATG
R1264		TAGCTCACACTCGCGTGCTC
F88	*NodD* (Macdonald et al., 2011)	TGCAGAGACGGGAGCTARTTC
R443		GACGCACACCAGTCTCTCTTCG
Sol_Forward	*Fusarium solani* (Willsey et al., 2018)	GCGCCTTACTATCCCACATC
Sol_Reverse		TTTTGTGACTCGGGAGAAGC
rpoB Uni_F	Amplicon sequencing (Z. Wang et al., 2020)	GGYTWYGAAGTNCGHGACGTDCA
rpoBUni_R		TGACGYTGCATGTTBGMRCCCATMA

2.4 Bacterial sample collection and isolation

2.4.1 Isolation of soil bacteria with CFC agar

Plants were removed from pots then soil loosely attached to the roots was shaken off. Roots were cut away using ethanol sterilised scissors and transferred to 50 mL falcon tubes containing 50 mL sterile phosphate-buffered saline (PBS; 8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4; pH 7.4). Tubes were vortexed at 4°C for 10 minutes. The resulting supernatant was collected and used to prepare 10-fold serial dilutions in PBS. Aliquots of 100 µL from each dilution were spread onto *Pseudomonas* Agar Base selective medium. Plates were incubated at 28°C until colony formation. From these, 192 representative colonies were randomly selected per harvest, streaked onto KB medium, and then preserved in glycerol at −80°C. These isolates were subsequently used for further genotypic and phenotypic characterisation (Pacheco-Moreno et al., 2024). For routine experiments, *Pseudomonas* spp. and other isolated bacteria were grown overnight at 28°C with shaking in LB.

2.4.2 Isolation of soil *Rhizobium spp.*

10 grams of rhizosphere soil was suspended in 20 mL PBS buffer; this mixture was shaken overnight at 28°C and 200 rpm. Subsequent serial dilutions were conducted up to 10^{-6}. Next, 100 µL aliquots of each dilution were plated onto *Rhizobium* semi-selective medium. After a four-day incubation period at 28°C, white-pink colonies were isolated from the plates using a sterile toothpick and inoculated into YMAA, LB agar and *Rhizobium* semi-selective media and incubated for 2 days at 28°C. Isolates that displayed no visible growth on LB agar were identified as negative LB, and were subcultured by streaking them a second time onto YMAA. To achieve pure cultures, confirmed negative LB colonies were subsequently grown on YMAA plates (Soenens & Imperial, 2018). For routine experiments, *Rhizobium* spp. was grown overnight at 28°C with shaking in TY.

2.4.3 Fungus spore harvest

Fusarium solani were grown on PDA plates under dark conditions at 25°C. After 10 days, *F. solani* was harvested using 5 mL sterile distilled water and gently scraped from plates with a sterile L-shaped glass spreader. A suspension of spores was filtered through a double layer of sterile Miracloth (Millipore, UK) to remove debris. The spore concentration was determined using a counting chamber. 10 µL of a 1:100 dilution of spores was added to the counting chamber and manually counted under a light microscope. Spore suspensions were stored at -20°C until use (Kjeldgaard et al., 2022).

2.5 Plant experiments and growth conditions

2.5.1 Soil collection

The soil used in this study was collected on three independent occasions in November 2021, June 2022 and October 2022 from a naturally grassed and unfertilised field site with no history of growing pea. The soil was taken from the JIC Field station, Church Farm, Bawburgh, Norfolk, United Kingdom (52°37'42.3"N 1°10'44.3"E). Covering grass was removed and soil collected from a depth of 30 cm. Rocks and visible plant residues were removed, the soil was mixed thoroughly and stored in closed storage boxes at 4°C until use. This soil is referred to throughout this thesis as “preharvest soil”, with each soil collection used to initiate a biologically independent plant experiment.

2.5.2 Pea cultivation condition.

Pea seeds (*P. sativum* cultivar Kahuna) underwent surface sterilisation with 5% NaClO for 1 minute and 70% ethanol for 2 minutes before thorough washing with sterile deionised water. Seeds were then germinated on water agar plates in a dark cupboard at room temperature for 4-5 days. The germinated peas were transplanted into 9 cm pots (135 mm height, 120 mm diameter), each containing approximately 380 g of collected field soil. Plant growth occurred in a controlled environment room (CER) maintained at 25°C under a 16-hour light and 8-hours dark cycle. Every two days, plants were watered with 300 mL filtered tap water. Water was sterilised using Fisherbrand^{TM} Vacuum Bottle Top Filter, 0.22 µm pore size. In addition, a separate close rotation experiment was performed in 2 litre pots (170 mm height, 120 mm diameter) for 3 months until mature pea was harvested. Plant growth occurred in the glasshouse at 16–24°C, with a day length of approximately 16 hours using high-pressure sodium lamps when necessary.

2.5.3 Close rotation experiment

To simulate a close-rotation system, sterile pea plants were cultivated in preharvest soil for 28 days, yielding "1st harvest-soil." Subsequently, the plants were removed from their pots, and their height, dry weight, and nodule number were evaluated. The soil was then thoroughly mixed, re-potted, and used for a "2nd harvest." This process was repeated after another 28 days, with the soil collected, mixed, and prepared for a "3rd harvest.", which was grown for a further 28 days before final plant sampling. Soil samples were collected from each harvest and used for soil nutrient and microbial analysis. Figure 2.1 summarises the close rotation protocol.

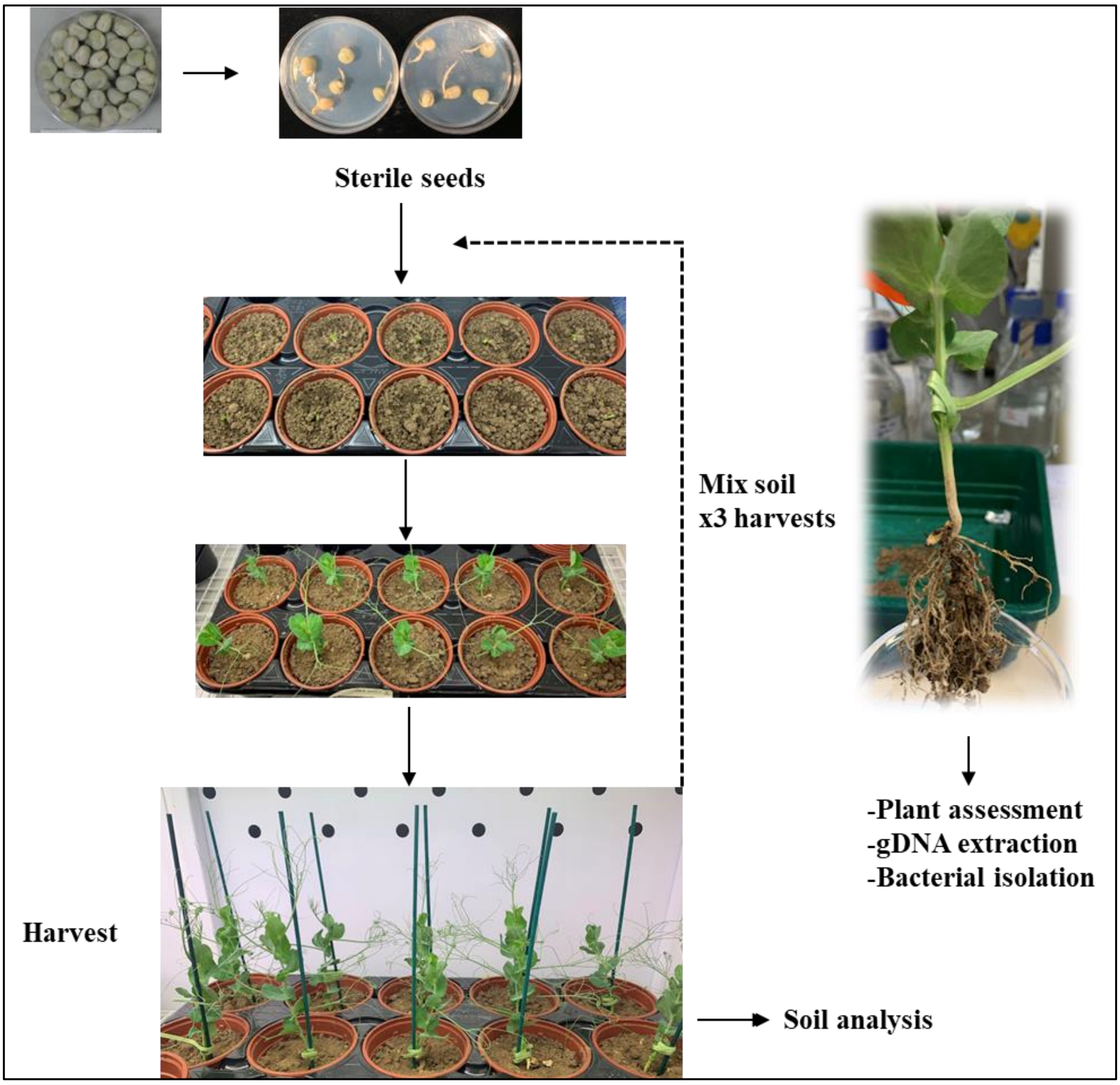


Figure 2.1 Diagram illustrates the close rotation experiment. Pea seeds were surface sterilised then germinated on water agar plates in the dark. Once germinated, peas were transferred to pots filled with preharvest soil (JIC field soil). Peas grown in at 25°C under a 16-hour light and 8 hours. After 28 days, peas were harvested and assessed, while rhizosphere gDNA were extracted and bacteria were isolated.

2.5.4 Soil analysis

pH, nutrient status (Mg, K, P and N) and organic matter analysis were conducted by the James Hutton Institute (James Hutton Limited, UK).

2.5.4.1 Soil pH measurement

For soil pH measurement, 10 g soil was combined with 25 mL deionised water to create a soil-water slurry. The mixture was thoroughly agitated on a roller for at least 30 minutes and then allowed to settle until a clear supernatant formed. The pH electrode probe was then inserted into the supernatant, and the pH was recorded.

2.5.4.2 Measurement of oxidisable organic matter

Samples were weighed into clean silica or porcelain crucibles and dried in an oven to constant weight before ignition. After reweighing, the samples were transferred to a muffle furnace to eliminate oxidisable material. Oven drying standardised the material to an oven-dry basis, minimising errors caused by non-homogeneous water content in the samples. Subsequent ignition provided an estimate of the oxidisable component. In practice, between 0.25 and 8 g of material was weighed into crucibles, which were then placed in a preheated oven and dried at 105°C for a minimum of four hours, typically overnight. Once dried, the crucibles were cooled, weighed, and transferred to a muffle furnace, where they were heated at 450°C for 12 hours. After cooling, the crucibles were reweighed to obtain the final measurement.

2.5.4.3 Estimation of soil nutrients

A 0.43 M acetic acid solution was used to extract the target analytes from the soil material, with subsequent quantification by inductively coupled plasma–optical emission spectroscopy (ICP-OES). For this procedure, 2.5–10 g of air-dried soil, sieved to <2 mm, was weighed into bottles and mixed with approximately 40 mL of 0.43 M acetic acid. The suspensions were agitated on an end-over-end shaker for two hours to facilitate extraction. Following shaking, the soil slurries were filtered through Whatman No. 542 filter paper into 100 mL volumetric flasks, with an additional 40 ml of acetic acid used to complete the filtration. The filtrates were left to drain overnight before being made up to volume with acetic acid. The resulting extracts were transferred to polythene bottles and analysed for analyte concentrations using ICP-OES.

2.5.5 Synthetic community (SynCom) and Natural community (NatCom) re-inoculation

To observe how rhizosphere consortia from different harvests of close rotation affect pea growth, sterile, germinated seeds were transplanted into 15 cells seedling trays half filled with medium vermiculite dimension 2.0-5.0 mm (Sinclair^{TM}) (see Figure 2.2), which was autoclaved twice to ensure complete sterility. 10 mL of rooting solution (1 mM $CaCl_2 \cdot 2H_2O$, 100 µM KCl, 800 µM $MgSO_4$, 10 µM FeEDTA, 35 µM H_3BO_3, 9 µM $MnCl_2 \cdot 4H_2O$, 0.8 µM $ZnCl_2$, 0.5 µM $Na_2MoO_4 \cdot 2H_2O$, 0.3 µM $CuSO_4 \cdot 5H_2O$, 6 mM KNO_3, 18.4 mM KH_2PO_4, and 20 mM Na_2HPO_4) was added to each cell, and the trays were monitored for the emergence of pea leaves.

SynComs were constructed by growing overnight cultures of 96 *Pseudomonas* / *Serratia* spp. alongside 96 *Rhizobium spp*. from each harvest individually in 96 well plates. After that, bacteria were combined in sterile tubes. Bacterial cells were centrifuged for 10 mins at RT, then washed with PBS buffer. The optical densities (OD_{600}) of each culture were adjusted to 0.1 with PBS buffer, then 1 mL from each was mixed. 2 mL of this SynCom culture was inoculated into each individual cell tray. PBS buffer was used as a control.

For the NatCom experiment, soil washes were obtained from pea rhizospheres of pre-close rotation and each harvest of close rotation. Harvested roots were gently removed from pots and then shaken to remove loosely attached soil and then vortexing in 30 mL PBS buffer for 10 minutes at 4°C. Samples were centrifuged at 1000 x g for 10 minutes at room temperature to pellet plant and soil debris, before supernatant was collected and decanted into a fresh tube. 5 mL of soil wash was inoculated into each individual cell tray in an experiment. Pea plants were arranged with each treatment group placed on separate trays under 16-hour light at 21°C and 8-hour at 17°C night cycle in a controlled environment cabinet. Watering with sterile water took place from the bottom of the tray, with 300 mL added per tray every 3 days. Plants were harvested 28 days post-inoculation, and height, weight, and number of nodules per plant were recorded.

Figure 2.2 Pea tray set up for Synthetic community (SynCom) and Natural community (NatCom) re-inoculation. Pea plants were grown in 15 cells seedling trays half filled with medium grain vermiculite.

2.5.5.1 Pea rhizosphere community challenged with *Fusarium solani*

Cell trays containing sterile vermiculite was inoculated with *F. solani* using 5 mL of $1x10^7$ spores per mL. Spore inoculum was added to the centre of an individual cell. A pipette tip was used to mix the suspension thoroughly once added to vermiculite. Pea plants were arranged with each treatment group placed on separate trays under 16-hour light and 8-hour dark cycle, 25°C in a CER. Plants were watered every 3 days by adding 300 mL sterile deionised water to bottom of the tray. Height, weight, nodules and disease severity were assessed after 4 weeks of inoculation. Disease severity score was assessed visually at 4 weeks after fungus inoculation (Trenk, 2025). Scoring system is shown in Figure 2.3

Figure 2.3 Disease scoring system. The disease scoring system assigns a value of 1 to indicate the absence of any visible infection, 2 to restricted lesions, 3 to expanding lesions 4 to softening root and 5 to substantial rot.

2.6 Amplicon sequencing, Bacterial genotyping and data analysis

2.6.1 Rhizosphere DNA extraction

To prepare the rhizosphere genomic DNA (gDNA) samples, plants were extracted from their pots and excess soil was shaken off from the roots. Roots were then cut using sterile scissors, placed into 50 mL falcon tubes, and rinsed with 10-20 mL sterile PBS. Roots were shaken at 200 rpm for 30 minutes at room temperature. The resulting supernatant was collected, and roots were transferred to a fresh 50 mL falcon tube. This washing procedure was repeated three times, each time with 10-20 mL fresh buffer. Collected supernatants were designated as wash 1, wash 2, and wash 3. For the final wash, 10–20 mL fresh buffer was added, tubes were vortexed twice for 30 seconds, and roots were then transferred to a new tube. The supernatant from this final wash (wash 4) was also collected. Wash 1 and wash 2 were pooled together and centrifuged for 30 minutes, 10,000 x g at 4°C. After discarding the supernatant, washes 3 and 4 were pooled and centrifuged. The resulting pellet was defined as the rhizosphere sample. Protocol was modified from prior literature (Pacheco-Moreno et al., 2024) and (Wen et al., 2023).

DNA extraction was performed using a FastDNA™ SPIN Kit for soil (MP Biomedicals, UK) according to the manufacturer's instructions. The quality of the DNA was evaluated by the 260/280 and 260/230 absorbance ratios using a NanoDrop spectrophotometer (Thermo Scientific, USA). A ratio of 1.7-1.90 was established as a minimum to pass the quality standard. The quantity was measured with a Qubit® 2.0 Fluorometer (Thermo Scientific, USA). The gDNA was stored at -20°C before transport for sequencing.

2.6.2 Amplicon sequencing

Genomic DNA (gDNA) samples were sent for library preparation and sequencing an Illumina Novaseq6000 with 250 paired-ends and 50000 reads (Novogene, China). The 16S rRNA gene was targeted to determine the bacterial composition and diversity using primers targeting the V4 region. Fungal population was targeted using ITS1-1F. The demultiplexed files supplied with primers and sequence adapters already removed were processed with LotuS2 (Özkurt et al., 2022) using the recommended pipeline for high-quality microbiota profile reconstruction. Briefly, reads were quality-filtered (minimum Phred Q20; removal of reads with ambiguous bases; length trimming to the modal insert size), Chimeras were removed de novo and by reference during denoising. Amplicon sequence variants (ASVs) were inferred with DADA2 within LotuS2. Taxonomy was assigned against SILVA database (Quast et al., 2012) for 16S, and UNITE for ITS (Abarenkov et al., 2023), using LotuS2's integrated classifiers. The pipeline returned a feature table, representative sequences, and taxonomy files; these were imported into R (version 4.5.1) for downstream ecology (alpha diversity: Observed, Shannon, Simpson; beta diversity: Bray–Curtis) and Principal Coordinate Analysis (PCoA). Statistical analyses were performed by ANOSIM and PERMANOVA (adonis function) (Dixon, 2003). The genera richness was estimated by calculating within-sample (α) diversity using vegan package version 2.6-10. The equations of diversity indices are:

The Shannon diversity index (H′) was calculated as:

$$H' = -\sum_{i=1}^{S} p_i \ln(p_i)$$

where $S$is the total number of taxa and $p_i$is the relative abundance of the i-th taxon.

The Simpson diversity index (D) was calculated as:

$$D = 1 - \sum_{i=1}^{S} p_i^2$$

where $p_i$represents the relative abundance of each taxon.

Observed diversity was calculated as the number of unique taxa (observed ASVs) detected in each sample:

$$\text{Observed diversity} = S$$

where Srepresents the total number of observed taxa in a sample.

For data visualization ggplot2 (version 3.5.2) and GraphPad Prism version 10.0 (GraphPad Software, USA) was used.

2.6.3 Bacterial genotyping

Colony PCR was performed by heating bacterial colonies at 95 °C for 10 minutes to lyse the cells, and the resulting lysate was used as the DNA template for amplification. The RNA polymerase B subunit (*rpoB*) gene was used as the target for sequence comparison. Amplification was performed in a 50 µL reaction volume containing 18 µL nuclease free water, 25 µL Taq green master mix (Promega, UK), 2.5 µL forward primer (10 mM), 2.5 µL reverse primer (10 mM), 2 µL template. PCR reaction was amplified using a PCR Thermocycler (BioRad). The program was set as follows: 90 seconds at 95°C as initial denaturation, followed by 30 cycles of 30 seconds at 95°C for denaturation, 30 seconds at 55°C as annealing, 90 seconds at 72°C for extension, and a final extension at 72°C for 10 minutes. PCR products were examined by electrophoresis at 100 V for 30 minutes in a 1% (w/v) agarose gel in 1x TAE buffer. Purified PCR products were sent for sequencing (Source Bioscience, UK). Cluster Omega was used to generate multiple sequence alignments of obtained DNA sequences (Madeira et al., 2024). Then, IQ-TREE (http://iqtree.cibiv.univie.ac.at/) was used for tree estimating utilising the Maximum-Likelihood method with a bootstrap value of 1000 (Trifinopoulos et al., 2016). The tree was visualised by I-TOL version 7.2 (Letunic & Bork, 2021). The reference strains used in the phylogenetic tree included *Clostridium botulinum, P. fluorescens, Stenotrophomonas maltophilia, Escherichia coli, Serratia marcescens* and *Serratia plymuthica*.

2.6.4 qPCR quantification of microbial abundance

Quantitative PCR (qPCR) was conducted to quantify gene abundance. Soil genomic DNA (gDNA) from each harvest was used for amplification with the specific primers provided in Table 2.1, above. DNA quantification was performed using a BioRad CFX96 instrument. Each 20 μL reaction mixture consisted of 1.5 μL gDNA (10 ng/μL), 10 μL 2x SensiFAST SYBR® No-ROX mix (Meridian Bioscience), 0.5 μL for each primer (10 mM). Samples were run on a programme cycle of 95°C for 2 minutes followed by 40 cycles of 5 seconds at 95°C for denaturation, 10 seconds at 65°C as annealing, 10 seconds at 72°C for extension. Standard curves were produced by dilution of *Rhizobium* DNA from 1/10, 1/100 and 1/1000. Amplification was performed in triplicate for all soil samples.

2.7 Bacterial phenotyping assays

Up to 192 isolates from each harvest were randomly picked and then subjected to phenotypic assays including polysaccharide production, siderophore production, protease activity, motility, hydrogen cyanide (HCN) production, indole-3-acetic acid (IAA) production and the *F. solani* inhibition assay. Figure 2.4 shows the ordinal scoring system for each phenotype.

Congo Red binding, siderophore production, protease and motility assays, were carried out in 500 cm^2 square plates (Thermo Scientific, UK) with 200 mL of KB-agar for *Pseudomonas* and TY agar for *Rhizobium*. Plates were dried for 1 hour in laminar flow cabinet. Overnight bacterial cultures were applied to the agar surface using an ethanol-sterilised replicator, ensuring even contact. After plates were allowed to dry for further 5 minutes. Plates were incubated at 28°C and assessed after 24 hours.

2.7.1 Congo Red binding

Congo Red binding was used to test for the production of polysaccharides. Final concentration of 0.005% Congo Red was added into KB agar. Congo Red binding was scored as follows: 0 for no binding and 1 for positive binding.

2.7.2 Siderophore production

Unmodified KB agar was used to evaluate siderophore production. Images were taken under UV light, with a transilluminator providing optimal fluorescence detection. Fluorescence emission by the colonies was scored as follows: 0 for no fluorescence, and 1 for positive fluorescence.

2.7.3 Protease activity

KB agar supplemented with 1% (w/v) skimmed milk powder was used to assess variations in protease production via a milk degradation assay. Media was prepared by dissolving 1 g milk powder into 10 mL warm sterile water and adding resultant solution to 90 mL KB agar. The milk supplemented KB agar was thoroughly mixed and poured into square plates. Images were taken under white light. Colonies were scored based on the presence of a clear degradation halo as follows: 0 for no halo and 1 for a visible halo.

2.7.4 Motility assay

0.5% KB agar plates were used to assess motility. Isolates were spotted on plate and incubated for overnight at room temperature and the extent to which colonies spread out from their initial point of inoculation was examined and was scored as follows: 0 for nonmotile and 1 for motile.

2.7.5 Hydrogen cyanide production

96-well plate with 150 µL KB medium per well was used to assess hydrogen cyanide (HCN) production. Soil isolates grown overnight in LB at 28°C were inoculated into the wells using a replicator, with 2 µL added per well. Feigl-Anger reagent was prepared in chloroform (Thermo Fisher Scientific, UK) using 3.33 µg/mL copper ethyl acetoacetate (Sigma-Aldrich, UK) and 3.33 µg/mL of 4-4'–methylene-bis-N, N-dimethylaniline (Sigma-Aldrich, UK) (Feigl & Anger, 1966). After inoculating the plate, 2 mL Feigl-Anger reagent was spread onto a piece of sterile Whatman™ filter paper cut to fit the underside of the 96-well plate lid and allowed to dry. The plates were incubated at 28°C for 24-48 hours under gentle agitation. HCN production was evaluated based on the intensity of blue colouration: 0 for no colour change (i.e., remains white) and 1 for blue (i.e., positive HCN production).

2.7.6 Indole-3-acetic acid (IAA) production

96-well plate with 150 µL YMB supplemented with 2 g/L L-tryptophan per well was used to assess indole-3-acetic acid (IAA) production. Soil isolates grown overnight in LB at 28°C were inoculated into the wells using a replicator, with 2 µL added per well. The plates were incubated at 28°C for 24-48 hours, shaking at 150 rpm at 28°c for 4 days. Bacterial growth was quantified via optical density at 600 nm (OD_{600}) before centrifugation at 4,000 x g for 20 minutes. Salkowski reagent was prepared in deionised water with final concentrations of 35% perchloric acid ($HClO_4$) and 10 mM $FeCl_3$ (Gang et al., 2019). Supernatants were transferred to a new 96-well plate and presence of IAA was quantified using colorimetric techniques using Salkowski reagent. Equal volume of Salkowski reagent was added to the supernatant and incubated in the dark at 28°C for 30 minutes. IAA concentrations were quantified using absorbance at 530 nm (A_{530}), thus indicating the production of IAA.

2.7.7 *F. solani* inhibition assay

90 mm Petri dishes with PDA media were inoculated with 6 mm agar plugs from the edge of the actively growing *F. solani* colonies grown on PDA. Plugs were positioned in the centre of the 90 mm Petri dishes. Soil isolates were grown overnight in 96-well plates using LB at 150 rpm at 28°C. Bacterial cultures were normalised to optical density of 0.1 at 600 nm (OD_{600}) using sterile PBS buffer, and 5 µL of normalised bacterial cultures were applied to sterile paper discs (Whatman™ diameter 6 mm). Bacterial-inoculated discs were placed equidistant from the central fungal plug. The plates were incubated in darkness at 25°C for 10 days to assess fungal growth inhibition by soil isolates. Fungal inhibition was evaluated based using a binary scale: 0 indicated no inhibition and 1 indicated inhibition of fungal growth. Protocol modified from prior literature (Lee et al., 2023).

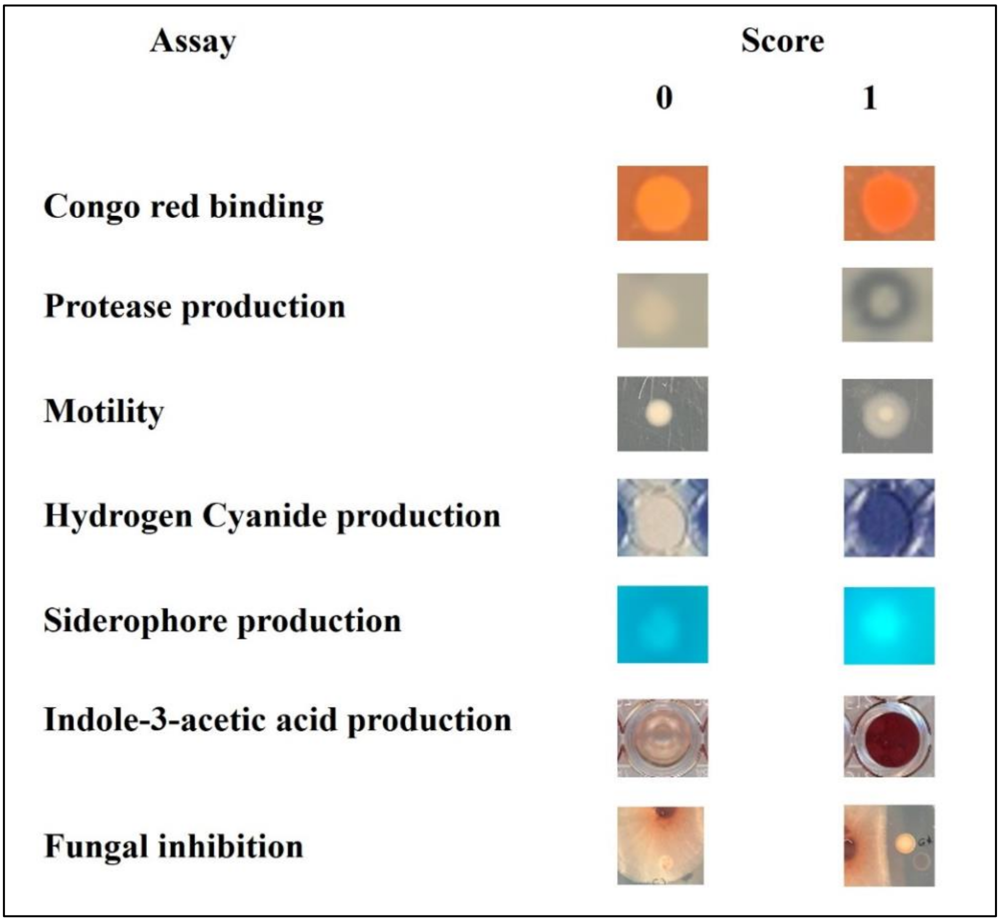


Figure 2.4 Scoring system for phenotypic assays. Isolates from preharvest, 1st harvest, 2nd harvest, and 3rd harvest rhizospheres were tested with different assays and scored as shown: 0 for no production and 1 for present production.

Chapter 3 –

Crop Rotation Impact on Plant Health and Soil Bacterial Communities

Chapter 3 – Crop Rotation Impact on Plant Health and Soil Bacterial Communities

3.1 Introduction

The use of close rotation practices is made necessary by a shortage of agricultural land and the growing population, with the purpose of enhancing crop production (Chen et al., 2009; Zhang et al., 2022). This practice is largely driven by a need for operational efficiency; by focusing on a single crop, producers can streamline resource allocation and maximize short-term yields. This extensive cultivation of a plant has encouraged the buildup of certain fungal infections which can reduce yield over time (Abawi & Ludwig, 2005; Liu et al., 2001). This phenomenon has been shown to cause a microbiome dysbiosis in several crops where the microbial population imbalances occurred (L. Bai et al., 2015; Gao et al., 2021; Xia et al., 2025). Similarly, decreased pea yield is also known to be associated with close rotation (Bainard et al., 2017; Knight, 2012; Nayyar et al., 2009). Even though peas play an active role in shaping the microbial communities around their roots (Chaudhari et al., 2020; Horner et al., 2019; Kalapchieva et al., 2023; Turner et al., 2013), limited studies have examined how close rotation affects pea phenotypes and their root-associated microbial communities.

