Host mediated microbiome selection to study the changes in the nutrient cycling, root exudation and bacterial population in the rhizosphere of soybean genotypes.

Deepa Manikkath Haridas

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Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

Deepa Manikkath Haridas

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Abstract

The soil microbiome is the principal reservoir of rhizosphere bacteria, and agricultural management has a significant impact on it. The aim of the study was to understand how changes in cultural practises, such as tillage levels and field cropping history, have influenced soil characteristics and extracellular enzyme activity and how the soil bacterial communities may be altered using the technique of host mediated microbiome selection. In host-mediated microbiome selection, the microbiome is selected based on specific plant traits (for example plant growth, disease resistance, flowering) and the microbiome associated with this trait is passed onto next generation of plants through multigeneration selection progressively enriching plants or their growth environment with microbiota associated with a specific plant trait. This facilitates the use of more complex communities instead of a single microbial strain and provides a potential platform for exploring plant–microbiome interactions. In this study, soybean (*Glycine max*) plants were grown in an autoclaved coir: sand mix that was inoculated with soil suspensions from the field soils, classified as untilled, tilled and legume soils based on management practices and cropping history. Plant height was used as a trait to select for a microbiome that can produce better plant growth under nutrient limiting conditions. The rhizosphere soil of plants from the first plant generation selected based on plant height (high growth) was used as inoculum for the growth of second generation of plants.

The initial field soils (untilled, tilled and legume) varied significantly in physical, chemical and soil enzyme activities. Absence of tillage for extended periods of time (more than 5 years) resulted in high organic matter content in untilled soils and the activity of both the soil enzymes, N-acetyl beta glucosaminidase (NAG) and phosphatase (PHOS), measured in this study were high in untilled soils. Next generation sequencing (NGS) analysis indicated significant differences in bacterial composition between the three field soils, and distance-based redundancy analysis revealed that organic matter content in soil had a major impact on bacterial composition. The results also showed that legumes nourish the soil by boosting the diversity of bacteria. In host mediated selection study, soil enzyme activity was found to be a strong indicator of biological activity in the rhizosphere and the activity of enzyme NAG showed significant positive correlation with plant height and above ground dry mass in this study. The results on root exudation

confirms that the soil type is a major factor influencing the bacterial community composition of rhizosphere soils and that plants can select for specific bacterial community to establish a mutual relationship with the help of their root exudates. The three field soils when mixed in equal proportions generated novel combinations of bacterial community with beneficial effects. The results from linear discriminant analysis (LDA) effect sizes (LEfSe) showed that the plants in each soil displayed significantly varied relative abundances of distinct bacterial species in their rhizosphere and many of these species were reported to play roles in soil nutrient cycling. This suggests that when plants are under nutrient stress, they recruit specific taxa in their rhizosphere that help the plants grow under nutrient deficient conditions, and their selection is significantly influenced by the source of inoculum. The findings of this study showed that host-mediated microbiome selection is a viable platform for studying plant-soil-microbe interactions, and that a microbiome rich in plant beneficial bacteria capable of stimulating plant growth in nutrient-limited conditions can be obtained even after two generations of selection.

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List of Abbreviations

4- MUB	4-Methylumbelliferone
ANOVA	Analysis of variance
C	Control
dbRDA	Distance based redundancy analysis
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid disodium salt dihydrate
GCMS	Gas chromatography- mass spectrometry
LB	Luria Bertani
LDA	Linear discriminant analysis
LEfSe	Linear discriminant analysis effect size
NAG	N- acetyl beta glucosaminidase
NGS	Next generation sequencing
NMDS	Nonmetric multidimensional scaling
OTU	Operational taxonomic unit
PCA	Principal component analysis
PCR	Polymerase chain reaction
PERMANOVA	Permutational multivariate analysis of variance
PHOS	Phosphatase
rpm	Revolutions per minute
S1	Untilled soil
S2	Tilled soil
S3	Legume soil
S4	Mixed soil
SPE	Solid phase extraction
TC	Total carbon
TOC	Total organic carbon

Chapter 1. Introduction

1.1. Present Agricultural Scenario

One of the most pressing issues of our time is ensuring enough food for the world's rising population. The population growth and increase in consumption will mean that global demand for food is expected to increase for another 40 years (Godfray *et al*., 2010). It is expected that today's population of about 7.7 billion is likely to rise to around 8.5 billion by 2030 and to over 9.7 billion by 2050 (United Nations, 2019). As a result, one of the most important challenges that will arise is the need to produce more food and bioenergy, preserving the environmental resources at the same time. Intensive agricultural production leads to overconsumption of non-renewable resources like fossil fuel and water. Excessive usage of fertilizers and pesticides have resulted in runoff of these chemicals contaminating the soil and water resources (Browne *et al*., 2013). The resulting contamination of soil results in various stress conditions like increased salinity, soil erosion and drought. Pests and illnesses will become more prevalent, reducing the productivity of agricultural eco systems. (Matson *et al*., 1997). Another important consequence of intensive agricultural practices is the production of greenhouse gases resulting in increases in global temperature (Tilman *et al*., 2002).