The pea–rhizobia connection is one of the most well-studied plant–microbe interactions (Oldroyd, 2013). These bacteria create nodules where they fix nitrogen (Zhang et al., 2024). Nodulation (*nod*) genes are conserved in all rhizobia and necessary for establishing symbiosis (Debellé et al., 2001). Therefore, understanding the dynamics of *Rhizobium* population together with the *nodD*-carrying population might reflect the soil alteration in the close rotation scenario.

Apart from soil microbes, nutrient availability is a cornerstone of plant health (López-Bucio et al., 2003). They are the building blocks for growth. Most of the physiological process from germination through flowering and fruiting depends on a steady supply of essential elements. Firstly, nitrogen (N) drives leaf and stem growth by being a key component of chlorophyll and amino acids (Farhan et al., 2024). Phosphorus (P) is critical for flowering, root development and energy transfer (Khan et al., 2023). Plants require potassium (K) to regulate water balance, enzyme activation, and stress resilience, (Johnson et al., 2022). In addition, micronutrients such as iron, manganese, zinc, serve as cofactors for enzymes. For instance, iron stimulates the formation of chlorophyll and play a role in plant respiration (Pietryczuk et al., 2025). Manganese is needed in only small amount by plants but it is critical to plant growth such as a vital component of the oxygen-evolving complex (OEC) within photosystem II (PSII) (Alejandro et al., 2020; Schmidt et al., 2016). Maintaining soil pH within the appropriate range is essential for optimal nutrient absorption and supporting a diverse microbial community (Fierer & Jackson, 2006). Optimal pH is necessary for effective legume nodulation and preserving soil structure (Msimbira & Smith, 2020; Wenyika et al., 2025). Soil organic matter is an important factor in determining multiple soil properties (Fierer & Jackson, 2006). It enhances water-stable aggregates, thereby enhancing soil structure, texture, and aeration (Tisdall & Oades, 1982). Organic matter influences the soil's water retention capacity which stimulate microbial activity (Maroušek et al., 2013). Furthermore, it acts as a reservoir for nitrogen and phosphorus (Weil & Brady, 2017). For these reasons, testing soil nutrient levels, soil organic matter and pH might provide evidence whether close rotation caused shifts in soil composition.

In this chapter, I aimed to understand how close rotation affects pea plants, the soil properties, and the dynamics in their rhizosphere microbial community.

3.2 Objectives

1. Investigate pea plant health by measuring height, weight and nodule number under three harvest close rotation
2. Examine the change in soil nutrient properties under pea close rotation
3. Evaluate the impact of close rotation on rhizosphere microbiome structure and composition
4. Quantify changes in total bacterial abundance, nodulation-gene expression, *Rhizobium* population density, and fungal community dynamics under close rotation.

3.3 Results

3.3.1 Plant health after close rotation

To understand how close rotation affects the overall health of pea plants across rotations, the effect of close rotation growth was mimicked under a controlled growth environment. This close rotation was performed in two scenarios where peas were grown for a short period (4 weeks) and a long period until peas were mature (3 months). Soil analysis was conducted to determine nutrient content (P, K, Mg and N), soil pH, and organic material in the short period experiments to investigate close rotation impacts on abiotic soil properties.

Firstly, pea seeds were surface-sterilised, germinated and planted in individual pots of naïve JIC field soil with no prior history of pea cultivation. This soil, prior to pea planting is referred to as "preharvest soil". After four weeks, ten to twelve pea plants were carefully removed from the soil, and their rhizospheres were collected for gDNA extraction and bacterial isolation. Plants were imaged at the time of harvest. The remaining "1st harvest soil" was collected, pooled and mixed in a sterile bag before being re-distributed to new pots for the next planting generation; "2nd harvest". After another four-week growth period, the same procedure was repeated to generate the "3rd harvest soil". The dry weight, height, and number of visible nodules were assessed at the end of every harvest. In total, three biologically independent experiments have been conducted with three close rotation harvests observed. A representative picture of pea plants from the 3rd harvest is shown in Figure 3.1.

Figure 3.1 Four-week-old pea plants from the close rotation experiment. Pea plant cultivation in a controlled environment room for 4 weeks in pea-naïve soil.

Figure 3.2 showed the measurement of pea plants after three close rotations. The statistically analysis showed that dry weight of 1st harvest peas was significantly greater than in the 2nd and 3rd harvests (Figure 3.2A). However, I observed no difference between pea plant weights in the second and third experiments Plant height remained consistent across continuous cultivation cycles in all three biological experiments (Figure 3.2B). Nodule counting also showed no significant difference between harvests. In the first experiment, nodule counts were not recorded, resulting in an incomplete dataset for this experiment. Moreover, plants exhibited no visible symptoms of fungal disease at any stage in all three experiments. Representative pea plants from each harvest of the 1st experiment are shown in Figure 3.3.

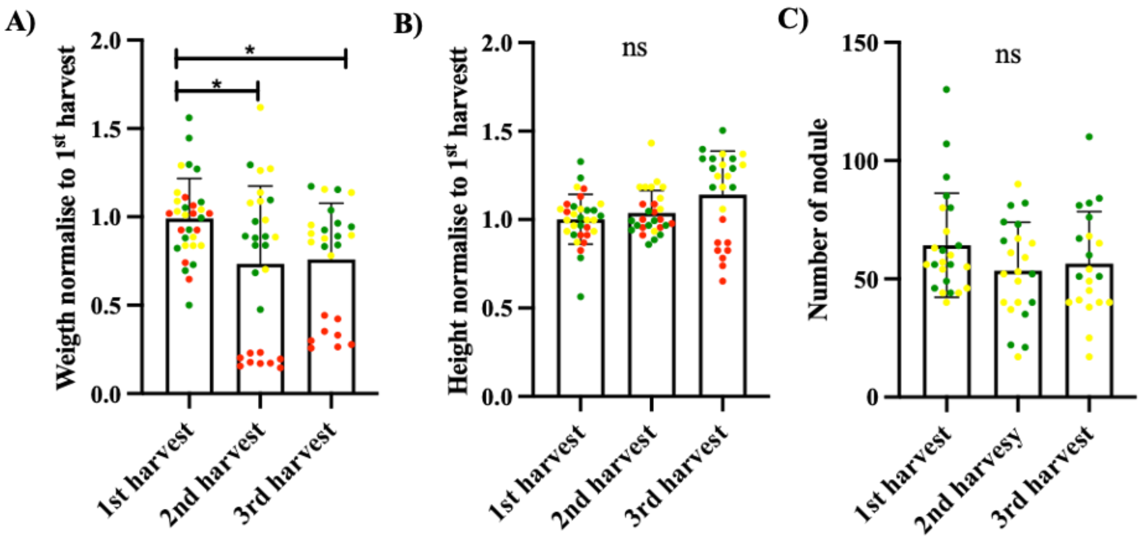


Figure 3.2 Determination of pea growth for three rotation cycles. Bar graphs showing the average of A) plant dry weight and B) plant height normalised to the average of the first harvest. C) number of nodules per pea plant. Ten to twelve pea plants were collected per harvest. The individual data points for each plant are shown as dots in different colours for independent experiments, first experiment (red), second experiment (green) and third experiment (yellow) and error bars represent the standard deviation. A mixed-effects model with Tukey multiple comparisons revealed a significant difference in pea weight, with significance indicated by asterisks: $p < 0.05$ (*).

Harvest	Representative images of pea plants from each rotation
1st	
2nd	
3rd	

Figure 3.3 The representative of plant sample in close rotation. Plants were cultivated in naïve soil for 4 weeks then removed from the pots for health assessment.

3.3.2 Soil nutrient properties following close rotation

In the first experiment, soil samples were collected at the end of each rotation and subjected to soil analysis. Analysis of soil nutrient levels across the pea close rotation showed that phosphorus, magnesium, and potassium concentrations did not differ significantly between harvests, indicating that the close rotation did not affect their availability (Figure 3.4). However, the lowest nitrogen level was detected in the preharvest. After planting, the nitrogen level peaked at the 1st harvest followed by a fluctuated pattern observed during close rotation. Critical soil nutrient ranges must be within a range for optimal plant growth including phosphorous (16–25 mg/kg), potassium (121–180 mg/kg), magnesium (51–100 mg/kg) and nitrogen (10-50 mg/kg) (Agriculture & Horticulture Development, 2022). The concentration of these critical soil nutrients from our close rotation experiments were determined and indicated their concentrations were sufficient for plant growth. Therefore, the availability of nutrients was not considered to be a critical factor affecting plant growth or health in this close rotation experiment.

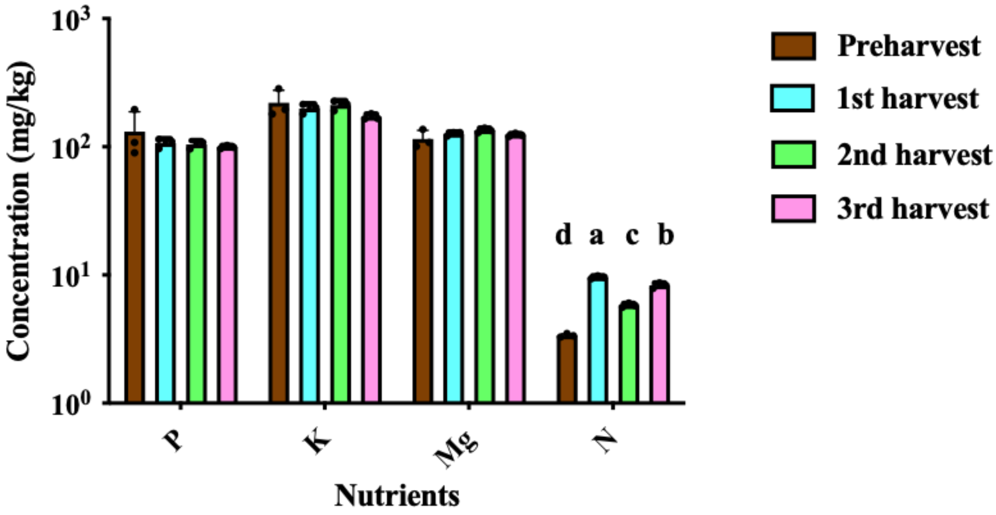


Figure 3.4 Examination of soil nutrient level after three close rotation cycles. Bar graphs showing average nutrient level in soil from first experiment including phosphorus (P), potassium (K), magnesium (Mg), and nitrogen (N). Bar colours represent different harvests: preharvest (brown), 1st harvest (blue), 2nd harvest (green), and 3rd harvest (pink). Soil samples were collected from three pots per harvest. Error bars represent the standard deviation. A one-way ANOVA was performed with Tukey multiple comparisons. Letters above plots (a, and b) indicate statistical groupings, with different letters indicating significant differences ($p<0.05$)

To investigate soil pH changes under close rotation, the pH of the soil samples was quantified. The result in Figure 3.5 indicates in the preharvest soil pH 7.8 was slightly alkaline. While in 1st harvest, pH 7.9 was a minimal change from preharvest. In 2nd harvest the pH remained stable and lastly 3rd harvest had the highest pH, notably more alkaline. Overall, there is a progressive increase in soil pH over successive harvests, especially notable by the 3rd harvest.

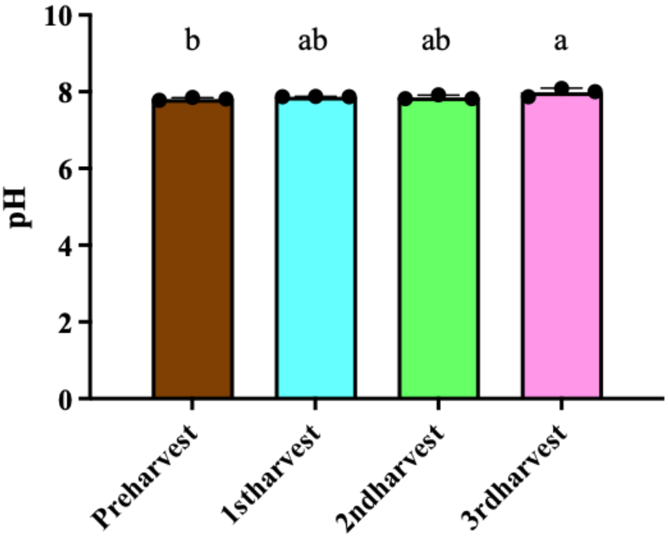


Figure 3.5 Examination of soil pH level after close rotation. Soil samples were collected from three pots per harvest. Significant difference was observed between preharvest and third harvest samples. A one-way ANOVA was performed with Tukey multiple comparisons. Letters above plots (a, and b) indicate statistical groupings, with different letters indicating significant differences ($p<0.05$).

To determine whether organic matter content changed over the course of crop rotation, soil samples were analysed after each harvest (Figure 3.6). The results showed no statistically significant changes, with values remaining stable between 4% and 4.6%. Although, organic matter did not change, further experiments were carried out to assess whether bacterial and fungal populations shifted across harvests.

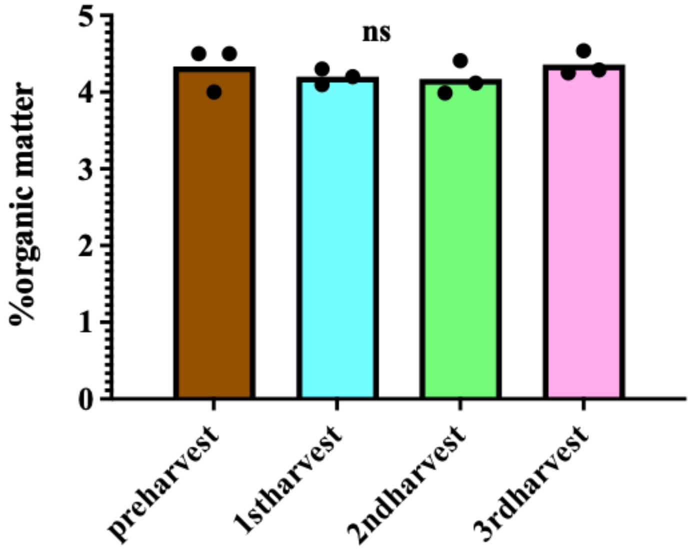


Figure 3.6 Percentage of organic matter after close rotation. Soil samples were collected from three pots per harvest. A one-way ANOVA was performed with Tukey multiple comparisons. No significant differences were observed between pea harvests.

3.3.3 Investigation of mature peas under close rotation

The experiments to this point focused on short-term observations (Figure 3.2), which may not fully capture the longer-term effects of close rotation on plant health. To address this, I investigated the effects of close rotation of mature pea plants. This experiment was conducted under controlled conditions in the JIC glasshouse. Surface-sterile seeds were planted in JIC field soil with no prior history of pea cultivation. This soil, collected before pea planting, was designated as “preharvest soil”. After three months of growth, the soil was harvested, pooled, and homogenised before being redistributed into pots for the subsequent planting, referred to as the “1st harvest”. Then, rotation started again and at the end the “2nd harvest” plants were harvested. Plant phenotypes were assessed at the end of both harvests by measuring biomass, plant height, pod number, and seed count. The results indicated no significant differences in yield parameters between peas grown in naïve soil and those subjected to close rotation (Figure 3.7). The results showed no differences between harvests for any measured parameter.

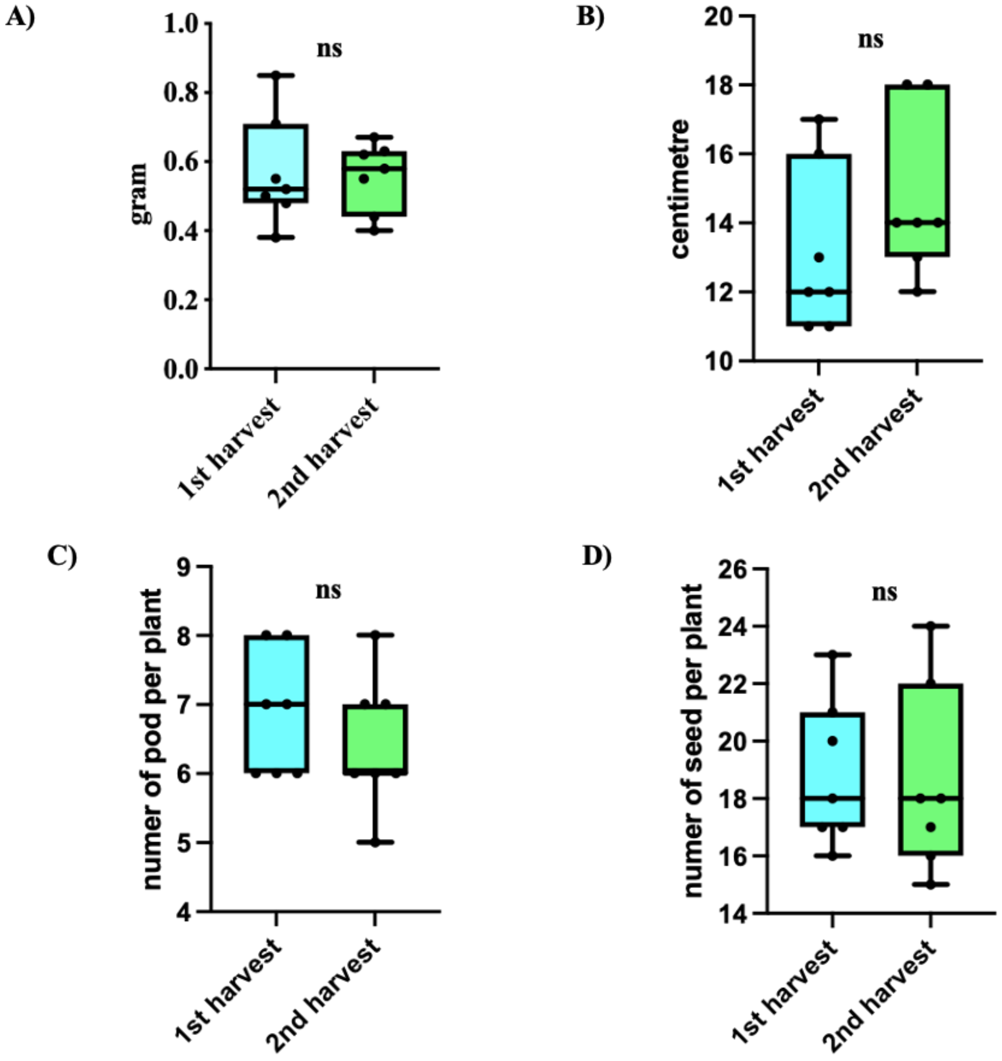


Figure 3.7 Pea yield from fully grown plants after one and two cycles rotation. Seven plants were measured per harvest. Boxes represent the interquartile range, the centre line indicates the median, and whiskers denote the range of the data. Individual points represent replicates. A) pea weight measured in grams. B) height measured in centimetres. C) number of pods per plant. D) number of seeds per plants. A t-test was performed; no significant differences were observed between pea harvests.

3.3.4 Pea rhizosphere bacterial recruitment during close rotation

After pea health assessment and soil chemical properties analysis, rhizosphere soil samples were collected from each pea plant. Genomic DNA was extracted and subjected to amplicon sequencing to profile the microbial communities associated with the rhizosphere in each harvest. The sequencing was based on the 16S RNA gene to identify the bacterial consortium, and internal transcribed spacer (ITS) to identify fungal populations in the pea rhizosphere. To illustrate the differences in microbial community composition between rhizosphere samples, a principal coordinate analysis (PCoA) with a Bray-Curtis distance matrix was conducted to measure the distance of the variation of bacterial taxonomic profiles between harvests (Figure 3.8). In the first experiment (Figure 3.8A), clear separation of the 1st and 2nd harvest samples was observed, while the preharvest and 3rd harvest were clustered together. This suggests that the microbial community was exposed to selective pressure by pea cultivation. However, they possibly partially reverted to the preharvest state after three pea rotations. In the third experiment (Figure 3.8B), the distance to the 3rd harvest was the furthest from the rest of the harvests, which indicates a later shift in community structure compared to the first experiment. In fungal community, overlaps between communities from each harvest were less distinct. The distance between preharvest and 3rd harvest samples was pronounced.

To identify significant differences between rhizosphere microbial communities, analysis of similarities (ANOSIM) was used. The closer of value to 1 means group are completely different. The analysis showed moderate between-group separation ($R = 0.3643$ for the first experiment, and $R = 0.495$ for the third experiment, both significant at $p < 0.001$). This indicated that bacterial community composition varied significantly among harvests. Similarly, in fungal communities' analysis, ANOSIM showed $R = 0.4063$ ($p < 0.001$). These results suggested the distinct microbe selection activity by pea in different harvests of the close rotation experiment.

To assess whether microbial community composition differed significantly between the harvests, the Permutational Analysis of Variance (PERMANOVA) was used. In the first experiment, bacteria community composition differed significantly across the four harvests ($R^2 = 0.267$, $p = 0.001$). This result suggested that the harvest accounted for approximately 26.7% of the total variation in microbial diversity. A subsequent Permutational Multivariate Analysis of Dispersion (PERMDISP) test confirmed that group dispersions were homogeneous (non-significant, $p = 0.213$), indicating that the observed differences were driven by distinct shifts in community member and abundance rather than differences in multivariate dispersion.

In the third experiment, the microbial community structure was significantly influenced by the harvest ($R^2 = 0.586$, $p = 0.001$). However, a significant PERMDISP result (*p* = 0.0008) indicated that multivariate dispersion was not homogeneous across groups. While this suggests that differences in group variance contribute to the PERMANOVA result, the high R^2 value (explaining 58.6% of the total variation) strongly suggests a substantial shift in community composition between harvests. Similarly, fungal microbial community composition was significantly affected by the close rotation ($R^2 = 0.358$, $p = 0.001$). A non-significant PERMDISP result, $p = 0.603$) confirmed the homogeneity of multivariate dispersions, indicating that the observed differences were driven by distinct taxonomic shifts rather than differences in group variance. The harvest stage accounted for 35.8% of the total community variation.

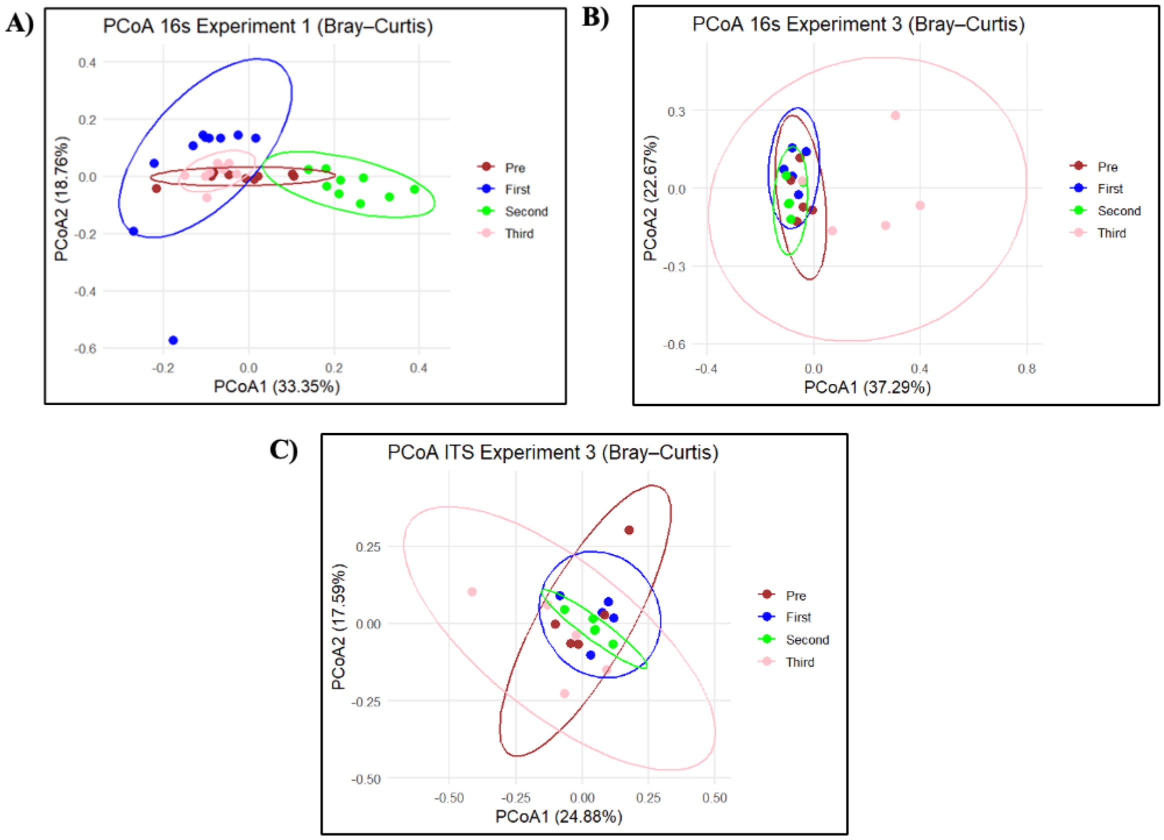


Figure 3.8 PCoA with Bray-Curtis distance matrix, comparison of Amplicon Sequence Variant (ASV) abundance for bacterial and fungal communities of close rotation rhizospheres. A) Bacterial community from first experiment. B) Bacterial community from third experiment. C) Fungal community from third experiment. Five to ten plant rhizospheres were used for each analysis. Each dot represents a rhizosphere sample, and

different colours represent different harvests. Preharvest (brown), 1st harvest (blue), 2nd harvest (green), and 3rd harvest (pink).

The bar chart (Figure 3.9) shows the taxonomic composition and relative abundance of bacteria and fungi in the pea rhizosphere for preharvest, 1st harvest, 2nd harvest, and 3rd harvest samples at the phylum level. In both experiments, preharvest soil contained a community that included 20%-30% of Proteobacteria. After the first pea cultivation, the abundance of Proteobacteria increased to 39.0% and sharply increased in the 2nd harvest to 67.5%. However, their abundance declined to 42.8% in the 3rd harvest. The trend of Proteobacteria also showed an increase in the third experiment (Figure 3.9B), thus increasing with each subsequent harvest, from 41.1% (1st harvest) to 45.8% (2nd harvest) to 54.9% (3rd harvest). On the other hand, Actinobacteria showed a consistent population proportion of around 13–20% throughout the close rotation of the first experiment. This pattern was not shown in the third experiment, as the population decreased from 41% to 17% at the end of close rotation. This trend also applied to phylum Acidobacteria. For the fungal phylum (Figure 3.9C), the main population was from the phylum Ascomycota, comprising 82% of the population since the preharvest, and the population increased to reach 92% after close rotation. In contrast, phylum Basidiomycota showed a decreased from 6.3% to 1.2%. Only a small portion (0.9%) of Mortierellomycota showed the increase in population to 1.5% after close rotation. Overall, the taxonomic structure showed remarkable consistency regardless of the starting soil profile. Proteobacteria consistently dominated the bacterial rhizosphere (42–50% abundance), while Ascomycota remained the primary fungal phylum throughout the duration of the pea rotation.

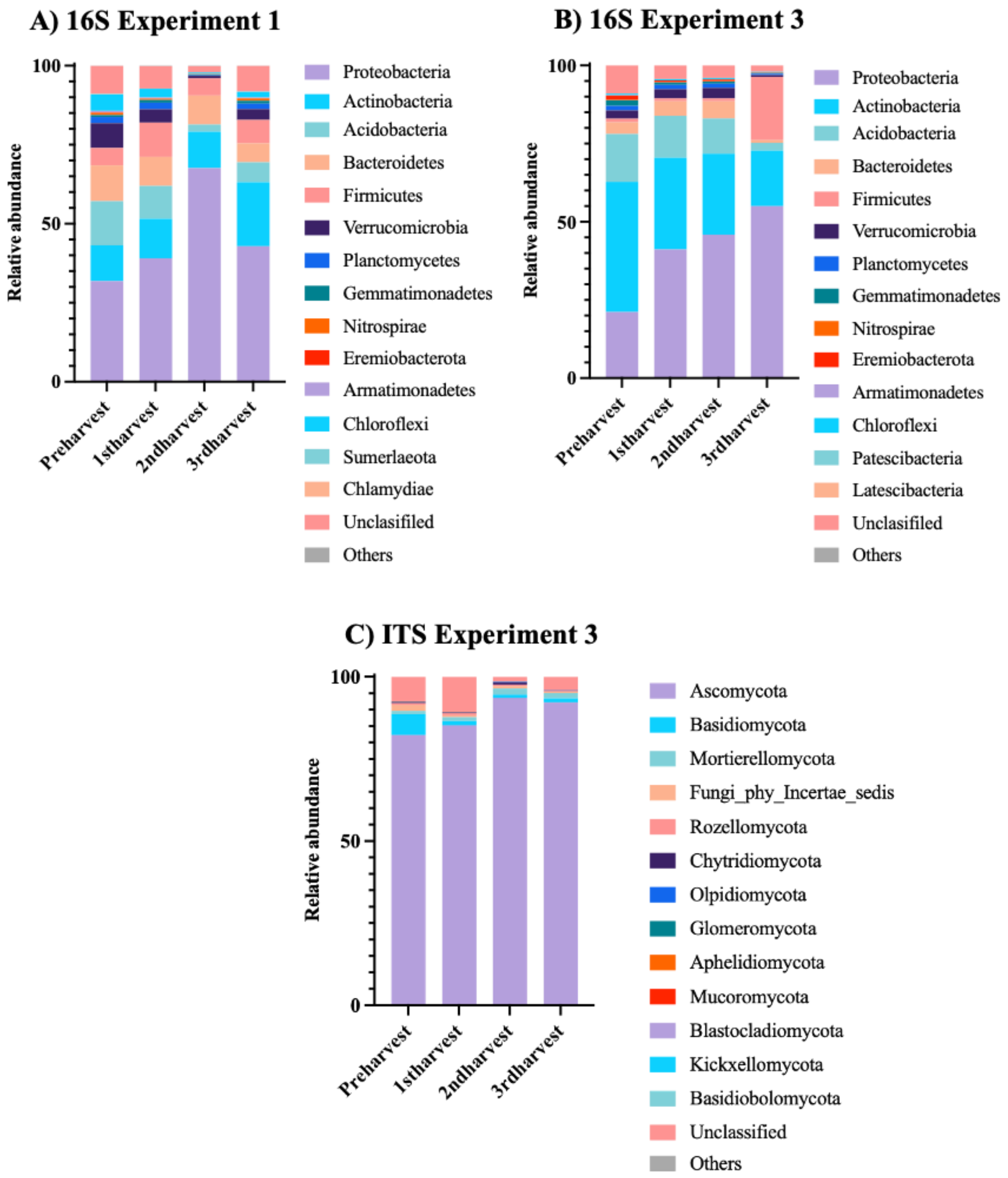


Figure 3.9 Relative abundance of bacterial and fungal taxa during close rotation at the phylum level. Bar graphs show A) Top 15 bacterial communities from the first experiment B) Top 15 bacterial communities from the third experiment C) Top 15 fungal communities from the third experiment across four harvests (preharvest, 1st, 2nd, and 3rd harvest). Five to twelve rhizosphere samples were analysed per harvest. Others referred to pooled low-abundance taxa and unclassified referred to unassigned taxa.

Figure 3.10 shows the relative abundance of bacteria and fungi in pea rhizosphere at the genus level. In the first experiment, genus *Gp6* (an unclassified subgroup within the phylum Acidobacteria) was the most abundant genus in the preharvest rhizosphere (3.4%). Other members of this phylum are *Gp4* (2.9%) and Subdivision3 genera incertae sedis (2.5%) which are both an unclassified subgroup of Acidobacteria. This group gradually declined and then was minimally present in the 3rd harvest. Proteobacteria, including *Pseudomonas*, which increased from rarely present (0.1%) in preharvest to 1.2% and peaked at 31.6% before decreasing in the 3rd harvest to 8.8%, were strongly selected in the close rotation rhizosphere. This phylum represented the dominant bacterial group in the experiment. Other members that were majorly present in the 2nd harvest also include *Massilia* (4.6%) and *Sphingomonas* (1.1%). In addition, members of Actinobacteria such as *Gaiella* (2.1%), *Pseudarthrobacter* (3.1%), and *Rubrobacter* (1.6%) showed the highest peak when they were recruited in the 3rd harvest. Amplicon sequencing analysis of the third experiment also showed the genus *Gp6* in preharvest (10%). Overall, this genus was more abundant in the pre- or 1st harvest but then declined and was replaced by Proteobacteria at the end of close rotation. Some of the Actinobacteria members remained present throughout the close rotation, including *Intrasporangium* (1.2-2.7%). In addition, *Rhizobacter*, *Massilia* and *Flavobacterium* showed the recruitment pattern after pea cultivation, as their abundance was sharply increased. The trend of *Pseudomonas* selection was increased to 2.7% after pea cultivation. Then, soil reuse influenced this genus recruitment in the 3rd harvest to reach 4.4%. This suggested that these two independent replications (first experiment and third experiment) exhibited the similar *Pseudomonas* recruitment phenomenon. Moreover, this experiment revealed the increase of *Agrobacterium* population in the 2nd harvest (2.2%) and the 3rd harvest (1.5%). Figure 3.10C showed top 15 fungal genera, which mostly comprised the phyla Ascomycota and Basidiomycota. The preharvest rhizosphere was majority dominant in *Gibellulopsis* with 23.8%. This genus gradually decreased and was replaced by *Fusarium.* They were rarely detected in preharvest, and 1st harvest, escalated in 2nd harvest (12.3%) and reached the peak at 25.3% in 3rd harvest. Some genera increased in population after pea cultivation such as *Plectosphaerella* and *Acrostalagmus*. In contrast, some disappeared after pea cultivation such as *Cladorrhinum* and *Entoloma.*

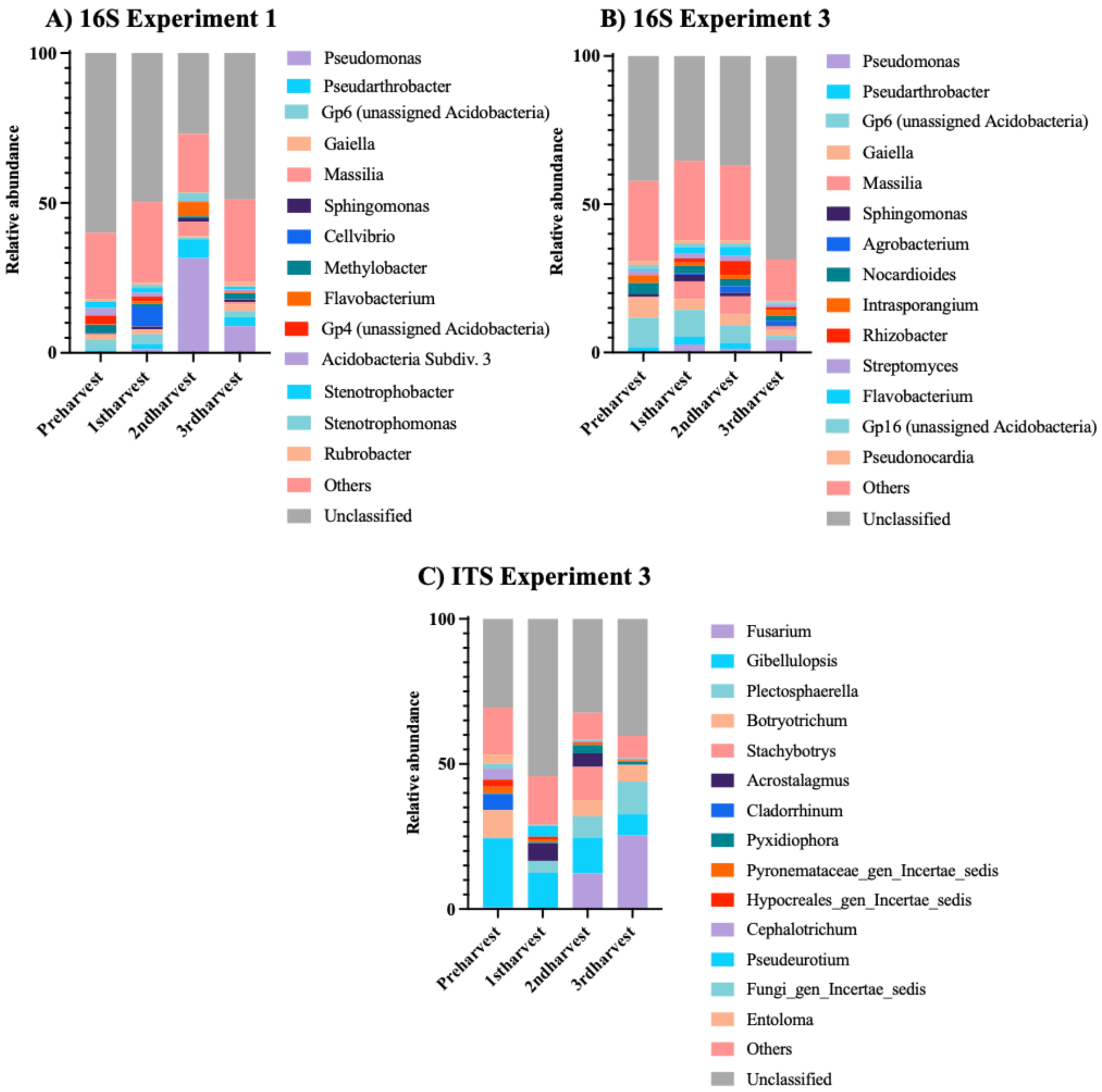
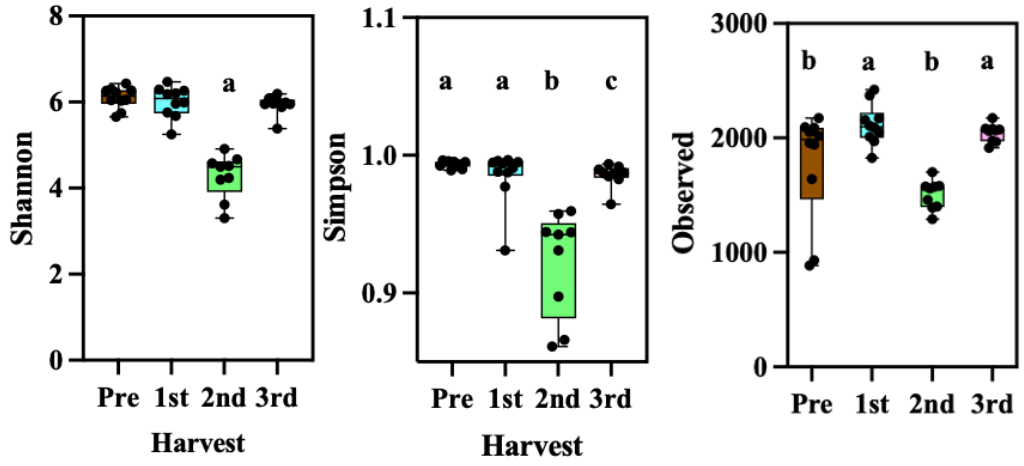


Figure 3.10 Relative abundance of bacterial and fungal taxa during close rotation at the genus level. Bar graphs show A) Top 15 bacterial communities from the first experiment B) Top 15 bacterial communities from the third experiment C) Top 15 fungal communities from the third experiment across four harvests (preharvest, 1st, 2nd, and 3rd harvest). Five to twelve rhizosphere samples were analysed per harvest. Others referred to pooled low-abundance taxa and unclassified referred to unassigned taxa.

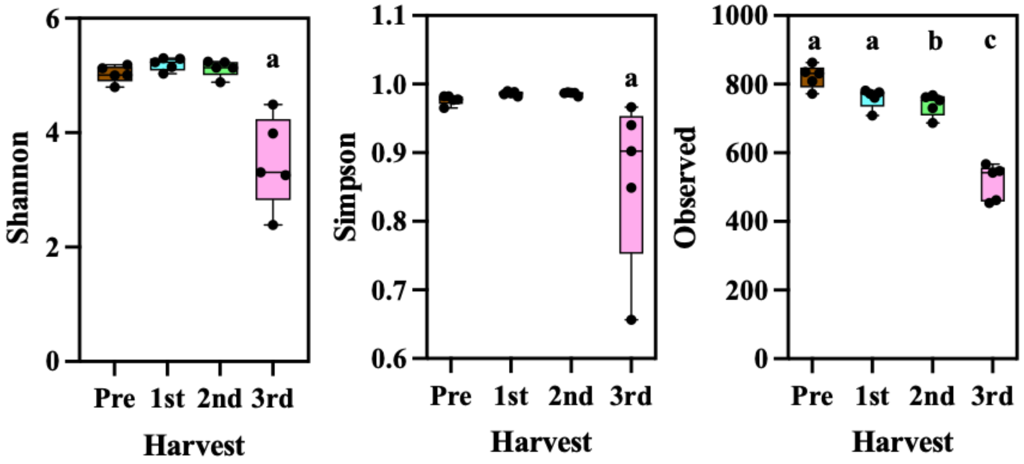
Alpha diversity for rhizosphere communities was measured in each harvest using three different methodologies (Figure 3.11). The Shannon index analysis was used to account for both rare and common species. Observed richness refers to the number of different species observed in a sample or community. The Simpson diversity is a measure of diversity that considers the number of species present, as well as the relative abundance of each species. The equations for each index are shown in the methods section. Figure 3.11A showed the result from the first experiment, which I observed a significant impact of pea rotation on microbial population. Overall, the Kruskal–Wallis test indicated significant differences among harvests for Shannon (p = 7.05x10^{-5}), Simpson diversity (p = 3.16x10^{-5}), and observed ASVs (p = 3.57x10^{-5}). Post-hoc Wilcoxon tests with Bonferroni correction revealed that diversity was significantly lower at the 2nd harvest compared with the other harvests. Similarly, in the third experiment (Figure 3.11B), significant differences among harvests for Shannon (p = 0.003), Simpson diversity (p = 0.002), and observed ASVs (p = 0.001). The 3rd harvest diversity was significantly lower than the other harvests in all three indices and observed richness also showed lower significance for the 2nd harvest compared to the preharvest and 1st harvest. The fungal diversity shown in Figure 3.11C was Shannon (p = 0.003), Simpson diversity (p = 0.007), and observed ASVs (p = 9x10^{-4}). The significant difference between harvests also showed that the 3rd harvest was the lowest and observed richness indicated that the 2nd harvest diversity was also lower than the preharvest, and 1st harvest. These results suggest microbial diversity remains relatively stable after the first pea cultivation, and then the community changed would take place later in either the 2nd or the 3rd time of pea rotation. In one experiment, diversity declined at the 2nd harvest but recovered by the third. In contrast, in another experiment, particularly for fungal communities, the lowest diversity was observed at the 3rd harvest. As the study concluded following this harvest, it remains uncertain whether this decline was permanent or if diversity would have recovered with further cropping, as observed in the first experiment.

A key limitation of amplicon sequencing is its inability to provide sufficient resolution for identifying species- or strain-level distinctions. In chapter 4, randomly selected bacterial isolates were sequenced, targeting the housekeeping gene *rpoB* to gain deeper insight into their identity and diversity.

A) Experiment 1 : 16S



B) Experiment 3 : 16S



C) Experiment 3 : ITS

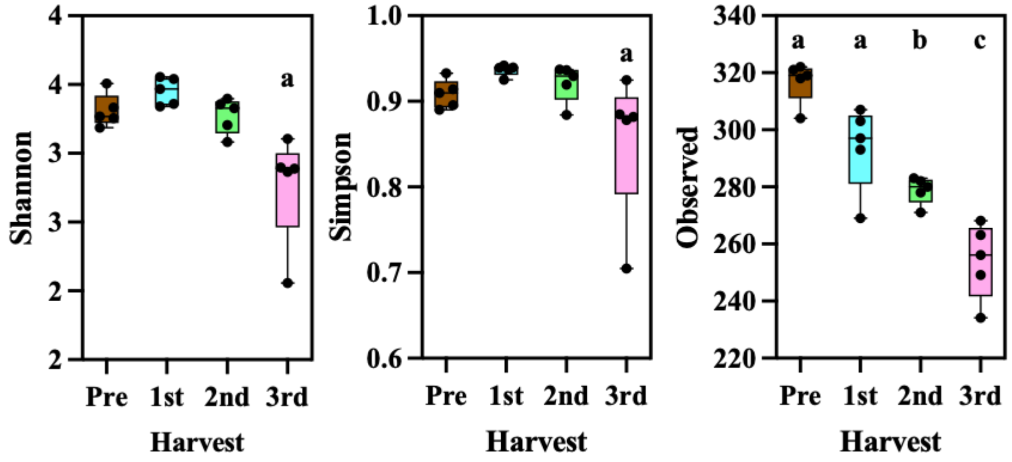


Figure 3.11 Boxplot of Alpha diversity comparison for rhizosphere communities measured by Shannon diversity, Simpson diversity and the observed richness. A) Bacterial diversity between harvest after close rotation in the first experiment B) Bacterial diversity between harvest after close rotation in the third experiment C) Fungal diversity

between harvest after close rotation in the third experiment. Different letters above boxes indicate significant differences between groups based on Kruskal–Wallis tests followed by pairwise Wilcoxon comparisons with Bonferroni correction ($p < 0.05$). Analysis performed with five to twelve rhizosphere samples per harvest.

3.3.5 Quantification of bacterial and fungal population after close rotation

qPCR was performed to quantify the abundance of bacterial species in the rhizosphere community under close rotation. Primers designed for 16s rRNA universal bacterial populations allowed us to detect changes in bacterial DNA abundance between soil rotations (Figure 3.12). For both the second and third experiments, fold change of bacterial population was significantly higher after pea cultivation. In first experiment, the 2nd harvest group exhibits the highest fold change, significantly greater than the preharvest, 1st harvest and 3rd harvest groups. This result suggests that plant cultivation stimulated an increase in the rhizosphere bacterial population, followed by further fluctuations during continuous cropping.

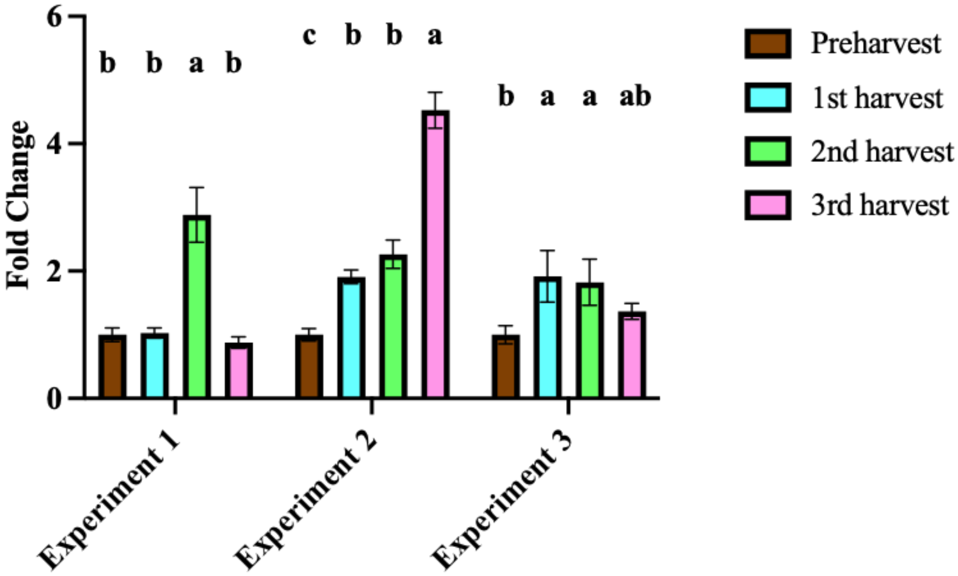


Figure 3.12 Quantification of bacterial population after close rotation. Rhizosphere samples from independent close rotation experiments (experiment 1,2 and 3) were investigated by qPCR. A one-way ANOVA was performed with Tukey multiple

comparisons. Letters above plots (a, b, c, d) indicate statistical groupings, with different letters indicating significant differences.

A high and competitive *Rhizobium* population is essential for effective nodulation and nitrogen input in legume crops like pea (Mendoza-Suárez et al., 2021). In this study, abundance of *Rhizobium* was followed throughout the close rotation using *Rhizobium* specific primers. In both the first and third experiments, the *Rhizobium* abundance in the first harvest differed significantly from preharvest (Figure 3.13). Also, the similar trend in following harvests of these two experiments was observed. These findings indicate that *Rhizobium* is rapidly recruited to the rhizosphere upon plant establishment. However, in the second experiment, the significance different of *Rhizobium* population was observed at the third harvest. Collectively, these observations imply that the impact of pea rotation on microbial communities might be characterised by high context-dependency such as starting microbial communities in preharvest soil. Thus, each biological experiment may respond differently to the same treatment conditions.

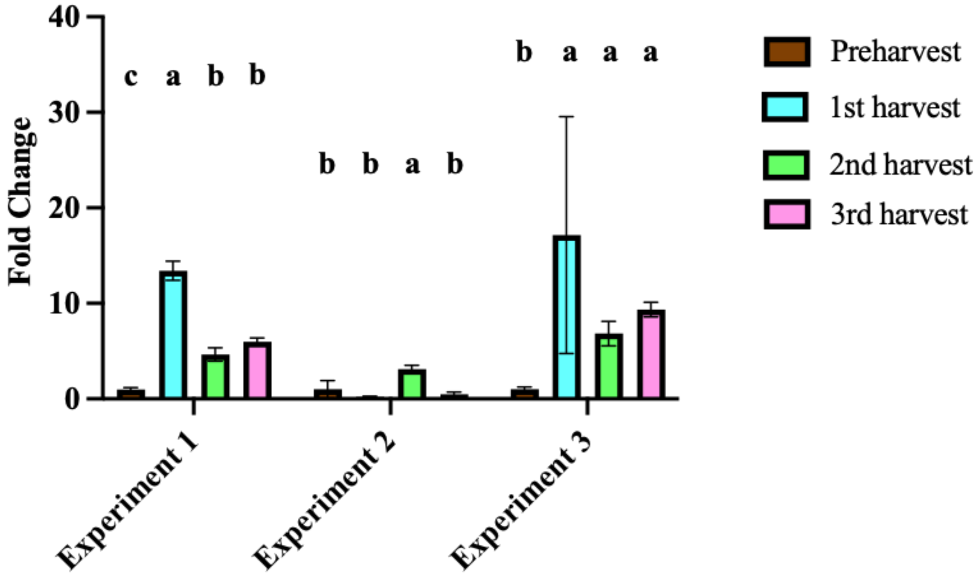


Figure 3.13 Quantification of *Rhizobium* population after close rotation. Rhizosphere samples from independent close rotation experiments (experiment 1, 2 and 3) were investigated by qPCR. A one-way ANOVA was performed with Tukey multiple

comparisons. Letters above plots (a, b, c, d) indicate statistical groupings, with different letters indicating significant differences ($p<0.05$).

The *nodD* gene is a key regulatory gene that is essential for establishing a symbiotic partnership with leguminous host plants. To test whether nodulation gene-carrying populations were enriched in the rhizosphere under close rotation, I quantified *nodD* gene by qPCR. Across the four harvest stages, results from all three experiments consistently showed that *nodD* gene abundance peaked at the first harvest (Figure 3.14). The abundance is also significantly increased in first and second experiment. Although there was a decline after the 1^{st} harvest, abundance levels remain relatively elevated through the 3^{rd} harvest. This result suggested that nodulation was selected across the close rotation.

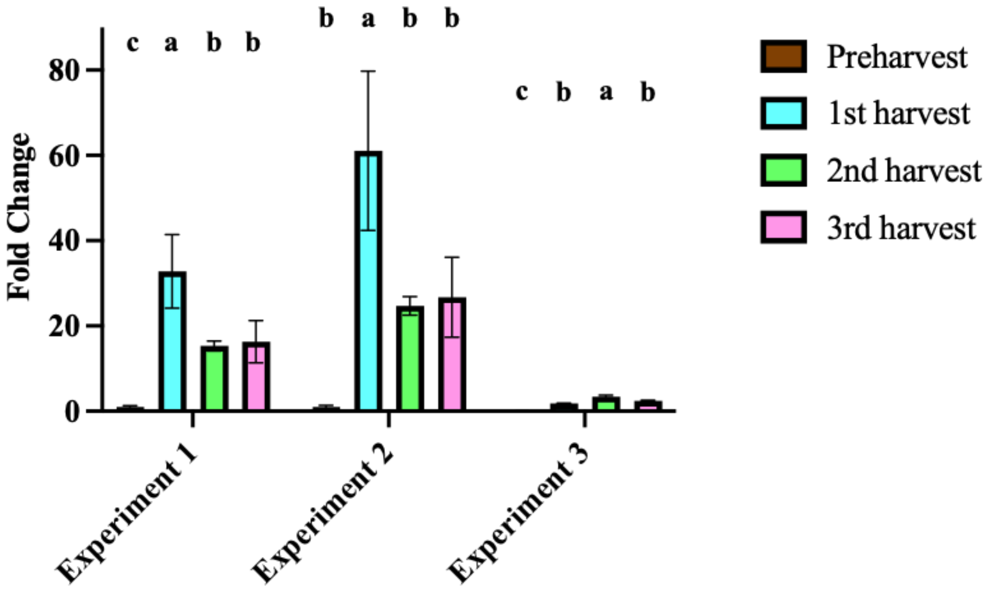


Figure 3.14 Quantification of *nodD* gene after close rotation. Rhizosphere samples from independent close rotation experiments (experiment 1,2 and 3) were investigated by qPCR. A one-way ANOVA was performed with Tukey multiple comparisons. Letters above plots (a, b, c, d) indicate statistical groupings, with different letters indicating significant differences ($p<0.05$).

3.3.6 Quantification of the fungal population after close rotation

Pea plant yields and seed quality can be reduced upon infection with several fungal diseases. Therefore, it is essential to determine whether the fungal population is impacted by the close rotation. *Fusarium* species was chosen as a representative of the most common fungi in the rot complex (Soylu & Dervis, 2011; Trenk et al., 2024). To assess the abundance and dynamics of fungal communities, qPCR targeting the *F. solani* was performed. Results (Figure 3.15) showed that a low amount of *F. solani* population was detected in all four harvests and three independent replicates. *F. solani* abundance peaked at the 1st harvest in the first experiment but drastically decreased at the 2nd harvest. At the 3rd harvest, there was a slight increase in abundance. A different pattern was shown in the second experiment, where populations grew gradually from preharvest to the 3rd harvest before reaching noticeably higher levels at the 2nd and 3rd harvests. This pattern was replicated in the third experiment, where levels peaked at the 3rd harvest after gradually increasing from their lowest levels at the 1st harvest. When combined, the findings imply that close rotation encourages a slow accumulation of *F. solani* in the rhizosphere. However, there were no obvious signs of root rot, which could indicate that the fungus population was still below is the levels usually linked to symptoms.

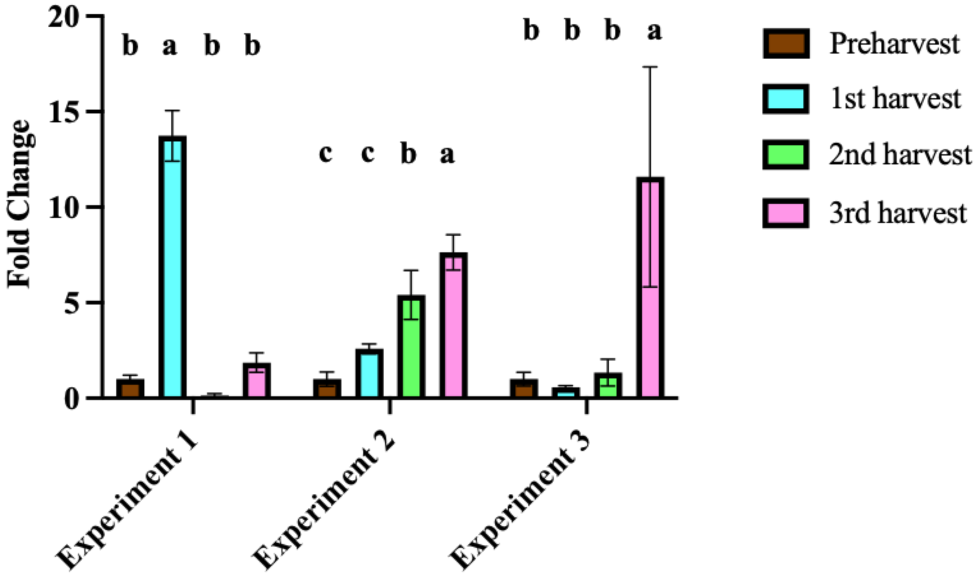


Figure 3.15 qPCR quantification of *F. solani* after close rotation. Rhizosphere samples from independent close rotation experiments (experiment 1, 2 and 3) were investigated by qPCR. A one-way ANOVA was performed with Tukey multiple comparisons. Letters above

plots (a, b, c, d) indicate statistical groupings, with different letters indicating significant differences ($p<0.05$).

3.4 Discussion

In this study, I hypothesised that close rotation affects the composition of the rhizosphere microbial community. Therefore, a close rotation experiment was designed so that all three pea harvests used the same seed cohort under identical growth conditions. Doing so, I can attribute the observed differences in plant growth to the effects of only soil material that was passed on from the previous harvest.

From the long-term experiments growing pea in the glasshouse, it was observed that plant weight was not significantly affected by close rotation. This observation may be due to spatially restricted growing conditions in the pot setup that may not accurately reflect the agricultural conditions. Peas growing in the field grow differently from those growing in a pot or container. It has been observed that the root structures are different between peas grown in open soil and those grown in pots as the pots limit root exploration (Poorter et al., 2012). Previously, studies examining peas during continuous cropping showed a change in plant morphology. A similar experiment to my study has been conducted by Ma et al. which pea plants were cultivated for 16 days in field-sourced continuous-cropping soil, whereas in our study, peas were grown continuously for four weeks in pots under controlled-environment conditions. This experimental setup might introduce variations that could lead to divergent outcomes compared to my findings. They found that the reduction of height, shoot and root length while planting with continuous cropping soil varies depending on the pea genotypes. Overall bacterial diversity remained steady, the relative abundance of certain microbial groups shifted notably with continuous cropping (Ma et al., 2023).

The results showed a high nutrient level of Mg and P during a close rotation, with no significant differences between harvests. The highest level was observed in K content, while N level showed a significant difference after pea planting. This result indicated the nitrogen fixation activity by pea plant as shown in literatures (Gou et al., 2023; Tripolskaja et al., 2023). My results showed the range of soil pH from 7.8 to 8.1, which means soil has significant increased between preharvest and 3rd harvest soil. A similar study showed results of soil pH increasing after pea continuous cropping from 7.8 to 8.0 (Ma et al., 2023). This finding might suggest the change in microbial composition. Several studies in other crops indicated that continuous cropping decreased soil pH (Lv et al., 2025; Pang et al., 2021; Wang et al., 2023). Bai et al. found that pH was not significantly correlated with bacterial abundance. Consequently, it may indirectly influence bacterial communities by altering other environmental factors (Bai et al., 2019).

In our lab-based experiment, the repeated tillage conducted after each harvest likely prevented an accumulation of organic matter. As the organic matter was exposed to the air, resulting in increased rates of decomposition. Rotating annual row crops with perennial grass or legume sods helps reduce erosion and increase organic matter in the soil as their root systems decompose (Cornell University Cooperative, 2008). Legume cover crops are commonly used to increase organic matter, as they enrich the soil with nitrogen (Kladivko, 2016). Organic matter levels between 3% and 6% are generally ideal for promoting the health and productivity of most agricultural crops (Cornell University Cooperative, 2008). It can enhance soil structure by promoting the formation of stable aggregates, increase soil holding capacity, and fuel soil microbial (Bot & Benites, 2005).

Amplicon sequencing was used to examine the effect of close rotation on the rhizosphere community. The results showed that both the bacterial and fungal communities responded to close rotation. The PCoA plot illustrates that microbial communities were moderately separated based on harvest groupings, a pattern that is unlikely to have arisen due to random variation ($p < 0.001$). Bacterial community analysis suggests that continuous cultivation might transiently favour fast-growing taxa like Proteobacteria (Kurm et al., 2017), whose abundance increased in the subsequent harvest before declining by the third harvest of the first experiment. However, in the third experiment the *Pseudomonas* population increased steadily along the harvest. Thus, these biological replicates showed the similar trend of Proteobacteria in particular, *Pseudomonas,* after close rotation. Acidobacteriota *(*genus *Gp4* and *Gp6)* were generally present in all harvests of close rotation. This genus is considered oligotrophs, meaning they thrive in nutrient-poor environments. Their adaptation enables them to endure and prevail in environments where other bacteria may encounter difficulties (Kielak et al., 2016). In the third experiment, the abundance of *Agrobacterium* was observed after pea cultivation. This genus belongs to the *Rhizobiaceae* family and is known to live in root surfaces and nodules (Miranda-Sánchez et al., 2016). This could potentially reflect enhanced nitrogen-fixing or symbiotic interactions around the rhizosphere. Both experiments contain common bacterial families that have been identified in pea such as *Comamonadaceae* and*, Sphingomonadaceae* (Chalasani et al., 2021). They are involved in nutrient cycling and stress adaptation (Schmalenberger et al., 2008)**.** In addition, I observed high abundance of genus *Massilia* after pea planting. This genus was reported to promote shoot growth and nitrogen accumulation (Han et al., 2024). The preharvest fungal community was majority dominant in genus *Gibellulopsis*. This genus was initially reported as a soil-borne fungus causing mild wilt symptoms (Melouk & Horner, 1974). Due to their attenuated virulence, it has been applied in several crops to induce resistance against Verticillium wilt, a soil-borne vascular disease (Ogundeji et al., 2022). Moreover, it is among the species recruited in suppressive soils (Prestt & Roberts, 2023; Xiao et al., 2025). This genus was gradually overtaken by *Fusarium* after close rotation, as determined by amplicon sequencing and qPCR abundance quantification.

My results showed a lot of experimental variability, with the first experiments showed that bacterial diversity decreased at the 2nd harvest but then recovered for the 3rd harvest. However, within the third experiment I found that the bacterial diversity reduced over time, with the lowest bacterial diversity being found within the 3rd harvest sample. It is worth mentioning that a factor that might influence the variation in this study is that preharvest soil for independent experiments was collected at different times of the year. This might contribute to the difference in starting soil microbiota in the first place. Despite the soil differences between these two biological experiments, there were some key trends that emerged in both. This phenomenon was also observed in the barley rhizophere microbiome study conducted in our lab (Pacheco-Moreno et al., 2024). In this experiment, that standard amplicon sequencing data were used to assess relative taxonomic composition. Absolute abundances could not be directly inferred from sequencing data alone. Because the PCR amplification introduces bias, different taxa have different gene copy numbers and sequencing depth varies between samples. Future studies could address this limitation by incorporating approaches such as the use of internal spike-in standards or combining amplicon sequencing with qPCR to quantify total microbial load. Normalising relative abundance data to independently measured gene copy numbers would allow more accurate estimation of absolute abundances and improve interpretation of treatment effects on microbial population size.

I found that both the overall bacterial population and *Rhizobium* population within the rhizosphere both increased immediately in abundance after pea planting. Previous work has shown that *Rhizobium* populations specifically follow this increased pattern (Hynes et al., 2001). The *nodD* gene, a central regulatory gene in the nodulation process, was monitored across four harvest stages to assess its expression dynamics. The result showed that *nodD* expression peaked at the first harvest, this suggests strong and immediate selection of nodulation activity by the plant. While its abundance declined slightly in subsequent harvests, the levels of *nodD* expression remained consistently higher than preharvest levels through to the third harvest. In some cases, the *nodD* gene copy number increased even though the overall *Rhizobium* population declined. This may be due to changes in strain composition, differences in gene copy number, or enrichment of symbiotic plasmids rather than an increase in total cell numbers (Pacheco et al., 2016).

Overall, close rotation did not affect visible pea phenotypes, and pathogenic fungal abundance remained at low levels across all harvests. This might suggest that although monocropping is often associated with increased disease risk, under certain conditions, it can lead to the development of disease-suppressive soils where diseases fail to persist or occur only at low levels despite the presence of pathogens (Peng et al., 2025). Plant root exudates, are able to recruit specific groups of microbes such as *Bacillus, Burkholderia, Chthonomonas, Pseudomonas*, and *Serratia* (Van Bruggen & Semenov, 2000). General suppression is a natural property of all soils, driven by the collective competitive activity of the soil's microbial communities (Raaijmakers & Mazzola, 2016).

This chapter examines the close rotation bacterial community in a non-culturable approach that showed consistent signs of community shift after close rotation. The bacterial diversity decreased and moved towards Proteobacteria, most obviously *Pseudomonas*. Similarly, the fungal diversity also decreased and shift toward *Fusarium.* Nitrogen fixing genes and species were strongly recruited after the pea planting. A deeper understanding of these communities requires resolving how bacterial populations differ across the preharvest, 1st, 2nd, and 3rd harvests. In the next chapter, I analyse soil bacterial isolates from each harvest to elucidate phenotype-based selection at each harvest.

Chapter 4 -
Analysis of bacterial population dynamics during close rotation

Chapter 4 - Analysis of bacterial population dynamics during close rotation

4.1 Introduction

Pseudomonas and *Rhizobium* species are particularly significant plant growth promoting bacteria (Etesami, 2025; Fahde et al., 2023) with importance to pea health. Examining how their populations change in close rotation is essential and will help us understand more about the microbiome alterations we observe. Because of the high diversity within the genus, *Pseudomonas* deploy multiple strategies to colonise the rhizosphere and antagonise plant pathogens (Loper et al., 2012; Raaijmakers et al., 2010). However, the genetic component of these beneficial activities remain only partially resolved (Pacheco-Moreno et al., 2024). Comparative genomics of the *P. fluorescens* complex reveals a small core genome (~1,334 coding sequences) alongside a massive pan-genome (>30,000 coding sequences). This suggests an extensive gene gain and loss across lineages and very high phenotypic diversity (Garrido-Sanz et al., 2016). To determine how close crop rotation shapes soil *Pseudomonas*, it is essential to accurately characterise isolate-level genotypic and phenotypic variation.

To successfully survive on the root surface, bacteria must move towards the root and form a protective biofilm. Biofilms formation occurs when bacteria assemble into structured communities encased in a self-produced polymeric matrix comprising an extracellular polymeric substance including exopolysaccharides (EPS), proteins, and DNA (Ramey et al., 2004), providing resistance to alterations in pH and protection from antimicrobial agents (Danhorn & Fuqua, 2007; Stoodley et al., 2002). Motility is also an important trait that facilitates the initial contact and adsorption of symbiotic rhizobia to the host root surface (Caetano-Anollés et al., 1988). Effective root colonisation depends on bacterial motility and chemotaxis toward root exudates including sugars, amino acids, and organic acids. Consequently, less motile pathogens can be competitively suppressed, and niches can be quickly occupied (Haas & Défago, 2005; Miller et al., 2007). Similarly, iron is micronutrient that is necessary for almost all living things but it is mostly found in soils in an oxidised ferric form (Fe^{3+}), which reduces its bioavailability (Li et al., 2023). To overcome this limitation, many soil and rhizosphere bacteria produce siderophores. These are a low-molecular-weight compound with a high affinity for ferric iron (Miethke & Marahiel, 2007).

Several bacterial species also produce hydrogen cyanide (HCN) which is an antagonistic metabolite that suppresses soilborne pathogens. HCN may additionally promote phosphate availability by chelating iron and preventing Fe–phosphate precipitation (Rijavec & Lapanje, 2016). *P. fluorescens* can also produce natural products that have nematocidal effects on plant-parasitic nematodes (Rahanandeh et al., 2024; Siddiqui & Shaukat, 2003). Moreover, they can produce indole-3-acetic acid (IAA) which is the main auxin that naturally occur in plants as a key regulator of plant growth and development (Woodward & Bartel, 2005). It is estimated that more than 80% of the bacteria isolated from the rhizosphere possess the ability to synthesise IAA (Patten & Glick, 1996). In addition, PGPR can reduce disease pressures by producing antifungal proteases to eliminate *R. solani* and *Fusarium* spp. (Nanadakumar et al., 2002; Yen et al., 2006).

Understanding how *Rhizobium* populations change over time is important because these bacteria support plant growth through their ability to fix atmospheric nitrogen (Oldroyd, 2013). Both species-level and strain-level differences can alter how well nodules form and how efficiently nitrogen is fixed. Furthermore, studies have shown that it is not just the number of microbes present that matters, but whether they are metabolically active (Harris et al., 2025). Active strains with beneficial metabolic traits are the ones most likely to colonise roots successfully and contribute to effective symbiosis. Overall, elucidating the dynamics of *Pseudomonas* and *Rhizobium* are crucial, to understand rhizosphere recruitment under close rotation.

In the previous chapter, the overall structure of the bacterial community was characterised. However, species-level identification and phenotypic characterisation remain necessary for a deeper understanding of microbiome change, as phenotypic characterisation reveals the functional traits that determine how microorganisms interact with plants, soil, and each other. In addition, the correlation between each phenotype will reveal what drives the selection of bacteria in certain harvests. Here, I aim to understand specifically how *Pseudomonas* and *Rhizobium* populations change, and the functional characteristics that are selected by the plant at an individual level.

4.2 Objectives

1. Determination of *Pseudomonas* and *Rhizobium* populations to observe number of species selected during close rotation.
2. Characterisation of the phenotypic traits of soil bacterial isolation, *Pseudomonas* spp. and *Rhizobium* spp., under close rotation.
3. Identification of differences in the phenotypic profiles of *Pseudomonas* spp. and *Rhizobium* spp. strains between harvests.

4.3 Results

4.3.1 Isolation of *Pseudomonas* spp. and *Rhizobium* spp. from each harvest

Rhizosphere samples were collected from each harvest (8-10 plants) to investigate differences in soil community composition across each close rotation harvest. To capture as much diversity as possible, these samples were cultured on 10% tryptone soy agar (TSA), Figure 4.1 shows the general culturable bacterial population from each harvest.

Pseudomonas spp. and *Rhizobium* spp. were selectively isolated as representative of key stone taxa in rhizosphere community. *Pseudomonas* was isolated on Cetrimide-fucidin-cephalosporin (CFC) agar and *Rhizobium* was isolated on *Rhizobium* semi-selective media (Figure 4.2). Collectively, 192 bacterial isolates were randomly picked per harvest. Isolates from different plants were combined to form a single representative sample for each harvest.

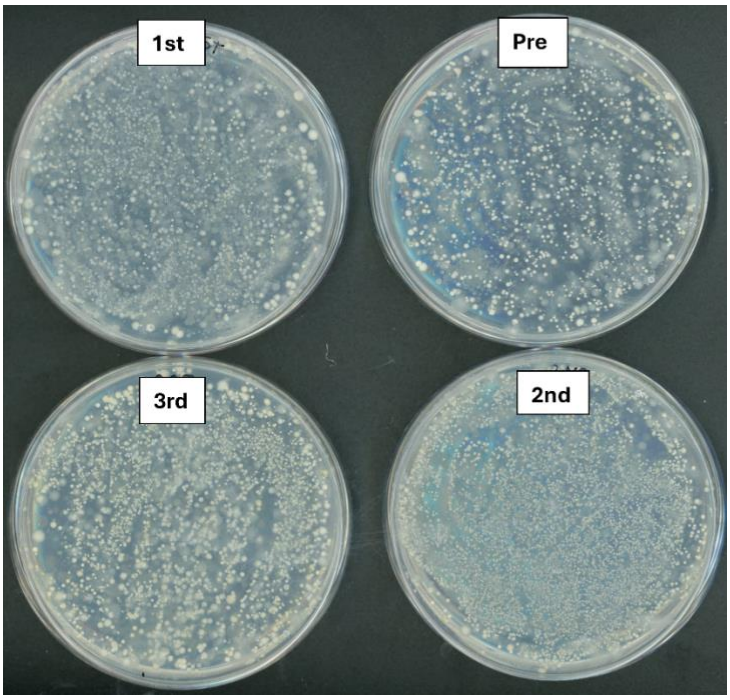


Figure 4.1 Soil bacteria from each harvest after close rotation. Bacteria were isolated on Tryptone Soy Agar (TSA) plate. Plates show preharvest isolates (top right),1st harvest isolates (top left), 2nd harvest isolates (bottom right) and 3rd harvest isolates (bottom left).

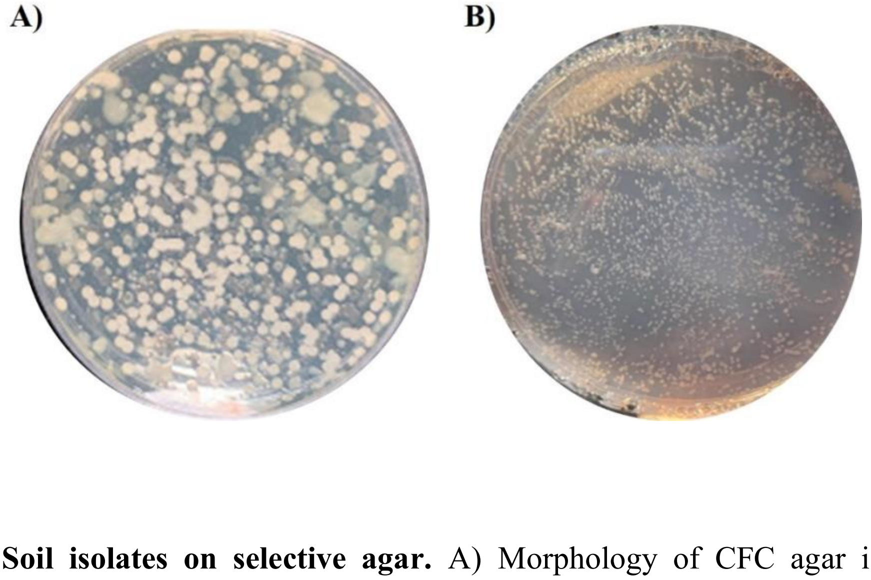


Figure 4.2 Soil isolates on selective agar. A) Morphology of CFC agar isolates. B) Morphology of *Rhizobium* selective agar isolates.

4.3.2 Phenotypic characterisation

To comprehend how different bacterial populations selected by plants throughout close rotation, high-throughput phenotypic screening was conducted. A total of 2,304 CFC agar isolates, and 1,152 *Rhizobium* selective agar isolates were examined. These bacteria were randomly picked across four harvests of three independent close rotation experiments. Isolates from each harvest were tested for biofilm formation, protease production, hydrogen cyanide production, motility, and siderophore production. An ordinal scoring system was used for each phenotypic trait where 0 means absence of the phenotype and 1 means presence of phenotype (Material and methods section (Figure 2.5)).

4.3.2.1 Phenotypes of CFC agar isolated bacteria

4.3.2.1.1 Production of exopolysaccharides measured by Congo Red binding.

Congo Red binding (CRB) was used to detect the presence of an extracellular matrix and biofilm production (Ma et al., 2006). Figures 4.3B shows that isolates from the first and second close rotations experiments, there was a steady increase in CRB across all harvests. These patterns suggest that relative to the start of close rotation, repeated planting selected for a higher proportion of biofilm-producing isolates. However, this increased trend showed a peak of exopolysaccharide producing isolates in the 2nd harvest of the second experiment followed by a significant drop in the 3rd harvest. Nonetheless, this result suggests that an ability to produce biofilm was not consistently selected for during close rotation.

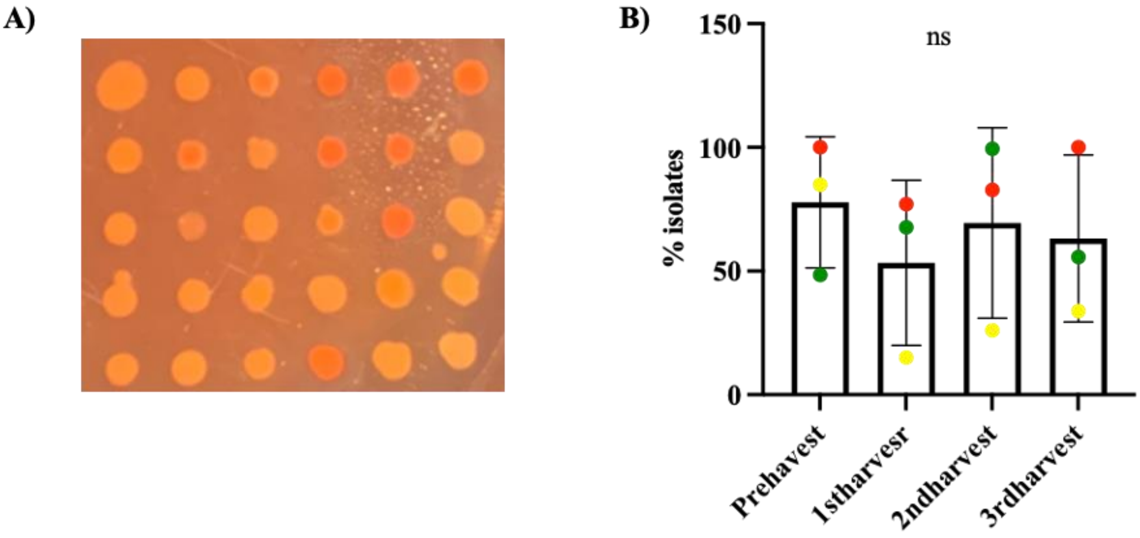


Figure 4.3 Congo Red binding of CFC agar isolated bacteria. A) representative image of CRB assay on KB agar. B) Percentage of CRB isolates across three independent experiments. Data points are presented as coloured symbols categorised by independent experiment, first experiment (red), second experiment (green) and third experiment (yellow). The error bars represent the standard deviation. For each harvest, 192 isolates were screened. Data is reported as the proportion of isolates (%). Group differences were evaluated using a mixed-effects model analysis with Tukey multiple comparisons.

4.3.2.1.2 Protease Activity

Agar plates supplemented with milk powder were used to assess extracellular protease production. As milk agar contains casein, the major milk protein, active strains produce a clear zone (halo) around the colony resulting from casein degradation (Figure 4.4A). The present of the halo serves as a measure of proteolytic activity (Smibert & Krieg, 1994). The first and second experiments showed a fluctuating pattern (Figure 4.4B), whilst this pattern was absent in the third experiment where less than 50% of population were able to produce protease. This result revealed no consistent trend in protease activity.

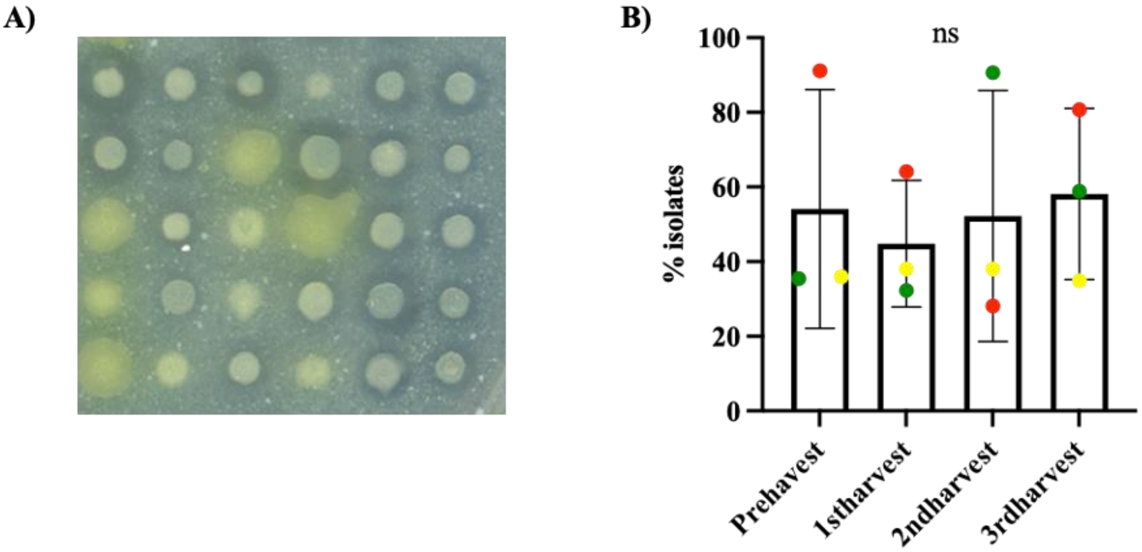


Figure 4.4 Protease activity of CFC agar isolated bacteria. A) representative image of protease production on milk agar. B) Percentage of protease producing isolates across three independent experiments. Data points are presented as coloured symbols categorised by independent experiment, first experiment (red), second experiment (green) and third experiment (yellow). The error bars represent the standard deviation. For each harvest, 192 isolates were screened. Data is reported as the proportion of isolates (%). Group differences were evaluated using a mixed-effects model analysis with Tukey multiple comparisons.

4.3.2.1.3 Motility

Low percentage agar plates (0.5% agar) were used to observed surface migration of bacteria to identify the population of motile bacteria in each harvest (Figure 4.5A). The first experiment showed a massive drop after planting while the third experiments showed a minimal drop (Figure 4.5B). The 2nd and 3rd harvests of the three experiment consistently included motile bacteria, constituting over 60% of the population. However, there was no significant increase in any harvest. Thus, this suggests that motility was not selectively favoured in the close-rotation rhizosphere.

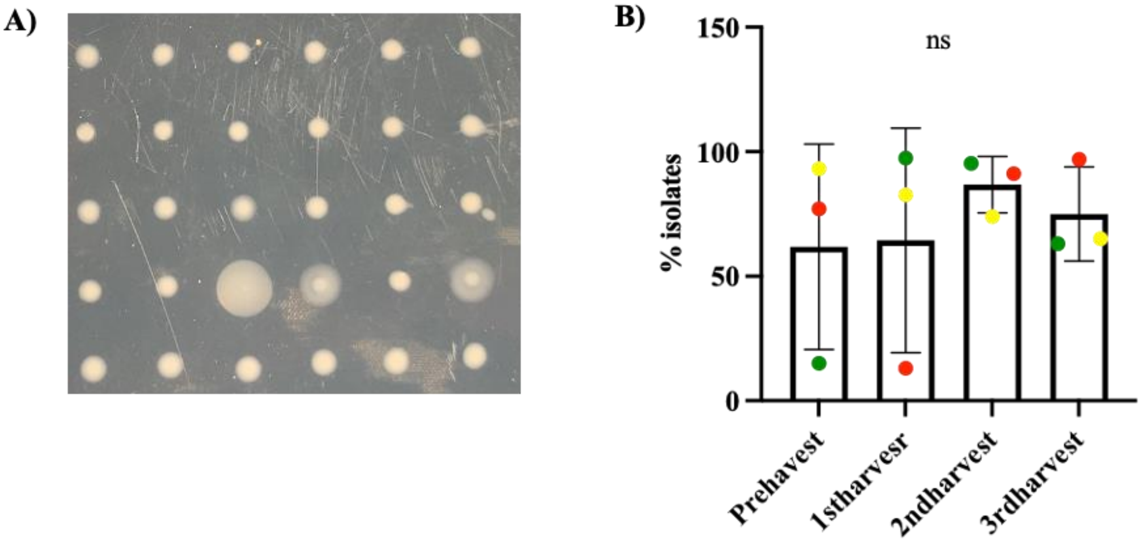


Figure 4.5 Motility of CFC agar isolated bacteria. A) representative image of motility assay on soft agar. B) Percentage of motile isolates across three independent experiments. Data points are presented as coloured symbols categorised by independent experiment, first experiment (red), second experiment (green) and third experiment (yellow). The error bars represent the standard deviation. For each harvest, 192 isolates were screened. Data is reported as the proportion of isolates (%). Group differences were evaluated using a mixed-effects model analysis with Tukey multiple comparisons.

4.3.2.1.4 Hydrogen cyanide (HCN) production

HCN-producing populations were identified using the Feigl and Anger colorimetric assay, a method that relies on detecting volatile HCN released during bacterial growth (Feigl & Anger, 1966). Overall, the population of HCN producers remained below 60%. While some fluctuations were observed in earlier harvests, a stable low proportion of HCN producers was maintained in the 2nd and 3rd harvests (Figure 4.6B). This data suggests that the production of HCN was not essential or selected within the pea rhizosphere.

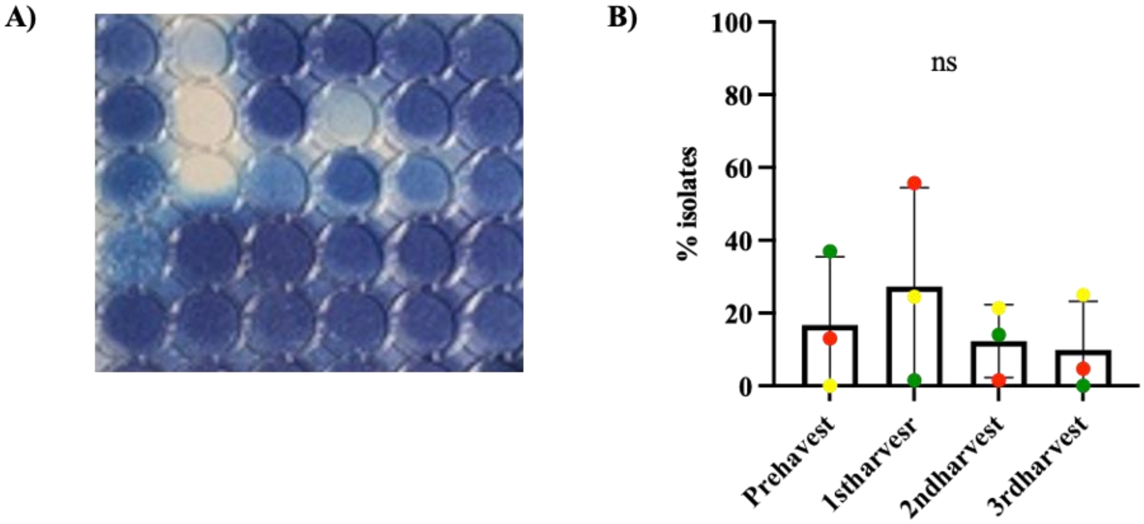


Figure 4.6 Hydrogen Cyanide production of CFC agar isolated bacteria. A) representative image of HCN production assay using Feigl-Anger solution. B) Percentage of HCN producing isolates across three independent experiments. Data points are presented as coloured symbols categorised by independent experiment, first experiment (red), second experiment (green) and third experiment (yellow). The error bars represent the standard deviation. For each harvest, 192 isolates were screened. Data is reported as the proportion of isolates (%). Group differences were evaluated using a mixed-effects model analysis with Tukey multiple comparisons.

4.3.2.1.5 Siderophore production

The fluorescence of bacterial colonies under UV illumination can suggest siderophore release because siderophores emit fluorescent light upon excitation with UV light (Meyer et al., 2002). The starting population in preharvest showed a huge variation with 10% of siderophore-producing isolates in the first experiment and close to 100% in the second experiment (Figure 4.7B). In addition, later harvests did not show a consistent pattern of selection. Thus, this trait might not be selectively advantageous after repeated close rotation with pea plants.

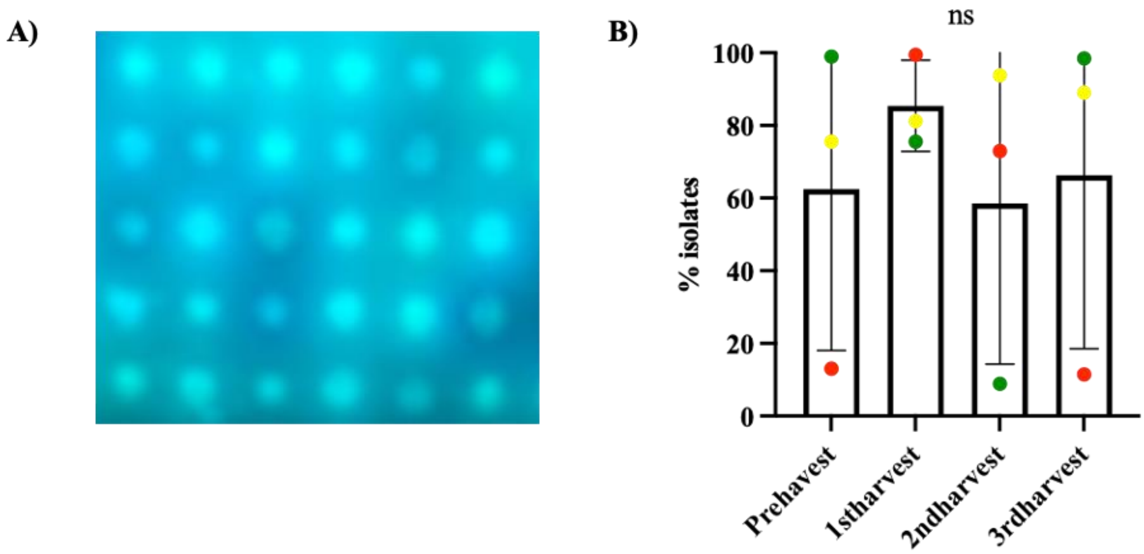


Figure 4.7 Siderophore production of CFC agar isolated bacteria. A) representative image of siderophore assay on KB media. B) Percentage of siderophore producing isolates across three independent experiments. Data points are presented as coloured symbols categorised by independent experiment, first experiment (red), second experiment (green) and third experiment (yellow). The error bars represent the standard deviation. For each harvest, 192 isolates were screened. Data is reported as the proportion of isolates (%). Group differences were evaluated using a mixed-effects model analysis with Tukey multiple comparisons.

4.3.2.1.6 Fungus suppression

In the soil, *Pseudomonas* bacteria produce a number of natural products including antifungals (Mehrabi et al., 2016). *Fusarium* species are selected as a representative of the most common fungi in the pea root rot complex. Bacteria isolated using CFC agar were evaluated for potential antifungal activity against *F. solani* on PDA agar plates as shown in Figure 4.8A. The result showed that the number of isolates which exhibited the fungal inhibition was less than 30% in all three independent experiments (Figure 4.8B). Additionally, there was no significant difference in antifungal activity between all harvests across all close rotation experiments. Thus, this suggests that close rotation did not enhance the recruitment of bacteria with antifungal properties.

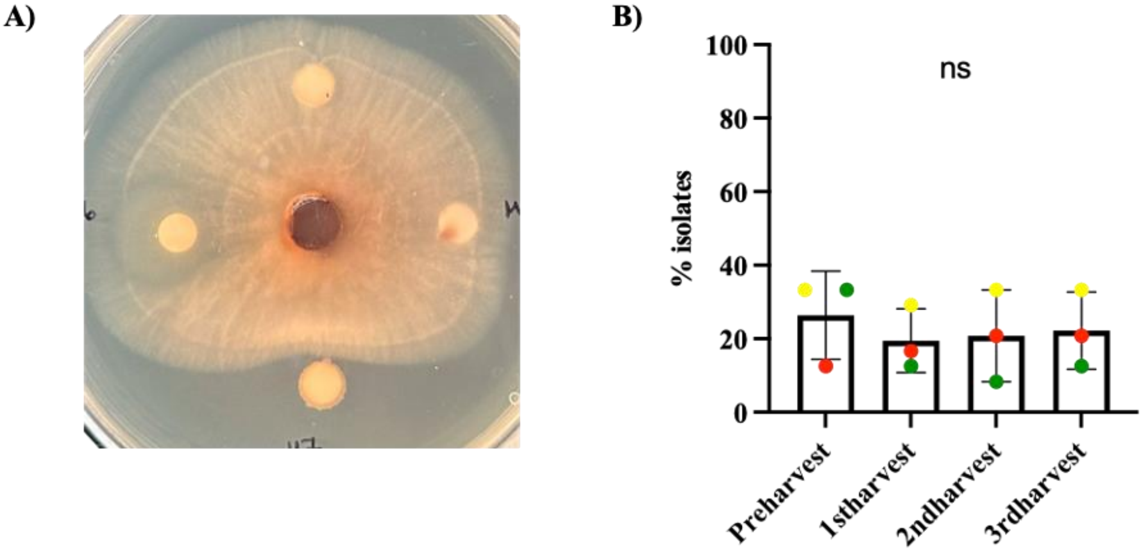


Figure 4.8 Fungal inhibition of CFC agar isolated bacteria. A) representative image of *F. solani* inhibition assay on PDA agar. B) Percent of isolates with antifungal activity across three independent experiments. Data points are presented as coloured symbols categorised by independent experiment, first experiment (red), second experiment (green) and third experiment (yellow). The error bars represent the standard deviation. For each harvest, 24 isolates were screened. Data is reported as the proportion of isolates (%). Group differences were evaluated using a mixed-effects model analysis with Tukey multiple comparisons.

4.3.2.1.7 Phenotypic profile of CFC agar isolates.

Radar charts were constructed to visualise how the general profile of bacterial traits changes across the close rotation experiment with respective to CFC agar isolates. Trait values were normalised to a 0–1 scale using min–max normalisation. Radar charts were constructed that compiled CRB, protease activity, motility and HCN across the three harvests within each experiment (Figure 4.9). The result indicated that the three independent biological experiments demonstrated variation in functional profiles.

In Figure 4.9A, the first experiment, preharvest (brown) and the 3rd harvest (pink) showed similar profiles, driven by protease and CRB and motility. The 1st harvest (blue) showed high levels of siderophore production, while motility and CRB binding were the most selected traits in the 2nd harvest (green). In the second experiment (Figure 4.9B), the preharvest, 1st and 3rd were defined by high siderophore production. The most diverse profile group was 2nd harvest, where CRB, motility and protease were selected. In Figure 4.9C, the third experiment, all the harvests displayed similar profiles, where bacteria generally showed high siderophore production and motility. In addition, the preharvest samples showed the strongest CRB phenotypes.

Overall, the first and second experiments demonstrated distinct, harvest-associated phenotypic patterns, while the third experiment showed relatively little harvest-specific phenotypic selection. These findings highlight that the trend that biofilm-forming predominated before cultivation. In contrast, isolates obtained from subsequent harvests showed increased phenotypic heterogeneity. To investigate how the phenotypic traits influenced bacterial selection across harvests under close rotation, a principal component analysis (PCA) was performed using same data from the three close rotation experiments based on five traits (Figure 4.10). The biplot revealed that PC1 and PC2 explain 34.9% and 22.9% of the total variance, respectively. Siderophore production and CB were the primary drivers of variance along PC1, while motility and HCN production influenced the distribution along PC2. Despite the different sampling times, isolates from preharvest, 1st, 2nd, and 3rd harvests showed substantial overlap. This suggested that the functional profile of CFC agar isolates community remains largely consistent throughout the close rotation. The results suggest that while the isolates are phenotypically diverse (forming distinct functional 'islands'), this diversity is maintained across all sampling time points rather than being driven by the harvest stage itself.

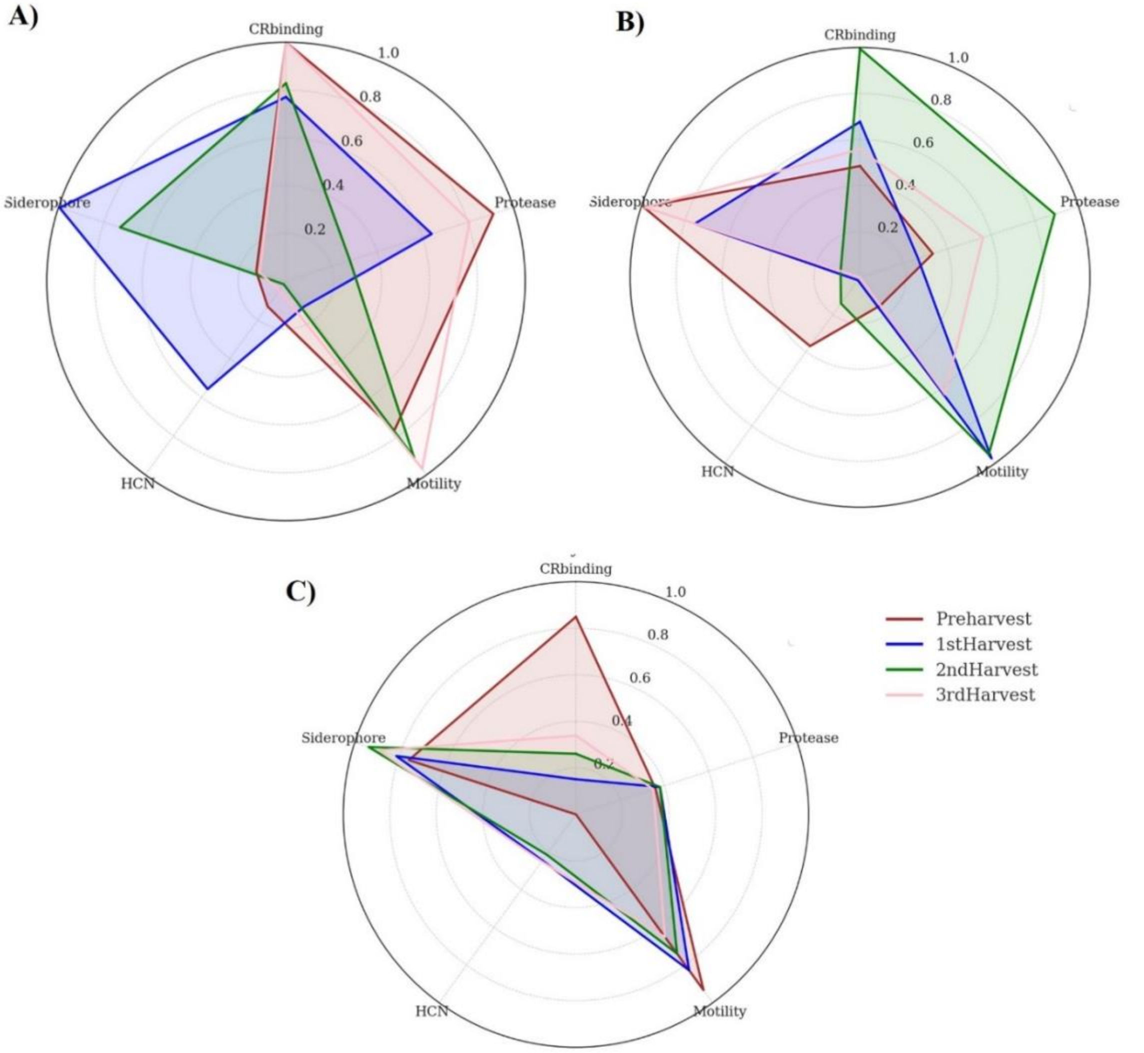


Figure 4.9 Radar chart of CFC agar isolates phenotype characteristic. A) Isolates from the first experiment B) Isolates from the second experiment C) Isolates from the third experiment. Phenotypic traits for Congo Red (CR) binding, protease, motility, HCN, and siderophore. Colour indicates the harvest stage: preharvest (brown), 1st harvest (blue), 2nd harvest (green), and 3rd harvest (pink).

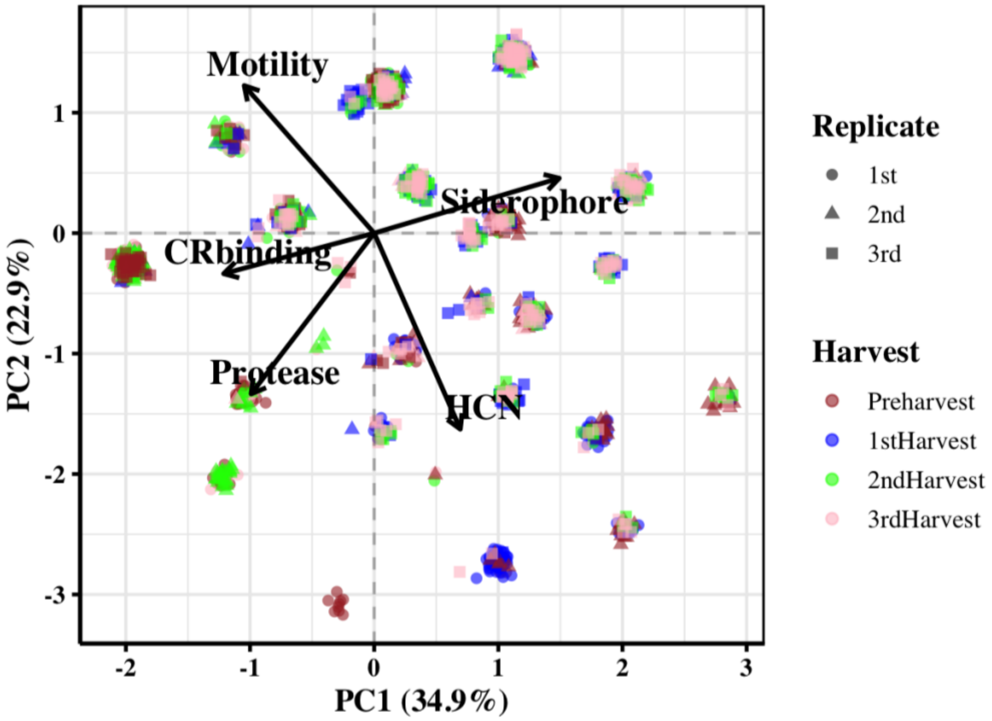


Figure 4.10 Principal component analysis of CFC agar isolates phenotypic traits. PCA biplot shows the distribution of strains based on their phenotypic traits for Congo Red (CR) binding, protease, motility, HCN, and siderophore production. Each dot represents one strain, and the colour indicates the harvest stage: preharvest (brown), 1st harvest (blue), 2nd harvest (green), and 3rd harvest (pink). Shapes representing the isolates from the first, second and third experiment. Arrows point in the direction of increasing values for each trait, and their lengths indicate their influence.

4.3.2.2 Phenotypes of the *Rhizobium* selective agar isolates.

4.3.2.2.1 Production of exopolysaccharides measured by Congo Red binding (CRB).

In the 1st and 3rd experiments, the highest proportion of CRB bacteria was observed in the 1st harvest (Figure 4.11B), which suggests that plant cultivation may influence the colonisation of biofilm formation bacteria. This population consistently decreased in subsequent harvests. However, there was no significant difference across harvests in the second experiment. Considering three experiments, biofilm formation was not a selected trait in close rotation.

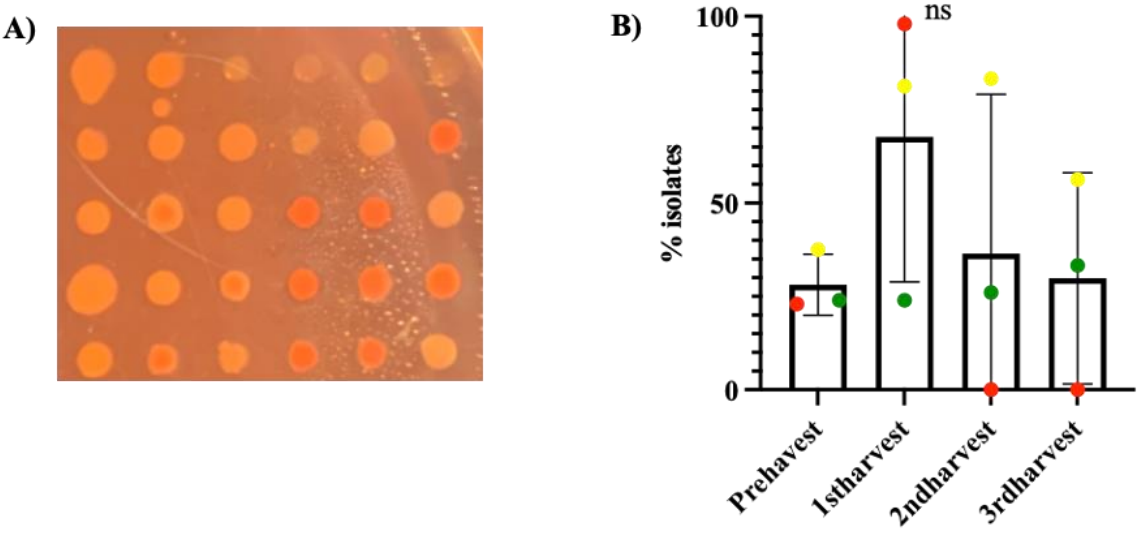


Figure 4.11 Congo Red activity of *Rhizobium* selective agar isolates. A) Example of CRB assay. B) Percentage of CRB isolates across three independent experiments. Data points are presented as coloured symbols categorised by independent experiment, first experiment (red), second experiment (green) and third experiment (yellow). The error bars represent the standard deviation. For each harvest, 96 isolates were screened. Data is reported as the proportion of isolates (%). Group differences were evaluated using a mixed-effects model analysis with Tukey multiple comparisons.

4.3.2.2.2 Protease activity

In first and second experiments, less than 5% of population had protease activity, a trait that exhibited no significant difference between harvests (Figure 4.12B). In contrast, the third experiment showed that up to 50% of the population could harbour the protease activity. This indicated that protease was not crucial for *Rhizobium* in pea rhizosphere as there is no pattern of recruitment.

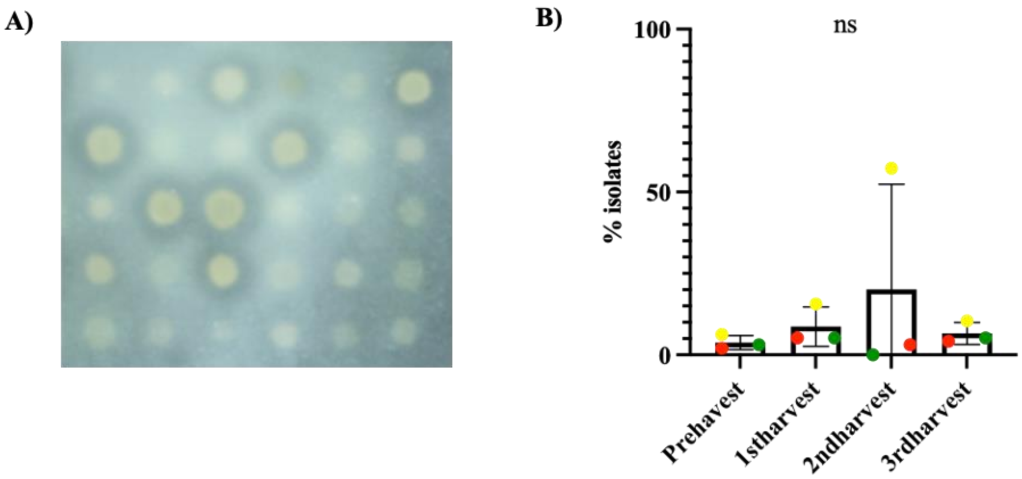


Figure 4.12 Protease activity of *Rhizobium* selective agar isolates A) representative image of Protease activity assay. B) Percentage of protease producing isolates across three independent experiments. Data points are presented as coloured symbols categorised by independent experiment, first experiment (red), second experiment (green) and third experiment (yellow). The error bars represent the standard deviation. For each harvest, 96 isolates were screened. Data is reported as the proportion of isolates (%). Group differences were evaluated using a mixed-effects model analysis with Tukey multiple comparisons.

4.3.2.2.3 Motility

Motility is a key trait that enables bacteria to move actively toward the rhizosphere during initial colonisation (Santoyo et al., 2021). However, three independent experiments showed that motility traits did not follow a consistent trend across harvests (Figure 4.13B). While the motile population increased in the 1st harvest during the first and second experiments, the third experiment maintained a motile population of more than 50%

throughout the closed rotation. This indicates that motility traits might not be essential for colonisation under closed-rotation conditions.

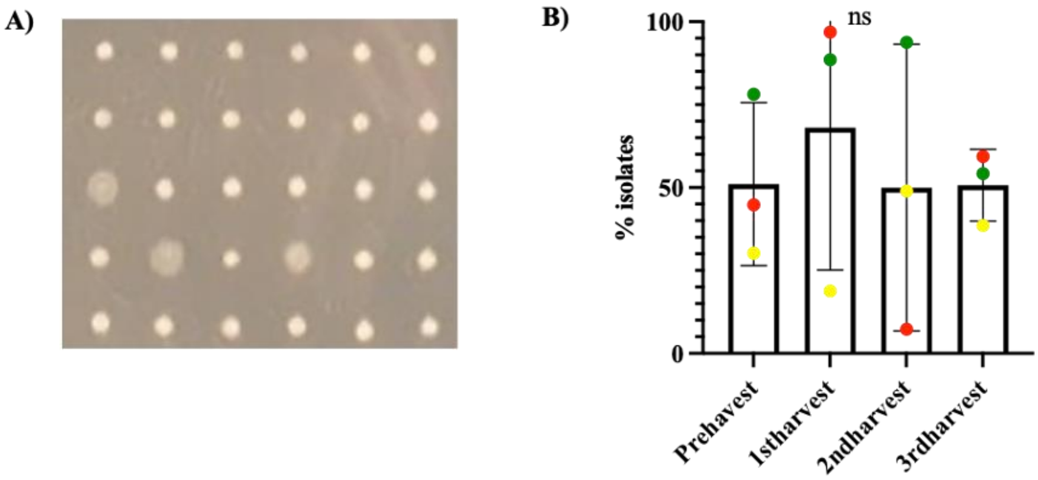


Figure 4.13 Motility of *Rhizobium* selective agar isolates A) Example of motility assay. B) Percentage of motile isolates across three independent experiments. Data points are presented as coloured symbols categorised by independent experiment, first experiment (red), second experiment (green) and third experiment (yellow). The error bars represent the standard deviation. For each harvest, 96 isolates were screened. Data is reported as the proportion of isolates (%). Group differences were evaluated using a mixed-effects model analysis with Tukey multiple comparisons.

4.3.2.2.4 IAA production

Rhizosphere-associated microorganisms, especially bacteria, exploit tryptophan released in plant root exudates as a primary precursor for IAA biosynthesis (Feng et al., 2024). Accordingly, tryptophan and Salkowski' reagent was added to the culture media in this assay. The pink colour shown in 96-well plates then indicated the production of IAA by the soil isolates (Figure 4.14A). In pre-, 1st and 2nd experiment, similar proportions of IAA-producing isolates were observed across all harvests (Figure 4.14B). In contrast, the 3rd harvest contained a significantly higher proportion of IAA-positive isolates, indicating that the IAA production trait was selected at later stages of close rotation.

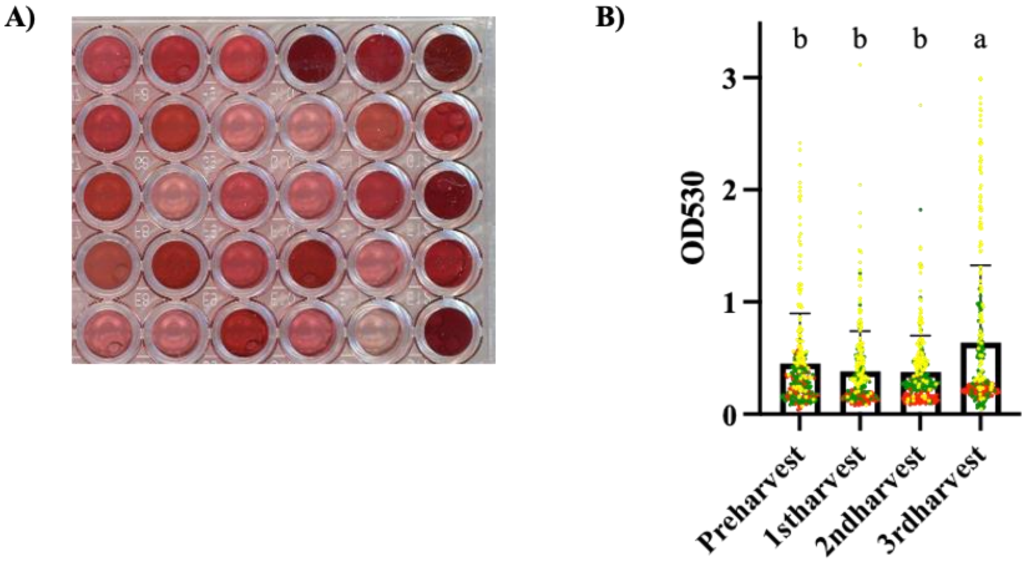


Figure 4.14 IAA production of *Rhizobium* selective agar isolates A) representative image of IAA production assay using Salkowski's reagent. B) OD_{530} of IAA producing isolates across three independent experiments. Individual points represent individual bacterial isolates and presented as coloured symbols categorised by first experiment (red), second experiment (green) and third experiment (yellow). The error bars represent the standard deviation. For each harvest, ninety-six isolates were screened. Group differences were evaluated using a mixed-effects model analysis with Tukey multiple comparisons, groups not sharing a letter differ significantly ($p < 0.05$).

4.3.2.2.5 Phenotypic profile of *Rhizobium* selective agar isolates.

Radar charts were constructed to visualise how the general profile of bacterial traits changes across the close rotation experiment with respective to *Rhizobium* selective agar isolates. Trait values were normalised to a 0–1 scale using min–max normalisation. Radar charts were constructed that compiled CRB, protease activity, motility, and IAA production across the close rotation experiments (Figure 4.15).

In the first experiment (Figure 4.15A), the preharvest and the 3rd harvest showed a similar phenotype profile, which contained a moderate population of CRB and IAA production. The second experiment (Figure 4.15B) showed that these two phenotypes were strongly selected by close rotation. An overall similar profile was observed in the third experiment (Figure 4.15C), but this one the IAA production was strongly selected in the

Rhizobium population in every harvest. PCA was performed using data from three close rotation experiments as well (Figure 4.16). The PCA biplot explains 57.8% of the total phenotypic variance (PC1: 34.9%; PC2: 22.9%). They showed that there was no distinct cluster by harvest which indicated the non-selected phenotype phenomenon. Overall, the results indicated that *Rhizobium* also showed no evidence of a strong selective phenotype, at least regarding the phenotypes that were tested.

Taken together, isolates recovered from CFC agar and *Rhizobium*-selective agar exhibited variable phenotypic selection across independent experiments. Among CFC agar isolates, none of the tested phenotypes showed consistent enrichment at any specific harvest during close rotation. In contrast, *Rhizobium* isolates displayed a clear harvest-dependent pattern, with IAA-producing isolates becoming increasingly enriched across harvests and reaching significantly higher abundance in the 3rd harvest.

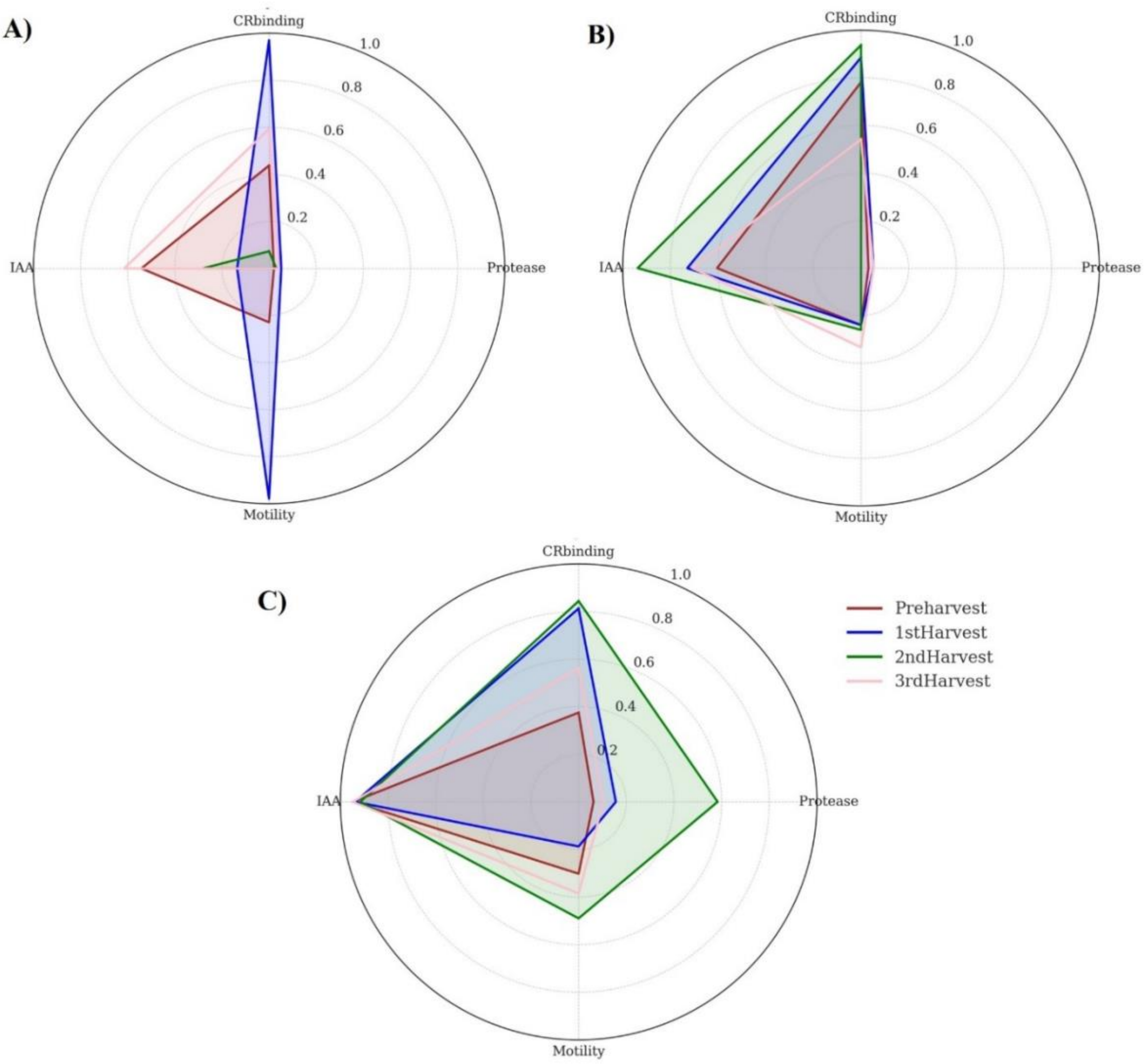


Figure 4.15 Radar chart of *Rhizobium* selective agar isolates phenotype characteristic. A) Isolates from the first experiment. B) Isolates from the second experiment. C) Isolates from the third experiment. Axes are the four assays (CRB, PA, MO, IAA), scaled 0–1. Colour indicates the harvest stage: preharvest (brown), 1st harvest (blue), 2nd harvest (green), and 3rd harvest (pink).

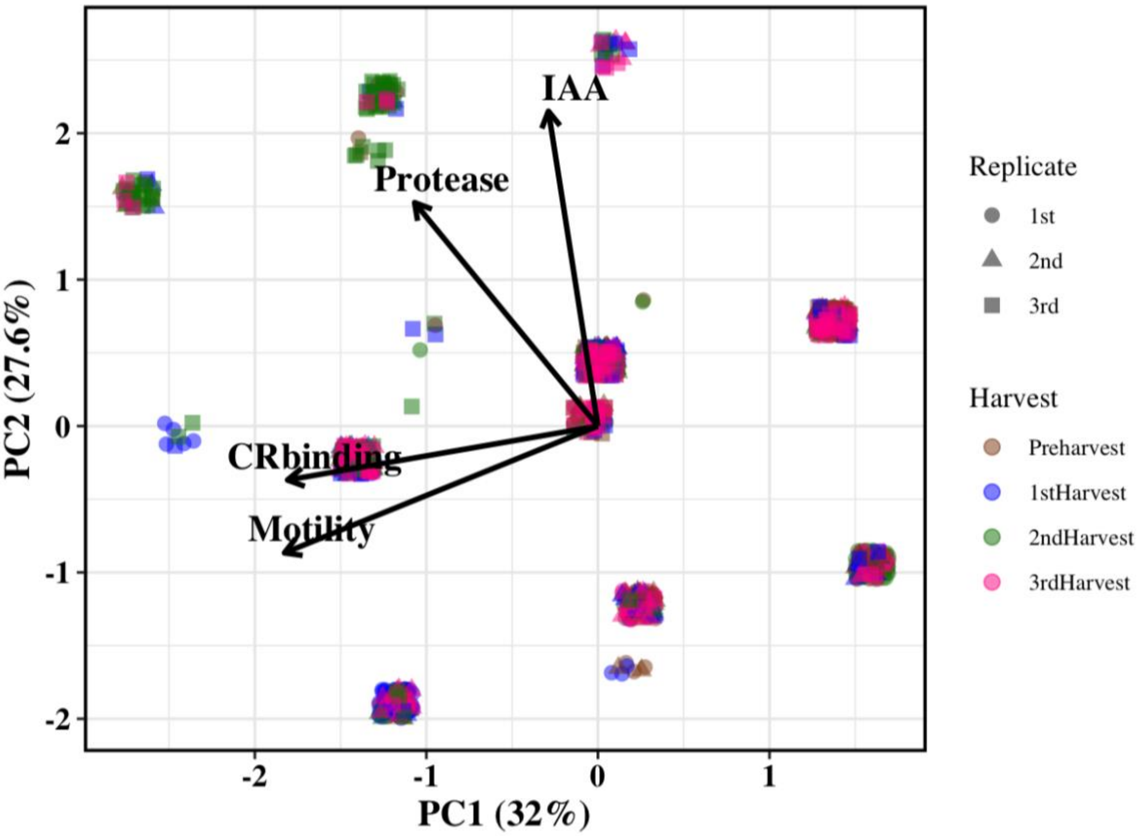


Figure 4.16 Principal component analysis of *Rhizobium* spp. phenotype traits. PCA biplot shows the distribution of strains based on their phenotypic traits for Congo Red (CR) binding, protease, motility, and IAA production. Each dot represents one strain, and the colour indicates the harvest stage: preharvest (brown), 1st harvest (blue), 2nd harvest (green), and 3rd harvest (pink). Shapes representing the isolates from the first, second and third experiment. Arrows point in the direction of increasing values for each trait, and their lengths indicate their influence.

4.3.3 Rhizosphere isolates genotyping.

The taxonomic identity of the soil isolates from selective agar was determined to observe how different species were selected over the course of the close rotation experiment. The phylogenetic tree was constructed by computing on an estimate divergence using the partial *rpoB* housekeeping gene, sequence. The phylogenetic trees were constructed to overview the genus selection across different biological experiments. The analysis focused on two close rotation scenarios: the first experiment and the third experiment. The underlying reason for this selection was to compare one experiment that showed the greatest distinction among groups (the first experiment) with others that demonstrated consistent plant health, in terms of height and weight (the second and third experiment). Thus, the first and third experiment were chosen for investigation.

For each harvest, 24 random isolates were selected for a total of 96 samples. Partial *rpoB* sequences were obtained for each following colony PCR, then used to construct a phylogenetic tree. Additionally, 10 partial *rpoB* sequences from other bacteria were included as a reference in the phylogenetic tree. The reference groups were selected from genera previously reported to be isolated using CFC agar. Additionally, an outgroup sequence was included that is clearly distinct from the other taxa. Phenotypic results for each isolate were included on the phylogenetic tree to visualise the dynamics of the phenotype selection.

From *rpoB* genotyping, I observed that the CFC agar isolated bacteria were in fact a combination of *Pseudomonas*, *Serratia* and *Stenotrophomonas.* Thus, this group of bacteria were then assigned as "CFC agar isolated bacteria" in further experiments (Chapter 5). Nevertheless, most bacteria isolate on the *Rhizobium* selective agar were identified as *Rhizobium*. Thus, isolates were assigned "*Rhizobium* spp.".

The phylogenetic tree in Figure 4.17 illustrated the genus selection in each harvest of close rotation. The reference strains are in white, preharvest isolates in brown, 1st harvest isolates in blue, 2nd harvest in green and 3rd harvest in pink. In the first experiment (Figure 4.17A), preharvest isolates were closely grouped and predominantly classified within the genus *Pseudomonas*. After 1st harvest, pea recruited bacteria from the *Serratia* genus. During the 2nd harvest, the recruited population was again *Pseudomonas*. Eventually, in the 3rd harvest, the pea selection switched back to the *Serratia* group. These selection patterns were mostly recapitulated in the third experiment (Figure 4.17B). Initially, *Pseudomonas* was also dominated in the preharvest population. Then the pea selection was observed in the 1st harvest, where the *Serratia* and *Stenotrophomonas* became dominant in the sample set. After that, 2nd harvest showed a *Pseudomonas* recruitment again and stayed dominant in the 3rd harvest community. Combining the phylogenetic tree with phenotypic data from the 1st harvest reveals a distinct grouping of multiple traits on the *Serratia* group. This suggested that the *Serratia* selected on CFC agar and their closely related species often share similar characteristics.

Overall, this experiment showed that even though *Pseudomonas* was the starting dominance group, close rotation can affect the rhizosphere recruiting for different bacteria group. However, correlation analysis was calculated (Table S.1), and there was no obvious phenotype that showed a consistent correlation in both independent experiments. This suggests that close rotation affects mostly genotype selection but does not show strong selected for the phenotypes I tested.

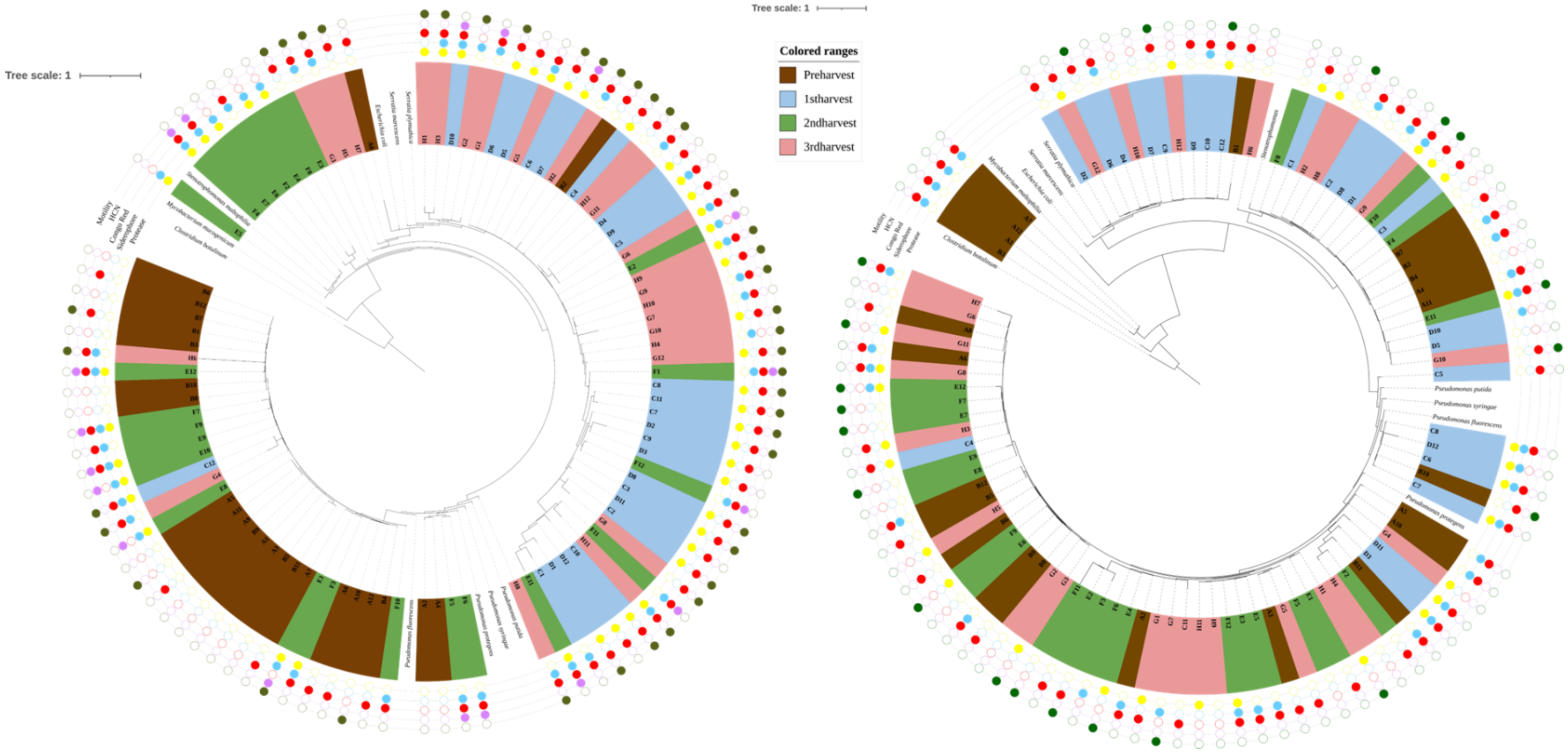


Figure 4.17 *rpoB* phylogenetics tree of soil CFC agar isolates from close rotation experiment. Isolated from the first experiment (Left) and isolated from the third experiment (Right). Colour represents different harvest: preharvest (brown), 1st harvest (blue), 2nd harvest (green), and 3rd harvest (pink). Phylogenetic tree is informatively presented by combining the results from phenotype characteristics including Congo Red binding (red dot), Siderophore (blue), HCN (purple) production, protease (yellow) and motility (green).

Rhizobium isolates from each harvest in close rotation of the third experiment were randomly selected. For each harvest, 10 random isolates were selected for a total of 40 samples *rpoB* sequencing. Phylogenetic trees illustrate that the preharvest population was diverse with both *Pseudomonas* and *Rhizobium* present (Figure 4.18). The population in the 1st harvest showed strong recruitment of a relatively defined *Rhizobium* genotype. Little subsequent refinement was observed between harvests since the isolates of diverse harvests were mixed. In addition, no biofilm formation was observed in any isolates, but IAA production was a common trait within this population. Correlation analysis (Table S.2) showed a positive correlation between protease activity and the 2nd harvest. Overall, this experiment showed that genotype selection in *Rhizobium* occurs only after the first pea planting, with little additional change during close rotation.

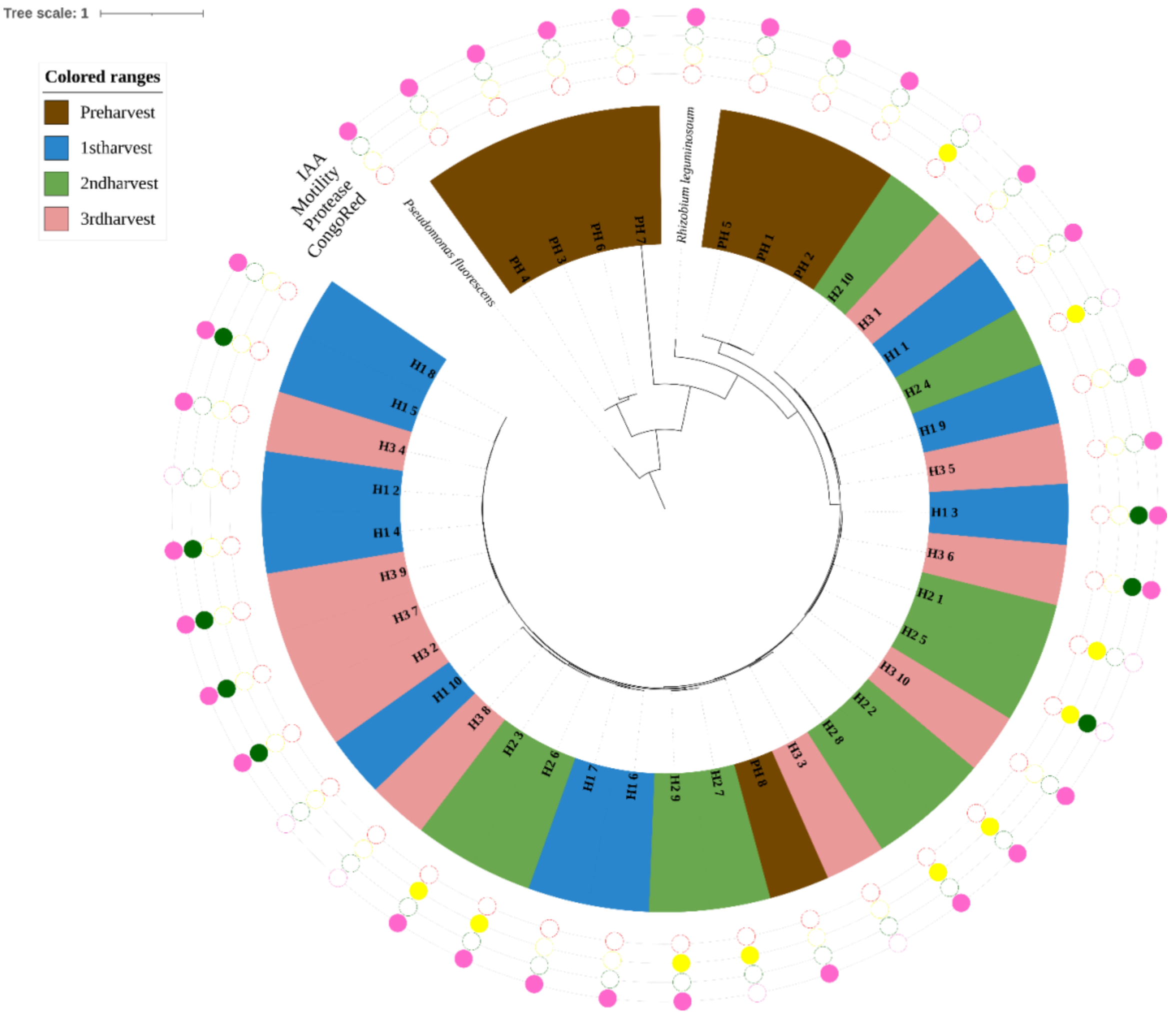


Figure 4.18 *rpoB* phylogenetic of *Rhizobium* spp. from rhizosphere of the 3rd close rotation experiment. Populations of *Rhizobium* spp. were isolated and genotyped. Colours represent preharvest (brown), 1st harvest (blue), 2nd harvest (green), and 3rd harvest (pink) samples. The phylogenetic tree is presented by combining the results from phenotype characteristics including CR binding (red dot), IAA production (pink), Protease (yellow) and motility (green).

4.3 Discussion

This chapter expands upon the previous one, in which I observed that the rhizosphere microbiome shifts suggest microbial changes associated with the close rotation. Nonetheless, they do not demonstrate a definitive or consistent effect specific to pea rotation across all experiments. To understand the selection of microbial populations at the strain level, in this chapter a culture-dependent approach was used for bacterial genotyping and phenotyping.

Firstly, *Pseudomonas* and *Rhizobium* bacteria were selectively isolated from the rhizosphere, assessed with several phenotypic assays and genetically characterised. The genotyping of the bacterial isolates was performed using the *rpoB* gene. *rpoB* evolves faster than 16S rRNA, it discriminates among closely related isolates and resolves species/subclade structure within genera, which 16S rRNA often leaves unresolved (Ogier et al., 2019). I conducted this genotyping in two biological experiments of close rotation, specifically the first and third experiments. Although, CFC agar is designed to recover presumptive *Pseudomonas* (Merck, 2019), as this method simply selects any microbes that are resistant to cetrimide, cephalothin and fucidin, the isolation procedure using selective agar did not eliminate all other soil bacteria. For this reason, half of isolates from the first experiment were identified as *Serratia*. This might also suggest that the underlying bacterial community of soil from first experiment was already enriched in resistant *Serratia*. Alternatively, the *Pseudomonas* population in this experiment may have been relatively less culturable than those observed in previous soil microbial isolation experiments (Pacheco Moreno et al. 2021, 2024).

Phylogenetic analyses showed the dynamic recruitment of different genera during each harvest in close rotation. While some harvest-associated clustering was observed, the lack of consistency across trials suggests that these shifts do not represent a strong initial selection or a clear directional recruitment of specific taxa. With soil samples collected across various times of the year, preharvest of different experiment soil was expected to display a high phenotypic diversity. HCN is a volatile compound that acts as a virulence factor to suppress other bacteria (Sehrawat et al., 2022; Zdor, 2015), its presence was found at a low percentage across the close rotation. These results are consistent with the statistically non-significant inhibition of *F. solani* observed across harvests. Although the two measured pathogen-suppressive traits remained stable during the rotation, no disease symptoms were observed in the plants. This suggests that the observed soil suppressiveness is not limited to these specific traits. Instead, plant health may be maintained by other microbial groups or alternative mechanisms that were not captured in these specific assays.

For *Rhizobium*, phenotypic assay demonstrated that the bacterial communities in the preharvest and the 3rd harvests were predominantly enriched with isolates capable of producing IAA. This plant-beneficial trait is known to enhance root development and suggest a potential growth-promoting role during these stages (Lebrazi et al., 2020). In the 3rd harvest, IAA production was significantly higher than other harvests. This suggests that the population with pea growth promoting traits was enriched after close rotation. It is possible that producing a beneficial trait for their host may secure their habitat and ecological niche. Similarly, a study involving *P. protegens*, which is initially antagonistic toward *A. thaliana*, found that within six plant growth cycles they can develop bacterial phenotypes that promote plant growth (Li et al., 2021).

This chapter suggests that close rotation may influence genotype selection in CFC agar isolates (*Pseudomonas* spp., *Serratia* spp., and *Stenotrophomonas* spp.) and the selection of plant growth-promoting traits, such as IAA production in *Rhizobium* spp. However, these observations remain tentative, as no comparisons were made with other plant species or with a soil-only control, making it difficult to attribute the effects specifically to pea rotation. The phenotypic selection patterns for other traits varied depending on the biological experiment. Thus, this result might suggest that close rotation does not affect phenotypic recruitment, at least for the phenotypes tested here. The next chapter focuses on a plant experiment designed to assess the influence of microbial community from close rotation on pea growth and health, and how this community responds to, and protects plants from fungal infection.

Chapter 5 -

Interactions Between Rhizosphere Bacterial and Fungal Inoculants Affecting Pea Plant Health

Chapter 5 - Interactions Between Rhizosphere Bacterial and Fungal Inoculants Affecting Pea Plant Development

5.1. Introduction

Crop production is influenced by the composition of the rhizosphere community, which is determined by rotation diversity and the identity of the previous crop (Benitez et al., 2021). For example, the selective pressure provided by the wheat variety grown in the first year affects both the overall abundance of *Pseudomonas* spp. in the soil and the structure of the *Pseudomonas* metagenome. This leads to variations in the abundance of individual genotypes within the population (Mauchline et al., 2015). In legumes, the interplay between host genotype and the microbiome is especially related to fungal pathogen resistance. Common bean cultivars that are resistant to the fungal root pathogen *F. oxysporum* were found to harbour a distinct microbiota compared to susceptible cultivars (Mendes et al., 2018). Plant genetics and the root-associated microbiome both affect peas' resistance to root rot. Microbial data can predict the disease outcomes more accurately than plant genotype alone, and some microbes in the pea rhizosphere were linked to either resistance or susceptibility (Gfeller et al., 2025). However, the understanding of bacterial and fungal communities under close rotations and pea productivity remains understudied.

To study these complex microbial interactions, researchers employ both natural microbial communities (NatComs) and synthetic communities (SynComs) (Armanhi et al., 2018; Herrera Paredes et al., 2018). A broad collection of microorganisms, obtained directly from natural settings such as soil or plant roots, constitutes NatComs (Bengtsson-Palme, 2020). NatComs are used as a "top-down" approach to elucidate how plants interact with both beneficial and harmful microbes that they encounter with in nature (Mawarda et al., 2020; Usero et al., 2021). For example, NatComs can be obtained by washing soil samples and propagating the raw microbial assemblage, thereby maintaining ecological complexity in experimental systems (Čaušević et al., 2022). To replicate the structure and function of microbiome, researchers use SynComs. Researchers construct SynComs by co-culturing various taxa under well-defined conditions (Y. Bai et al., 2015; Bodenhausen et al., 2014). While NatComs are more complex and resilient, allowing for studies that more accurately reflect natural dynamics, SynComs offer higher reproducibility because of their limited microbial community complexity (Großkopf & Soyer, 2014; Mehlferber et al., 2024). These complementary approaches are increasingly informing techniques for crop management based on the microbiome. It is crucial to keep in mind that microorganisms find it difficult to colonise new soils and exhibit their functional abilities in different environments, unlike native microorganisms which are typically well suited to establish themselves in their original soils and maintain key functions (Calderón et al., 2016; Harris Jennifer et al., 2025; Wippel et al., 2021). A recent study showed that native (local) microbiomes performed better by thriving in their original soils and promoting positive plant–microbe interactions. Their robust colonisation, neutral assembly and growth-promotion capabilities highlight their value for developing next-generation bioinoculants tailored to local soils (Zhou et al., 2024).

Pseudomonas synthetic communities have been studied in several crops. For example, *Pseudomonas* was identified as a key PGPR candidate when Zhuang *et al.* examined garlic rhizosphere microbiome succession across growth stages and soil types. They validated a six-strain SynCom that enhanced radish seedling growth (Zhuang et al., 2021). Dobrzyński et al. showed that wheat seed yield and rhizosphere nutrient status improved using bacterial consortium, combining *Pseudomonas* sp. G31 and *Azotobacter* sp. PBC2, while creating little disturbance to the local bacterial ecology (Dobrzyński et al., 2025). In addition, in barley, cultivars typically perform better when exposed to *Pseudomonas* SynComs and soil washes taken from the same cultivar (Pacheco-Moreno et al., 2024).

Microbial inoculation has been extensively assessed across many crops (de Andrade et al., 2019; Rani et al., 2021; Snak et al., 2024). In peas, inoculation with single strains or consortia of *Azotobacter chroococcum* and *Bacillus megaterium* improved phenotypic traits including plant height, root length, and yield components (Mahmoud et al., 2023). In addition, *B. simplex* inoculation enhanced protein levels in pea plants exposed to lead (Pb) stress due to their efficient phosphate solubilisation, siderophore production, and lead bio-sorption abilities (Chamekh et al., 2022). Recent research identified *Bacillus* and *Pseudomonas* as highly effective biocontrol agents against pea downy mildew. Foliar application of either bacterium reduced *Peronospora viciae f. sp. pisi* sporulation by 85–98%, while soil drenching with bacterial broths achieved up to ~90% pathogen suppression. Moreover, the dual application of both microbes produced a synergistic effect. They provided an additional 27–47% reduction in disease severity compared with single inoculations (Okechukwu et al., 2025). These findings highlight the promise of microbial inoculation strategies for disease biocontrol. Taken together, SynComs and NatComs represent promising approaches for studying the effects of microbial communities.

I aimed to investigate the close rotation effects on bacterial communities on pea plants health by measuring height, shoot weight, root weight and nodule number, using SynComs and NatComs from different harvests of close rotation.

5.2 Objectives

1. Investigate the effects of close rotation on synthetic CFC agar isolates-*Rhizobium* community inoculations for pea plant health (height, shoot weight, root weight, root-shoot ratio and nodule number).
2. Examine the effects of close rotation on natural microbial community inoculations for pea plant health (height, shoot weight, root weight, root-shoot ratio and nodule number).
3. Explore the effects of close rotation on natural microbial community inoculations in suppressing pathogenic fungi on pea plant (height, shoot weight, root weight, root-shoot ratio and nodule number).

5.3 Results

5.3.1 Effect of close rotation CFC agar isolates and *Rhizobium* SynCom on pea health.

CFC agar isolates and *Rhizobium* isolates were obtained by randomly selecting isolates from soils collected at different harvests under pea close rotation. They were characterised both phenotypically and genotypically through *rpoB* gene analysis, as previously described in Chapter 4. To investigate the impact of close rotation on these bacterial communities in supporting pea health, SynComs were established using isolates from CFC agar. First, each isolate was cultured individually. To form the SynCom, 96 isolates of CFC agar isolates, and *Rhizobium* strains were combined. This inoculum was applied to sterile vermiculite, into which surface-sterilised, germinated pea seeds were sown.

After 28 days of growth, plant performance was evaluated by measuring root and shoot biomass, nodule formation, and plant height (Figure 5.1).

The SynCom experiment was conducted in three biological replicates, and the data is shown in different colours. Root weight varied significantly across treatments. Compared to the control, preharvest, and 1st harvest, roots from the 2nd and 3rd harvest treatments were significantly heavier (Figure 5.1A). In contrast, shoot weight showed no statistically significant differences among treatments (Figure 5.1B). The second biological replicate (green data points on Figure 5.1B) exhibited a lower shoot weight compared to the other two biological replicates. This effect may have occurred due to an additional unknown stress or technical difference during this replicate that lowered the shoot weight. According to the variability in the second biological replicate root weight, a distinct higher root-to-shoot ratio was observed (Figure 5.1C). However, when considering only the first and third replicates, shoot biomass remained stable, while roots responded strongly to treatments from the 2nd and 3rd. This trend may reflect an investment in root biomass relative to shoots during later harvest close rotation. Plant height showed no significant differences across treatments with all groups (Figure 5.1D). However, nodules were only observed in the first replicate, and there was no significant difference between treatment groups (Figure 5.1E). This result demonstrates that CFC agar isolates (including *Pseudomonas*, *Serratia* and *Stenotrophomonas*) and *Rhizobium* close rotation significantly increased root biomass in pea plants, while shoot biomass and plant height remained unchanged. The results suggest that microbial effects, particularly with additional rotation, can enhance below-ground growth rather than above-ground traits.

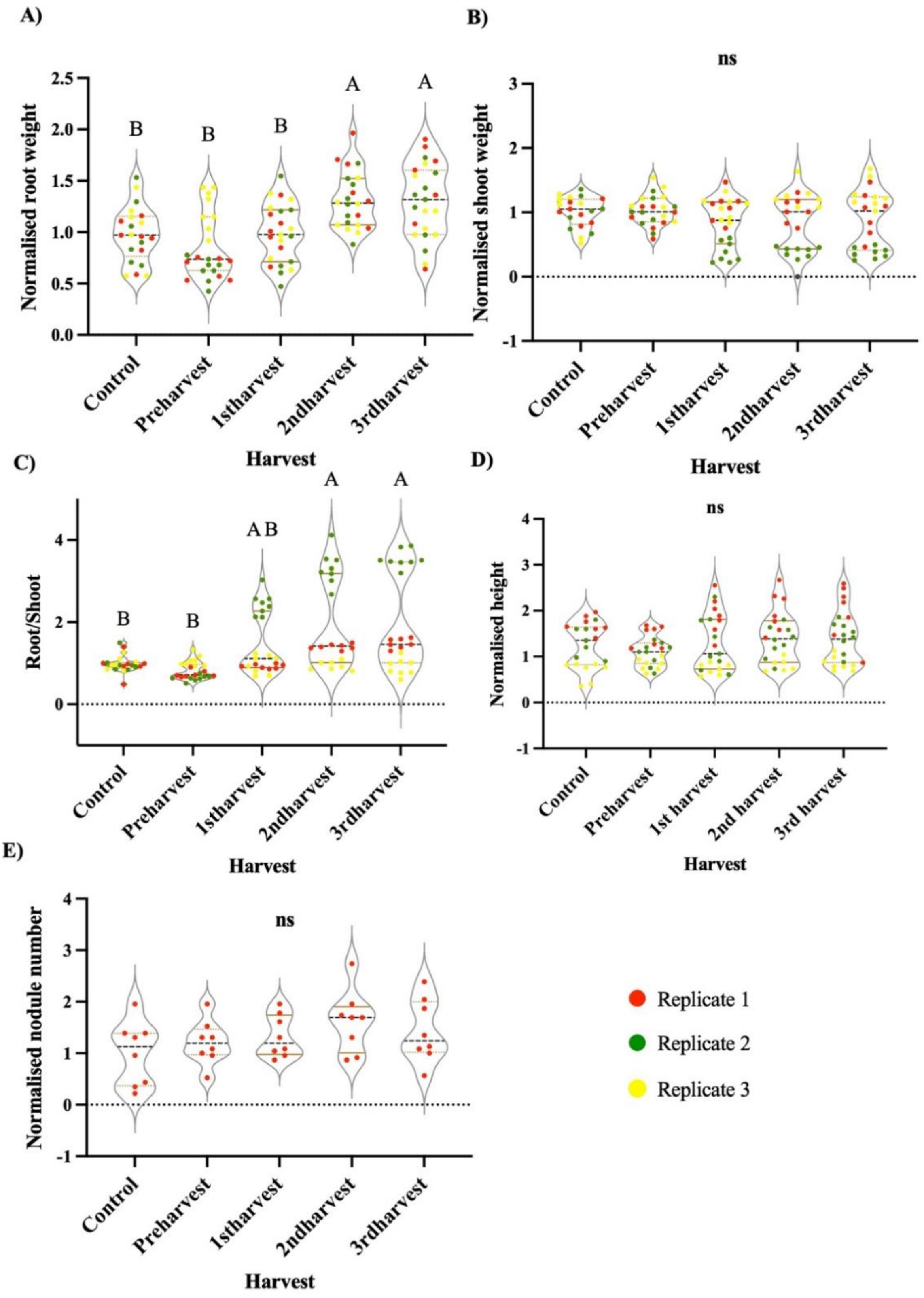


Figure 5.1 Effect of SynCom of CFC agar isolates and *Rhizobium* inoculation on pea plants. Violin plots comparing pea plant growth traits. The data are normalised to control (uninoculated). Specifically, individual values were divided by the mean of the control group, effectively setting the control mean to 1.0.A) normalised root weight. B) Normalised

shoot weight. C) Normalised height. SynComs from close rotation harvests were used for inoculation, control plants received no inoculum. Each dot represents individual replicate values (coloured red, green, or yellow for replicate sets). A mixed-effects model analysis was performed with Tukey multiple comparisons. Letters above plots (A, B, C, D) indicate statistical groupings, with different letters indicating significant differences.

5.3.2 Effect of close rotation NatComs inoculation on pea plant health.

To examine the impact of natural rhizosphere microbial community from close rotation on pea health, I conducted an inoculation experiment using NatComs. This study included two sets of experiments. First is the tray experiment and second is the large pot experiment. NatComs were obtained by extracting whole microbial consortia from soils collected from different harvests under close rotation. The inocula were applied to sterile vermiculite in which surface-sterile, germinated pea seeds were sown, allowing the establishment of native microbial interactions involving bacteria, fungi, and other root-associated microorganisms. In addition, *F. solani* suspension was applied to simulate fungal infection. In the tray experiment, after 28 days, plant growth parameters including root and shoot biomass, nodulation and plant height were assessed. Figure 5.2 presents overall pea health under NatCom inoculation in three replicates. While representative pea plants and pea leaves are shown in Figure 5.3 and 5.4.

Figure 5.3 shows representative pea plants after NatCom inoculation. In the no fungus group, plants displayed healthy growth, characterised by green foliage, compact shoot architecture, and well-developed root systems across all harvest stages. Even when inoculated with microbes from repeated cultivated soil, plants retained substantial biomass with minimal signs of senescence, indicating sustained growth potential. In contrast, plants exposed to fungal treatment exhibited pronounced reductions in overall health relative to untreated controls. Overall, these observations indicate that fungal infection adversely affected plant growth and regeneration capacity, leading to diminished biomass accumulation and accelerated senescence. In addition, representatives of pea leave under NatCom inoculation are shown in Figure 5.4.

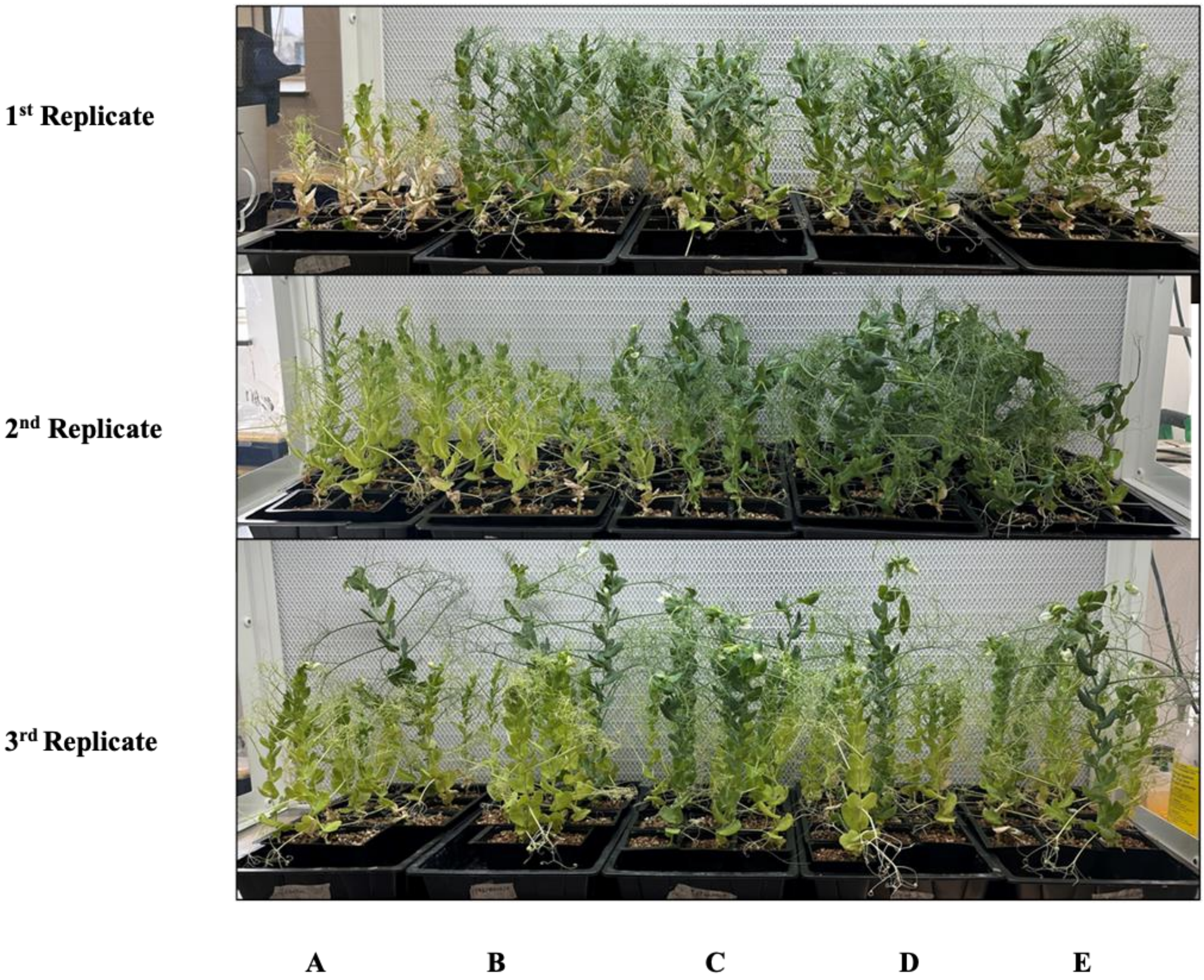


Figure 5.2 Pea plants inoculated with NatComs derived from different soils under close rotation. Pea plants were grown in the same condition with different inoculation A) uninoculated (control). B) preharvest. C) 1st harvest. D) 2nd harvest. E) 3rd harvest. Rows 1 through 3 represent the first, second, and third replications, respectively.

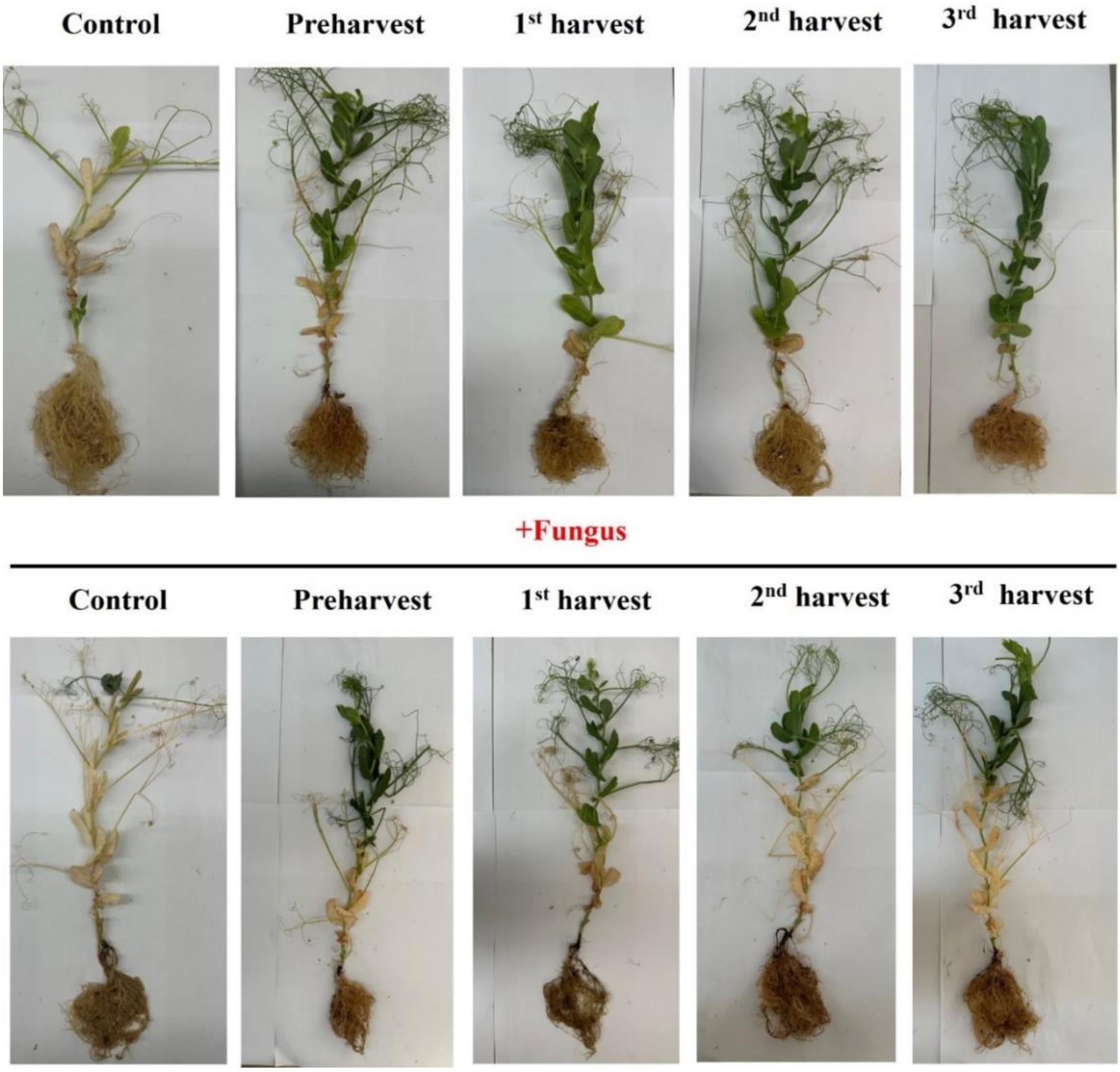


Figure 5.3 Representative pea plants inoculated with NatComs derived from different soils under close rotation. Representative pea plants (from 1st replicate) grown under two conditions, without fungal treatment (top row) and with fungal treatment (bottom row). Plants were cultivated in sterile vermiculite and inoculated with NatComs derived from different close rotation harvests (Control (uninoculated), Preharvest, 1st harvest, 2nd harvest, and 3rd harvest).

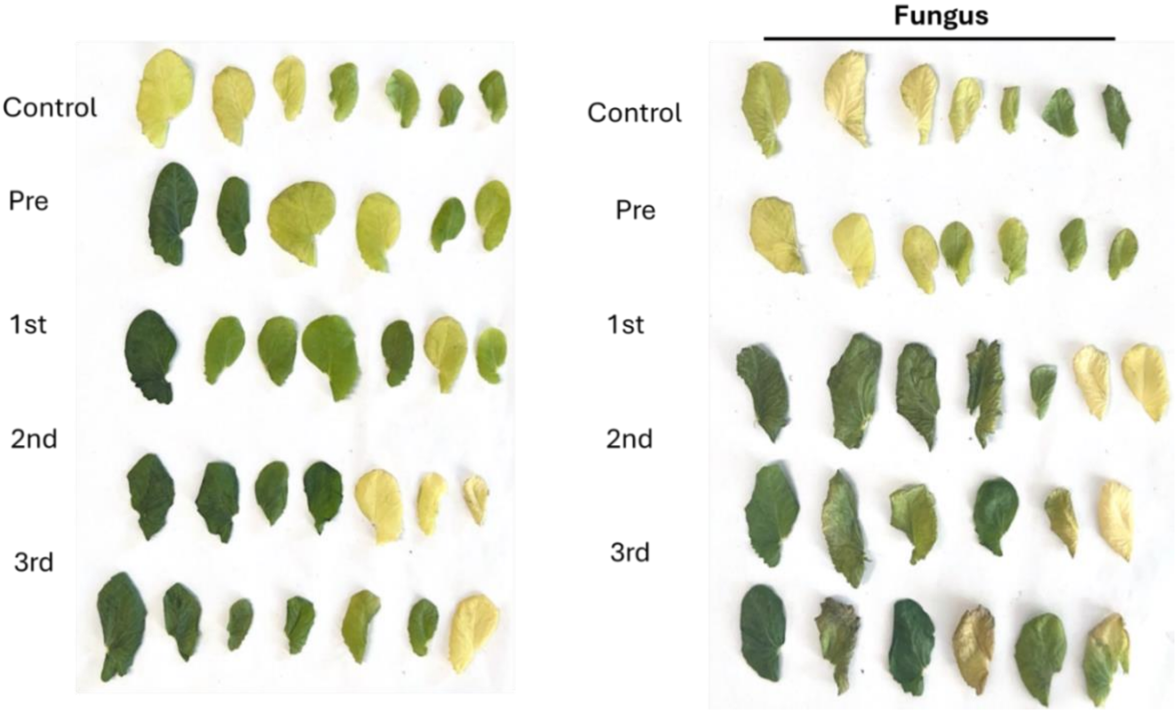


Figure 5.4 Leaf morphology of pea plants inoculated with NatComs obtained from different soils under close rotation. Representative pea plants (from 1st replicate) were grown under two conditions, without fungal treatment (left group) and with fungal treatment (right row).

Inoculation with harvest-derived NatComs had a significant effect on pea growth traits, both in the absence and presence of fungal communities (Figure 5.5). No significant differences were detected in the fungus-free NatCom group; however, root weight was significantly lower in the plants inoculated with the 2nd and 3rd harvests compared with plants without NatCom (Figure 5.5A). Shoot weight was significantly greater when the NatCom from soil harvest was inoculated (Figure 5.5B). Specifically, shoot weight was greater when plants were inoculated with NatCom from the 2nd and 3rd harvests. Consequently, this led to a reduction in the root-to-shoot ratio (Figure 5.5C) where the plants without NatCom had a higher root to shoot ratio compared to plants with NatCom. Plant height was also consistently greater in inoculated groups, with significant increases in the 1st and 3rd harvest treatments. In the fungus-inoculated group, control plants were significantly shorter than 1st, 2nd and 3rd harvest inoculated plants (Figure 5.5D). Nodulation responded most strongly (Figure 5.5E), with inoculated treatments of the 1st, 2nd and 3rd harvests showing significantly higher nodule numbers than the uninoculated control. Similarly, in fungus infection condition, more nodule number were observed when inoculated with NatCom of 1st, 2nd and 3rd harvests.

The results suggested that the addition of fungal communities did not abolish these beneficial effects of NatCom but might increase plant growth variability. Collectively, these results show that NatCom inoculation enhance pea nodulation, height, and biomass accumulation, with positive effects maintained even under fungal pressure. Results from SynComs, which were composed primarily of only four genera, showed increased root biomass over successive rotations (Figure 5.1), but this was not replicated when tested with the whole microbial community, which was consist of more diverse and complex members.

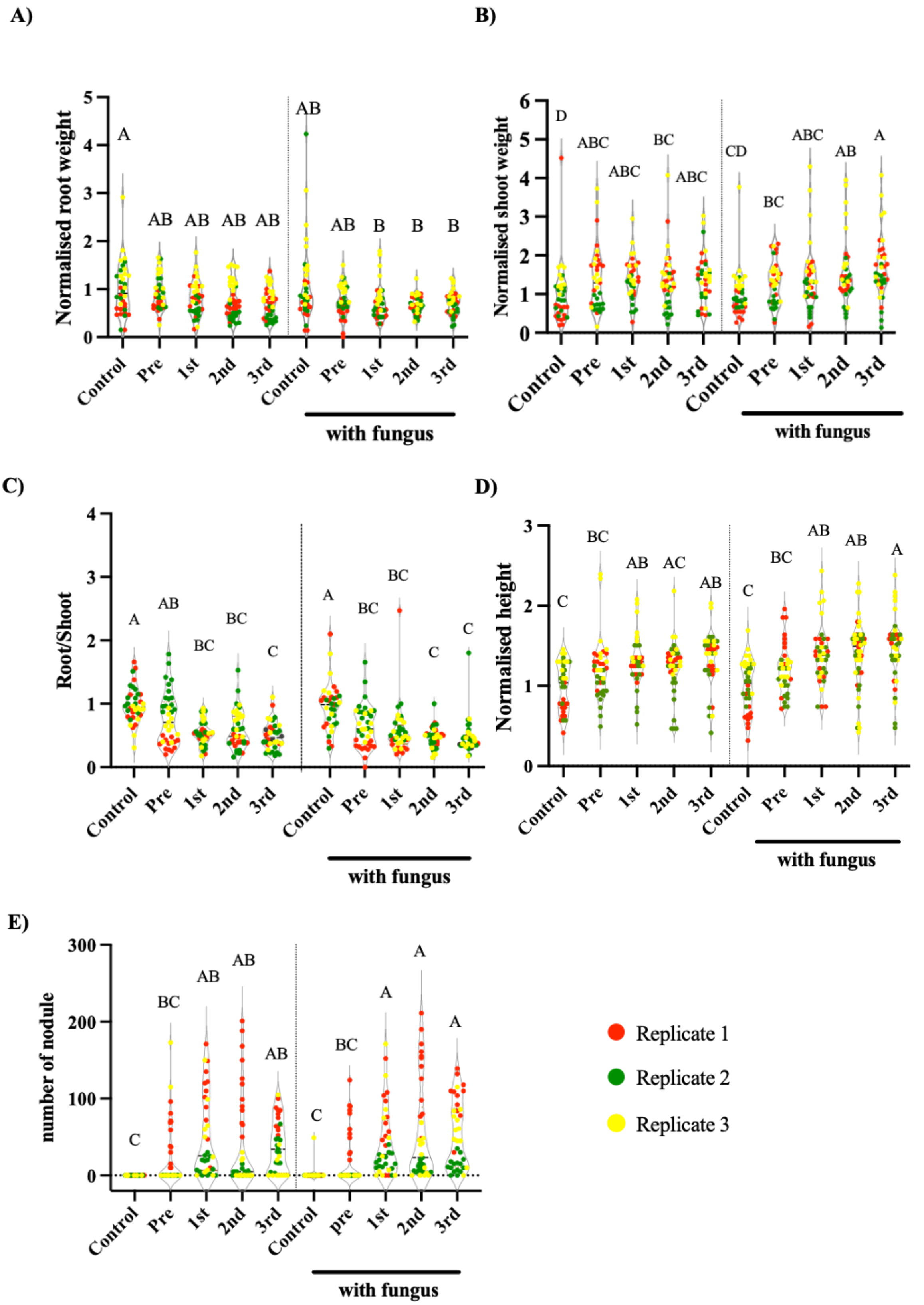


Figure 5.5 Effect of NatComs inoculation on pea plants. Violin plots comparing pea plant growth traits. The data are normalised to control (uninoculated). Specifically, individual values were divided by the mean of the control group, effectively setting the control mean

to 1.0. A) normalised shoot weight number of nodules B) normalised root weight C) ratio of root to shoot D) normalised height E) nodule number. NatComs from close rotation soils were used for inoculation, control plants received no inoculum. Each dot represents individual replicate values (coloured red, green, or yellow for replicate sets). A mixed-effects model analysis was performed with Tukey multiple comparisons and showed a significant difference between pea harvests with multiple comparisons. Letters above plots (A, B, C, D) indicate statistical groupings, treatments sharing the same letter are not significantly different, while different letters mean significant differences.

In addition to plant growth traits, fungal disease severity was assessed. Disease symptoms varied among microbial inoculation treatments across different harvests (Figure 5.6). In the absence of fungal infection, plants in both the control and preharvest groups mostly exhibited low severity scores (0–1), indicating minimal disease development. However, in the 1st and 2nd harvest groups, a marked shift toward higher severity score (scores 3–5) was observed. By the 3rd harvest, severity remained elevated in some replicates but showed greater variability, with both moderate and severe scores represented. Under fungal inoculation, the preharvest treatment was better at suppressing disease compared to 1st harvest. Also, the 2nd and 3rd harvest showed more suppression compared to 1st harvest. These results suggest that the close rotation microbial slightly helped reduce the pathogen symptoms. However, in the 3rd experiment (Figure 5.6C) fungal infection also occurred in the no-fungus treatment group, and therefore the results may be confounded by the presence of some background pathogen activity.

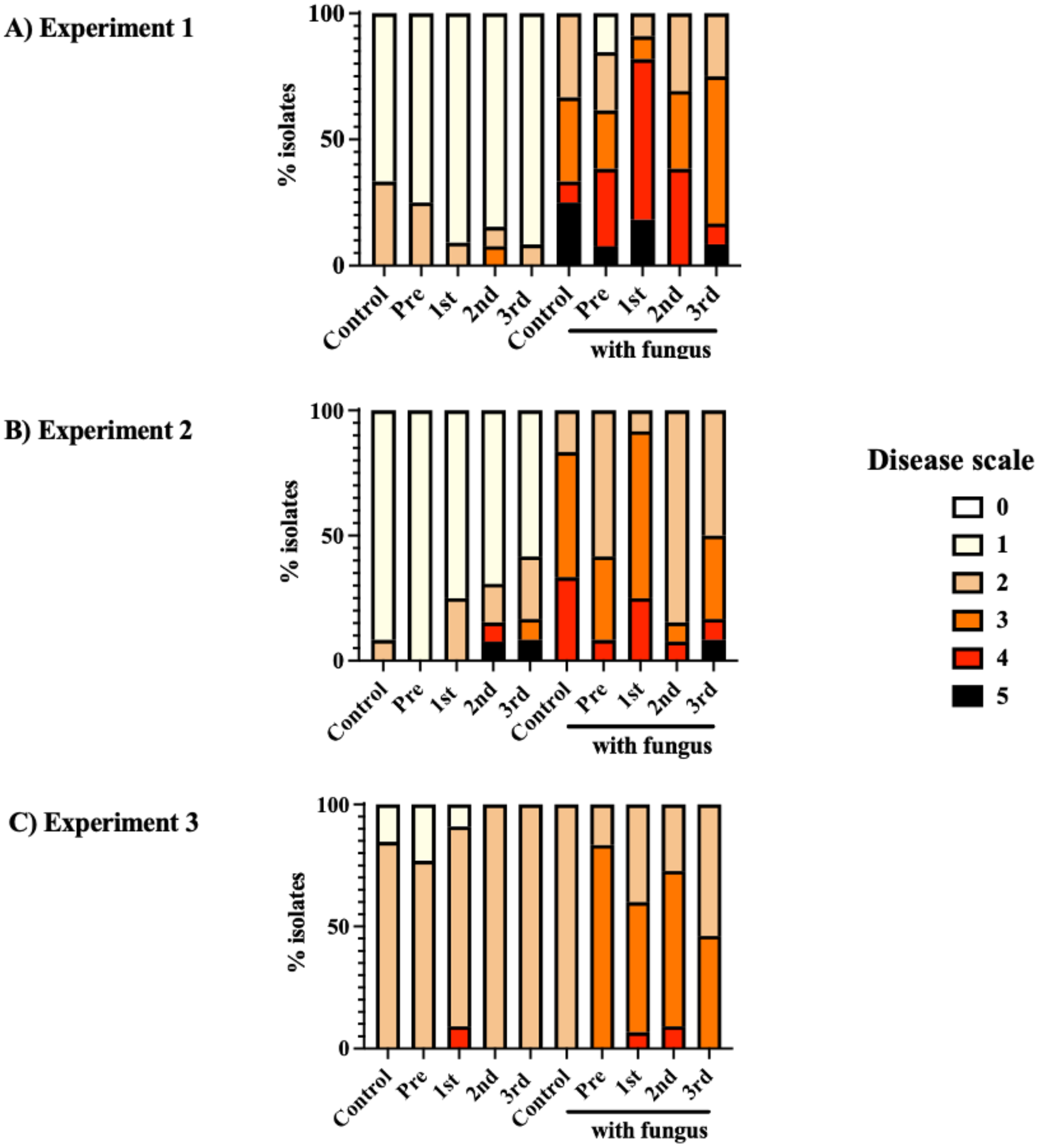


Figure 5.6 Fungal disease severity across different NatCom inoculations. Bar graphs show the relative abundance of different disease severity in A) 1st replicate B) 2nd replicate C) 3rd replicate, with and without fungus treatment. Fungus infections were scored using a disease severity index from 1 to 5, 1 = no visible infection, 2 = restricted lesions, 3 = expanding lesions, 4 = softening roots and 5 = substantial rot.

5.3.3 Effect of close rotation NatCom and fungus inoculation on mature pea.

Previous experiments demonstrated that NatCom inoculation influenced several pea growth traits. Nevertheless, yield remains the principal parameter for evaluating agricultural success, as it most directly reflects both productivity and sustainability (Manish Lad et al., 2022). To assess the effects of microbial inoculation on mature pea health, NatComs were applied to sterile soil in which surface-sterilised, germinated pea seeds were sown, under both fungus-free and fungus-challenged conditions. After three months, plant growth parameters and pea seed weight were measured. Representative plants are shown in Figure 5.7, and overall pea health assessments are presented in Figure 5.8.

In the NatCom group without fungal inoculation, plants exhibited relatively healthy growth across harvests, characterised by green foliage, elongated shoots, and multiple lateral branches. Plant health appeared broadly comparable across the different inoculated conditions. In contrast, plants subjected to fungal treatment showed clear reductions in overall health, with symptoms becoming more pronounced in later harvests. Plants displayed symptoms of disease, including chlorosis, tissue necrosis, and reduced foliage density. Growth was stunted in later harvests, with thinner shoots and limited branching compared with the fungus-free group.

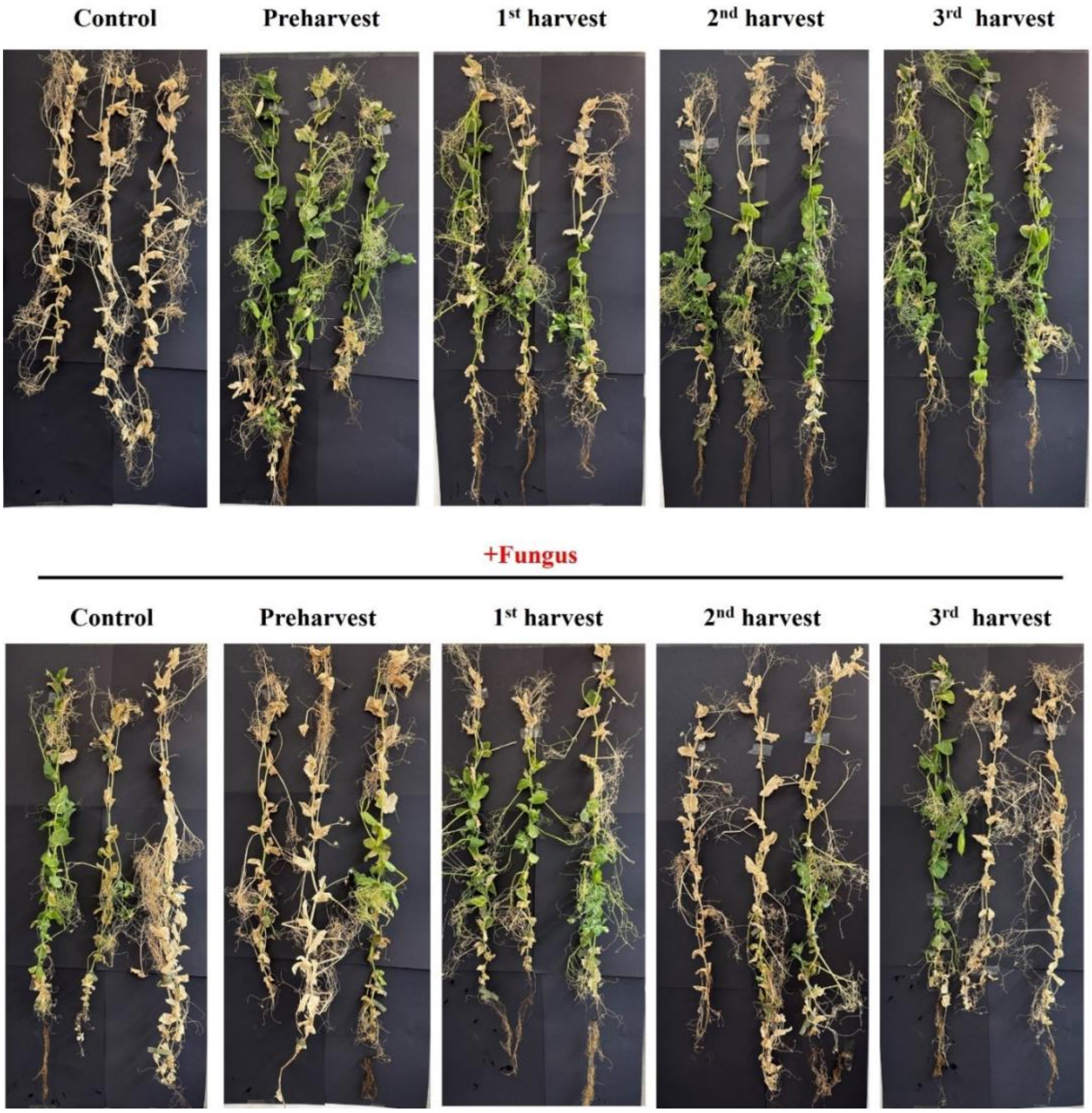


Figure 5.7 Pea plant health assessment after three months inoculated with NatCom from close rotation. Peas were inoculated with harvest derived NatCom from different harvest in close rotation (first row), and peas inoculated with NatCom and fungus (second row).

Root weight was not significantly different among treatments, regardless of fungal inclusion (Figure 5.8A). In contrast, shoot weight responded strongly to inoculation (Figure 5.8 B). In the absence of fungus, inoculation with preharvest, 1st, 2nd and 3rd harvest NatComs significantly increased shoot biomass compared to the control, with the highest values observed for the 1st harvest inoculum. When fungus was included, shoot weight did not differ significantly between inoculation treatments, although a decreasing trend was observed in infected plants. The root-to-shoot ratio remained unaffected by treatment in both fungal and non-fungal groups (Figure 5.8C). Similarly, nodule numbers did not differ significantly between treatments (Fig. 5.8D) No significant differences were observed in plant height across treatments, with or without fungal presence (Figure 5.8E). Likewise, total weight of seed per plant remained unchanged by either harvest-derived inocula (Figure 5.8F). Also, there was no significance different in seed weight. Among measured traits, only shoot biomass responded significantly to NatCom inoculation, with strong increases in the 1st harvest treatments under fungus-free conditions. All other traits including root biomass, nodulation, height, root-to-shoot ratio, and yield, showed no significant differences. This suggests that close rotation NatCom effects were restricted to shoot development and were diminished by fungal co-inoculation.

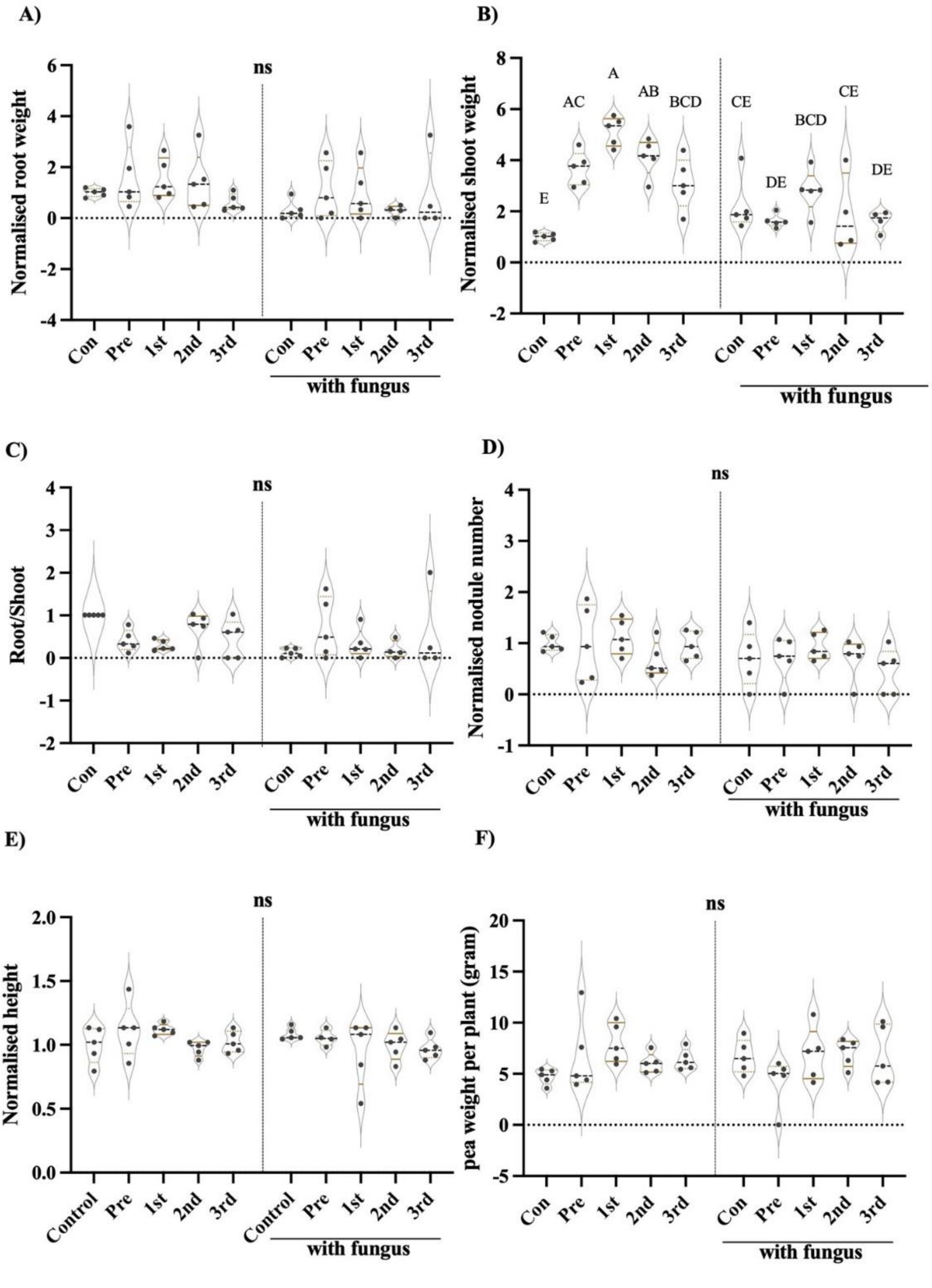


Figure 5.8 Growth and yield parameters of pea plants under NatCom inoculation. Violin plots comparing pea plant growth traits. The data are normalised to control (uninoculated). A) number of nodules B) normalised height C) normalised shoot weight D) normalised root weight E) ratio of root/shoot F) weight of total seeds per plant. NatComs

from close rotation soils were used for inoculation, control plants received no inoculum. Each dot represents an individual plant. A one-way ANOVA was performed with Tukey multiple comparisons and showed a significant difference between pea harvest with multiple comparisons. Letters above plots (A, B, C, D, E) indicate statistical groupings, treatments sharing the same letter are not significantly different, while different letters mean significant differences and not significant (ns).

5.3 Discussion

Re-inoculating microbial communities taken from close rotation into pea plants grown in sterile environments serves as a powerful approach to assess the functional contribution of the rhizosphere microbiome. This experiment enabled us to directly evaluate the biological importance of bacterial communities in facilitating plant growth. The interactions of a SynCom composed of CFC agar isolates and *Rhizobium* with pea plants, as well as those of NatComs, were evaluated under two scenarios including short-term growth and extended growth until pod harvest.

The results exhibited the increased root-to-shoot ratio observed in pea plants inoculated with the CFC agar isolates-*Rhizobium* SynComs from the 2nd and 3rd harvests (Figure 5.1). This data suggested stress-induced biomass reallocation patterns. Environmental stresses such as drought or nitrogen deficiency frequently result in an elevated root-to-shoot ratio, thought to be caused by increased carbon allocation to the roots (Seidel et al., 2024). Crop plants have been shown to maintain or increase their root-to-shoot ratio in response to drought by allocating more resources toward root development to enhance nutrient uptake potential (Kalra et al., 2024; Kou et al., 2022). The SynComs isolated in Chapter 3 likely contained some plant-pathogenic strains, such as members of the *Serratia* and *Stenotrophomonas* genera, alongside commensal and beneficial microbes. This might explain why SynCom inoculations cause some stress to plants.

The NatCom inoculation experiment showed a broader range of positive plant responses compared to those treated with the SynCom that was made solely of *Pseudomonas*, *Serratia*, *Stenotrophomonas* and *Rhizobium*. This increase could be a result of the boost in functional and taxonomic diversity that exists in NatComs. Then, the effects on plant growth and development are more extensive than those produced by the smaller two-four genus SynCom. This is due to the complementary traits, such as stress tolerance, pathogen inhibition, and nutrient solubilisation, contributed by various strains (Hu et al., 2016). Furthermore, NatCom offers versatility that simplified inoculants cannot reproduce. Thus, the diverse core microbiomes are acknowledged to be crucial to plant health and resilience in agroecosystems (Toju et al., 2018). Based on my results, inoculated plants exhibited significantly greater nodulation, height, and biomass (in some treatments) than uninoculated controls. Root-to-shoot ratios were significantly lower in inoculated plants, which reflects a shift in biomass allocation toward shoots. The fungus infection did not eliminate these impacts, but it made the impacts more variable and changed the resource allocation patterns even more. Overall, these results demonstrate that close rotation NatComs consistently enhance pea growth and nodulation, with robust effects maintained in the presence of fungal communities. However, no significant differences in these traits were observed between harvests. The degree of pea disease was documented, revealing varying patterns among tests. In both the first and second replicates, plants inoculated with fungus at the first harvest exhibited greater disease severity compared with the preharvest group. This might suggest that fungal pathogens can gradually assemble a disease-conducive microbial community (Pathobiome) that increases susceptibility to pea root rot (Hossain et al., 2021).

However, the close rotation effect was not observed in mature pea. The result indicated that only shoot biomass responded significantly to NatCom inoculation. In contrast, root biomass, nodulation, plant height, root-to-shoot ratio, and yield did not change significantly between treatments. This pattern showed that the benefits of NatCom were mostly limited to above-ground growth in mature peas. The effect was compromised when fungal co-inoculation was present. This suggested that fungi were more able to compete in long-period cultivation. However, some plants experienced a severe fungal infection which hindered attempts to prolong the experiment. Also, time constraints limited this large pot experiment to a single replicate. Due to a small sample size, these findings must be interpreted cautiously and validated through further experimentation.

Fungal infection slightly increased variability and altered biomass allocation patterns, this phenomenon happened in both NatCom experiments conducted in pea trays and large pots (Figure 5.5 and 5.8). The observed variation of NatCom benefits in the presence of fungal co-inoculation suggests that soil-borne pathogens can compromise the effectiveness of beneficial microbial consortia. This interference is likely attributable to direct competition for root colonisation sites and nutrient resources, together with disruption of induced systemic resistance (ISR) pathways. ISR is a plant defence mechanism triggered by certain non-pathogenic rhizobacteria (van Loon et al., 1998). Competition from pathogens can reduce PGPR rhizosphere colonisation potential and functional efficacy, which is critical for their performance (Santoyo et al., 2021).

Taken together, findings in this chapter highlight that close rotation shapes microbial community to enhance pea health. The simplified CFC agar isolates–*Rhizobium* SynCom demonstrated a shift to root biomass. NatCom inoculations exhibited broader and more consistent enhancements in growth traits, particularly nodulation, height, and shoot biomass. The co-inoculation of fungus and microbes showed that close rotation might select suppressive bacteria that can then reduce pea disease severity.

Chapter 6 -

General Discussion

Chapter 6 - General Discussion

6.1 Introduction

Food insecurity is still an issue around the world today. Millions of people still lack dependable access to adequate healthy food (World Food, 2025). Sustainable agriculture is a positive way to move forward (Hiywotu, 2025) as it focuses on protecting the environment and making food production more resilient. Legumes like peas play a key part in cropping systems to create sustainable agriculture and satisfy the growing demand for plant-based protein and food in general (Yanni et al., 2023). The close rotation of pea has been introduced to enhance crop production (Zhang et al., 2022). However, the intensive cultivation of a vulnerable host such as pea facilitates the proliferation of particular plant diseases. This may disrupt the balance of beneficial and pathogenic taxa in the rhizosphere soil and increased disease incidence and severity in the long term (Bainard et al., 2017). Thus, understanding the composition and biological activity of the rhizosphere microbiome is an important task that might lead to better crop productivity in the future (Compant et al., 2005; French et al., 2021).

In this thesis, I aimed to investigate the effects of pea close rotation on the microbiome, and whether this process leads to microbiome dysbiosis. To do this, I set up a series of lab-based experiments to mimic close rotation under a controlled growth environment. I used culture dependent and independent methods including amplicon sequencing and high-throughput phenotypic characterisation, to elucidate the impact caused to the microbial community. In addition, I conducted plant assay inoculations with synthetic community and natural community to examine the impacts of different microbial communities on plant health and disease control.

6.2 Close rotation affects the pea rhizosphere microbial community.

Plant microbiome has been studied widely in recent years due to its crucial role in plant health, nutrient uptake, and stress resilience (Tiwari et al., 2025). Plants primarily gather their microbes from surrounding soil (Bulgarelli et al., 2015). Their signalling molecules and immune system selectively recruit these soil bacteria, fungi and other microorganisms (Xiong et al., 2021). Previous studies on field soil samples suggested that close rotation affects these microbial communities, and leads to an increase in the harmful fungal population (Ding et al., 2024; Gao et al., 2019).

To mimic the close rotation, I performed three independent experiments of three harvests with soil with no pea cropping history. I observed no significant difference in pea health or soil nutrients caused linked to the close rotation experiments. However, I demonstrated that the close rotation of peas can affect the rhizosphere community. Using amplicon sequencing, a change in the bacterial community was indicated, shifting to Proteobacteria dominance. In the independent experiments, the genus *Pseudomonas* was the most recruited genus after the repeat planting. In addition, the shift of fungal communities toward *Fusarium* was verified with both amplicon sequencing and population quantification. A limitation of this experimental design is the absence of a non-planted soil control, which would have allowed for a direct comparison between baseline shifts in the soil microbial community and the cumulative effects of successive rotation. Therefore, future experiments incorporating fallow soil controls are necessary to validate the specific impact of the rhizosphere effect and to determine if the observed population fluctuations are truly driven by plant-microbe interactions.

Microbial diversity remained relatively stable after the first pea cultivation; then the community diversity significantly changed in either the 2nd or the 3rd harvest. I also observed that microbial community reversion (phylum level) to a composition similar to the 1st harvest soil occurred in the 3rd harvest. Bacterial mechanisms of rhizosphere colonisation are complex, as I demonstrated here, there are back-and-forth selection dynamics of close rotation microbial communities. These findings implied that the taxonomic composition also varies at the species or strains levels due to selective pressures imposed by close rotation. For a better understanding, the examination of the species level using the *rpoB*, housekeeping gene was performed. I observed the similar back-and-forth pattern in the genus level population. This emphasized that amplicon sequencing alone might not be able to capture a complex dynamic. To better understand rhizosphere microbiomes adaptation, the parallel genome sequencing can be used to reveal bacterial genetic dynamics. The use of this technique has been shown in previous work in our lab (Pacheco-Moreno et al., 2024; Pacheco-Moreno et al., 2021). In addition, the molecular response to close rotation could, in future be quantified using metatranscriptomics. This technique has been used to investigate active transcripts within the microbial communities (Shakya et al., 2019; Urich et al., 2008). Furthermore, incorporating a fourth rotation would clarify whether the community in the third experiment follows the same recovery pattern observed in the second experiment.

In chapter 4, the phylogenetic analysis indicates that genotype selection at the genus level was most pronounced following the first pea cultivation. Thereafter, a back-and-forth pattern of selection persisted among bacteria isolated on CFC agar throughout the close rotation. While amplicon sequencing showed a high population of *Pseudomonas*, the phylogenetic analysis showed a marked rhizosphere recruitment of *Serratia* instead. This disparity between the results might be explain by several assumptions. It is possible that a subpopulation of *Serratia* exhibited resistance to the antibiotics employed for *Pseudomonas* selection. Alternatively, although amplicon sequencing indicated a high relative abundance of *Pseudomonas* within the rhizosphere, these populations may be less amenable to cultivation compared with *Serratia*. A further consideration is that amplicon sequencing itself may have limitations in efficiently amplifying or accurately resolving taxa assigned to the genus *Serratia*.

Taken together, I demonstrated the impact of close rotation to rhizosphere microbial community using lab-based experiments. It would be compelling to establish pea cultivation in a field with no prior history of pea planting and manage the crop under two contrasting approaches: (i) a diverse crop rotation that includes peas alongside other crops, and (ii) a close rotation where peas are grown consecutively. Microbiome data collected from these systems could then be compared with the results presented in this thesis to determine whether similar dynamics can be observed. Additional experiments to increase the frequency of harvests in close rotation may yield significant data to elucidate the longer-term trends of this process on the rhizosphere microbiota. It is important to note that the controlled close rotation experiment presented here was conducted under conditions where other sources of variation were excluded. However, in the field, diverse factors such as weather conditions, soil disparity, and pest outbreaks (Lundberg et al., 2025). These may influence the experimental outcome.

6.3 Analysis of bacterial population dynamics during close rotation

To successfully colonise the plant rhizosphere, bacteria must adapt their lifestyles. Motility is important for moving toward the food source (Santoyo et al., 2021). Biofilm formation is vital for the effective colonisation of surfaces such as plant roots, and survival in competitive and hostile environments (Danhorn & Fuqua, 2007). In addition, microbes can synthesise numerous different antimicrobial compounds and proteins, including HCN and proteases (Neeraja et al., 2010). To facilitate plant growth, siderophores can be produced in response to iron deficiency. Also, auxin may be produced to enhance plant growth and root elongation (Crowley, 2006; Gallavotti, 2013; Mercado-Blanco & Bakker, 2007). Moreover, plants also benefit from interactions with *Rhizobium*, which are symbiotic nitrogen fixers (Pankievicz et al., 2019). These phenotypes can establish the beneficial relation between PGPR and the plant host (Mmotla et al., 2025).

This study focusses on phenotyping members of gamma- and alpha- Proteobacteria, *Pseudomonas* and *Rhizobium,* which are identified as well- known and important PGPR. I saw little evidence of phenotypic selection after close rotation in my experiments. The production of biofilm, HCN, protease and fungal suppression activity did not appear to be important for colonisation of the pea rhizosphere. Also, the motility of bacterial population. For *Rhizobium,* the population with IAA production enriched in every harvest and the number of IAA+ isolates peaked after three rotations. It remains to be determined whether further addition of the rotation would result in an even higher IAA+ population or if the community has already reached its peak abundance. Producing more phytohormone leads to root development, this can benefit bacteria to establish and expand their habitat and resources (Duca et al., 2014). Moreover, enhancing IAA under salinity stress reduces the uptake of toxic ions, resulting in enhanced plant development (Chakraborty et al., 2011). While the phenotype preference observed here, on the other hand, the *rpoB* gene showed that genotype selection for *Rhizobium* only occurred once after first pea planting. This observation is consistent with the population quantification by qPCR, where the first pea planting strongly influenced the increasing of *Rhizobium* abundance. After that the presence of *Rhizobium* stayed relatively steady throughout the close rotations. This initial selection did not appear to undergo additional refinement over successive harvests, unlike the *Pseudomonas* and *Serratia* populations which kept adapting as the environment changed over time.

Further research with additional phenotypic assays should be included in future experimental work, such as suppression activity against other soil pathogens. Also, when the results showed no phenotypic selection, it is possibly because some of the phenotypic characteristics may not be suitable for expression under laboratory conditions. Although I did not observe any *Rhizobium* genotype-level selection, the enrichment of the IAA trait suggests that some form of selection occurred within the population. Because only core genes were sequenced, this analysis likely missed finer-scale selection acting on accessory genomic elements, including those responsible for IAA biosynthesis. To elucidate this process, again, the parallel genome sequencing can be used for comparing the presence or absence of accessory genes across strains. This technique will enable the identification of genomic loci associated with adaptation under close rotation. Moreover, metatranscriptomics could be employed to complement and enhance our understanding of the gene expression associated with rhizosphere colonisation.

6.4 Influences of close rotation-derived microbial community on pea health.

The microbial community protects plants against pathogen invasion by direct activation of plant immunity or by enhancing disease resistance, as well as by indirectly suppressing pathogenic bacteria (Lee et al., 2021). They can also produce phytohormones and solubilise phosphates to help improve nutrient uptake and plant health (Feng et al., 2021; Maldonado et al., 2020; Timofeeva et al., 2023). SynComs and NatComs were used in this study to investigate close-rotation bacterial communities, how they affect plant health, and how they suppress pathogens.

I demonstrated that SynComs inoculation composed CFC isolates and *Rhizobium* was not sufficient to maintain healthy pea growth. Peas showed increased root biomass, which may represent a stress adaptation response. The most striking result was that close rotation shapes the overall microbial community to enhance pea health. When the whole microbial community was tested, they exhibited a wider range of traits in growth enhancement, including nodulation number, height, and shoot biomass. This suggests that the NatCom may consist of diverse strains conferring beneficial traits, such as pathogen inhibition and nutrient solubilisation. When *Fusarium* was added to this system, the close rotation-derived microbial community showed a trend of reduced pea disease severity. These findings suggest that close rotation in pea cultivation may contribute to the selection of bacterial populations with fungal-suppressing potential. I hypothesised that close rotation shaped the soil microbial community to become suppressive to fungal infection. This finding relates to the result presented in chapter 3, where no disease symptoms were observed on pea plants under close rotation, possibly because the underlying microbial community had been trained to respond to pathogen infection. In addition, these trained bacteria isolates might explain why the microbial population underwent back-and-forth dynamics. Could this be all because they were trying to respond to disease infection?

However, experimental systems involving soil, plants, and microbes are complex. It is laborious to translate from the lab to the field in a replicable and reproducible manner (Lin et al., 2024; Parnell et al., 2016). Thus, further study needs to be conducted. The 3rd harvest NatCom can be tested for their suppressive activity by soil inoculation, seed coating or spraying in a disease field. Also, this inoculant should be thoroughly tested to determine what the underlying mechanism is. The root exudates can be investigated to look for antifungal metabolites.

6.5 Concluding remark and future directions.

In this thesis, the findings indicate that repeated pea cultivation may contribute to shifts in rhizosphere microbial communities. However, the extent and persistence of these changes may be influenced by rotation length and environmental conditions. In the absence of extended monitoring or mechanistic evidence, it is possible that the observed changes reflect natural variation rather than a consistent effect of cropping. Genotype selection dynamics was also evident during close rotation, which might indicate the stress adaptation from the increased of pathogens caused by close rotation. However, the selection of *Rhizobium* populations at the genotypic level only occurred during the first pea planting. This is because of their conserved beneficial nitrogen fixation trait. Moreover, plant growth–

promoting traits, such as phytohormone production, were selected in the *Rhizobium* population. After three rotations, the shaped microbial population can enhance plant growth and exhibited a trend of reduced fungal disease symptoms. Taken together, the close rotation might activate pathogen-suppressive microbes, resulting in a better adapted microbial community against root rot infection.

The findings from this thesis provide encouragement to utilise trained microbial communities as biocontrol agents to enhance agricultural sustainability. To achieve this, the results must be translated from pot experiments to field conditions. Field-scale trials with larger plant populations are therefore required, along with optimisation of the application method. Analysis of the trained bacterial isolates genome can suggest the novel biosynthetic gene clusters encoding antibiotics. Furthermore, pea breeding efforts could be directed towards genotypes that favour the establishment of suppressive microbial consortia in the rhizosphere. Those genotypes may selectively recruit or support beneficial microbial populations capable of inhibiting pathogens and promoting overall plant health. Altogether, this work highlights the potential of sustainable agricultural practices to support resilient and productive cropping systems for the future.

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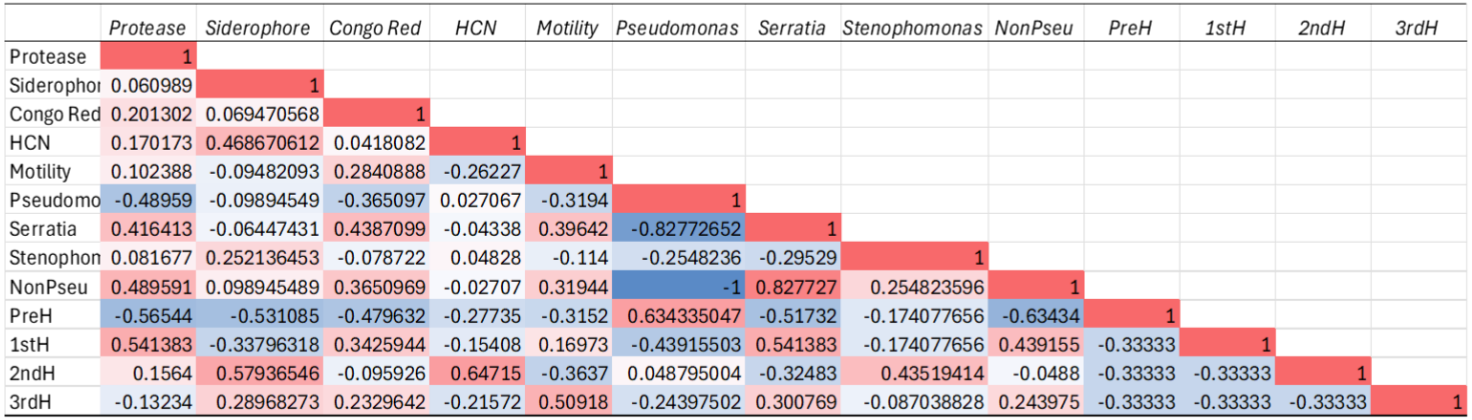
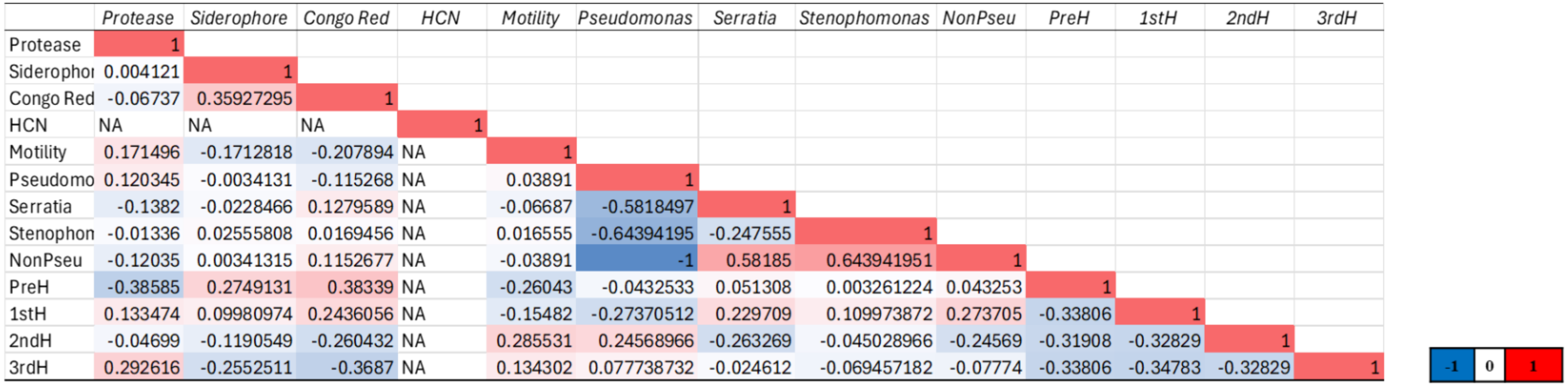
Appendix

Experiment1



	Protease	*Siderophore*	*Congo Red*	*HCN*	*Motility*	*Pseudomonas*	*Serratia*	*Stenophomonas*	*NonPseu*	*PreH*	*1stH*	*2ndH*	*3rdH*
Protease	1												
Siderophor	0.060989	1											
Congo Red	0.201302	0.069470568	1										
HCN	0.170173	0.468670612	0.0418082	1									
Motility	0.102388	-0.09482093	0.2840888	-0.26227	1								
Pseudomo	-0.48959	-0.09894549	-0.365097	0.027067	-0.3194	1							
Serratia	0.416413	-0.06447431	0.4387099	-0.04338	0.39642	-0.82772652	1						
Stenophon	0.081677	0.252136453	-0.078722	0.04828	-0.114	-0.2548236	-0.29529	1					
NonPseu	0.489591	0.098945489	0.3650969	-0.02707	0.31944	-1	0.827727	0.254823596	1				
PreH	-0.56544	-0.531085	-0.479632	-0.27735	-0.3152	0.634335047	-0.51732	-0.174077656	-0.63434	1			
1stH	0.541383	-0.33796318	0.3425944	-0.15408	0.16973	-0.43915503	0.541383	-0.174077656	0.439155	-0.33333	1		
2ndH	0.1564	0.57936546	-0.095926	0.64715	-0.3637	0.048795004	-0.32483	0.43519414	-0.0488	-0.33333	-0.33333	1	
3rdH	-0.13234	0.28968273	0.2329642	-0.21572	0.50918	-0.24397502	0.300769	-0.087038828	0.243975	-0.33333	-0.33333	-0.33333	1

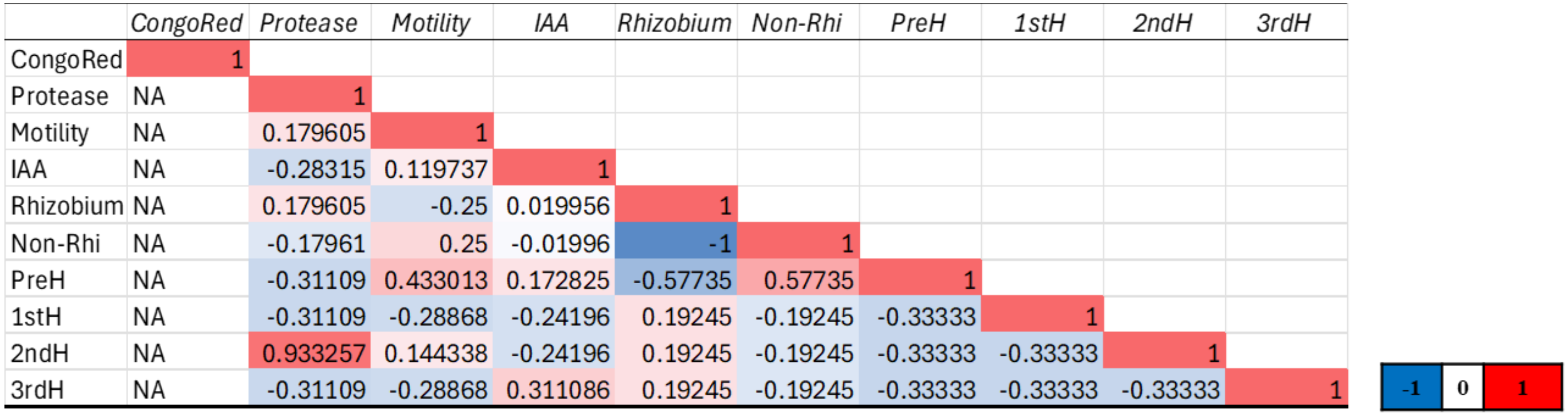
Experiment 3



	Protease	*Siderophore*	*Congo Red*	*HCN*	*Motility*	*Pseudomonas*	*Serratia*	*Stenophomonas*	*NonPseu*	*PreH*	*1stH*	*2ndH*	*3rdH*
Protease	1												
Siderophor	0.004121	1											
Congo Red	-0.06737	0.35927295	1										
HCN	NA	NA	NA	1									
Motility	0.171496	-0.1712818	-0.207894	NA	1								
Pseudomo	0.120345	-0.0034131	-0.115268	NA	0.03891	1							
Serratia	-0.1382	-0.0228466	0.1279589	NA	-0.06687	-0.5818497	1						
Stenophon	-0.01336	0.02555808	0.0169456	NA	0.016555	-0.64394195	-0.247555	1					
NonPseu	-0.12035	0.00341315	0.1152677	NA	-0.03891	-1	0.58185	0.643941951	1				
PreH	-0.38585	0.2749131	0.38339	NA	-0.26043	-0.0432533	0.051308	0.003261224	0.043253	1			
1stH	0.133474	0.09980974	0.2436056	NA	-0.15482	-0.27370512	0.229709	0.109973872	0.273705	-0.33806	1		
2ndH	-0.04699	-0.1190549	-0.260432	NA	0.285531	0.24568966	-0.263269	-0.045028966	-0.24569	-0.31908	-0.32829	1	
3rdH	0.292616	-0.2552511	-0.3687	NA	0.134302	0.077738732	-0.024612	-0.069457182	-0.07774	-0.33806	-0.34783	-0.32829	1

-1	0	1

Table S.1 Correlation analysis of *CFC* agar isolates (1st and 3rd experiment) across phenotypic assays, experiments, and harvests. 96 isolates from each experiment were used to construct phylogenetic trees (Figure 4.17) and Pearson's correlation was calculated. -1 indicates negative correlation and 1 indicates positive correlation. For some phenotypes, there was no within-harvest variance (e.g., all observations were positive); consequently, correlations involving that phenotype are reported as NA.



	CongoRed	*Protease*	*Motility*	*IAA*	*Rhizobium*	*Non-Rhi*	*PreH*	*1stH*	*2ndH*	*3rdH*
CongoRed	1									
Protease	NA	1								
Motility	NA	0.179605	1							
IAA	NA	-0.28315	0.119737	1						
Rhizobium	NA	0.179605	-0.25	0.019956	1					
Non-Rhi	NA	-0.17961	0.25	-0.01996	-1	1				
PreH	NA	-0.31109	0.433013	0.172825	-0.57735	0.57735	1			
1stH	NA	-0.31109	-0.28868	-0.24196	0.19245	-0.19245	-0.33333	1		
2ndH	NA	0.933257	0.144338	-0.24196	0.19245	-0.19245	-0.33333	-0.33333	1	
3rdH	NA	-0.31109	-0.28868	0.311086	0.19245	-0.19245	-0.33333	-0.33333	-0.33333	1

Table S.2 Correlation analysis of *Rhizobium* spp. (3rd experiment) across phenotypic assays, experiments, and harvests. 20 isolates were used to construct phylogenetic trees (Figure 4.18) and Pearson's correlation was calculated. -1 indicates negative correlation and 1 indicates positive correlation. For some phenotypes, there was no within-harvest variance (e.g., all observations were positive); consequently, correlations involving that phenotype are reported as NA.