There is need for environmentally sustainable agricultural intensification. Soil fertility is an important factor that plays a major role in crop production. The dependence on inorganic fertilizers to improve fertility has caused detrimental effects on the environment. Soil erosion, increased salinity, and nutrient depletion are the three factors that have dramatically decreased soil productivity. To achieve environmental and food security, therefore, it becomes essential to restore natural biological processes in soil. For sustainable food production, it is important to have energy efficient methods for nutrient recycling and pest control. Many of these services are rendered by microbes (Zolla *et al*., 2013) and their appropriate management can help us solve many sustainability issues (Barea, 2015). The use of metagenome sequencing to monitor a soil microbial community indicated a drop in a specific microbial population (decline in Verrucomicrobia in Prairie soils) due to excessive usage of fertiliser supplements (Fierer *et al*., 2013). This shows the

importance of below ground microbial communities in the ecosystem functions. The genetic variability of a healthy soil out surpasses the total genetic variability of plants and animals it supports and it is essential to make use of this variability for sustainable food production.

1.2. The soil microbiome and its role in host growth and development

Microbial communities play a vital role in every biogeochemical process taking place on Earth. They play an important role in the carbon and nutrient cycles and thus contribute to ecosystem functioning and productivity. Animals and plants are constantly inhabited by microbes and these host associated microbial communities, referred to as the microbiome, influence the development, physiology, and stress response of their hosts. Zilber-Rosenberg and Rosenberg (2008) proposed a hologenome theory of evolution based on four generalizations:

1. All plants and animals establish a symbiotic relationship with microorganisms.
2. These symbionts are transmitted between generations.
3. The association between host and symbionts affect their fitness in the environment.
4. The changes in the microbiota with changes in environment is rapid as compared to the changes in the host genome. So, the symbiotic association helps the holobiont to adapt and survive under changing environmental conditions.

Thus, we can say that the "holobiont" is an assemblage of the individual host and its symbionts functioning as a single unit (Theis *et al*., 2016). The hologenome is the complete genetic content of the host genome including the mitochondrial and cytoplasmic genome (Bordenstein and Theis, 2015).

Microbiomes can encompass a hundred-fold more genes than host genomes (Morowitz *et al*., 2011], and this 'hologenome' of a host–microbiome association can vary over space and time (Zilber-Rosenberg and Rosenberg, 2008, Vandenkoornhuyse *et al*., 2015). This means that the microbiome can adjust to changes in the environment more easily than the host genome, and the signal from the microbiome affects the host's physiology and hence phenotype. Thus, it can be said that phenotypic expression of host traits is determined by the presence or absence of certain host associated microbes.

Conventional intensive agricultural practices that depend on inorganic fertilizers, pesticides and other chemical inputs have increased yield but also contributed to soil degradation, loss of biodiversity, increased susceptibility of crops to pests/pathogens and negative environmental impacts which, together, have significant consequences for human health and food security (Tilman *et al*., 2002). In this context, it has become imperative to consider a more sustainable approach. One way to develop a sustainable crop production method is to harness the beneficial effects of the plant associated microbiome. For a sustainable ecosystem, it is important to find out efficient methods for recycling nutrients, controlling pests and for alleviating the impact of abiotic stress factors. This can be achieved by the proper management of beneficial microorganisms and their functions (Zolla *et al*., 2013).

1.2.1 Microbiome in health and disease

In humans, microbes have a tremendous influence on physiology, health, and disease. They protect against pathogens, educate the immune system, and, through these basic functions, affect directly or indirectly most of our physiological functions (Shreiner *et al*., 2015). Gut microbiota is integral to host digestion and nutrition, and they can generate nutrients from substrates that are otherwise indigestible by the host. For instance, xyloglucans are commonly found in vegetables such as lettuce and onions, and the capacity for microbial digestion of xyloglucans was recently mapped to a single locus in a certain species of *Bacteroides* (Larsbrink *et al*., 2014). This study demonstrated that a common dietary fibre is metabolized by a small number of bacterial species that are commonly present in the human gut microbiota, illustrating the concept of human-microbe symbiosis. The research into the link between microbiome and cardiovascular disease (CVD) is progressing. Artherosclerosis, including acute coronary syndrome (ACS) and stroke, heart failure, and hypertension, may be influenced by changes in the diversity and composition of the gut microbiome and its associated metabolites, according to growing data in CVD (Ahmad *et al*., 2019). The role of microbiota in irritable bowel syndrome is a topic of great interest. Results from 16S rRNA based microbiota profiling showed both qualitative and quantitative changes of mucosal and faecal gut microbiota in patients with irritable bowel syndrome (Simren *et al*., 2013). Therapies that alter the

microbiota, including dietary changes, probiotics, and antibiotics, have shown encouraging results in this regard (Simren *et al*., 2013).

While human microbiome research has progressed, the microbiome of plants has received less attention. (van der Heijden and Hartmann, 2016). It is now a well-established fact that plants form close associations with microbes and many studies have reported the beneficial interactions among plants and microorganisms in the environment and linked those to ecosystem functions such as nutrient recycling and stress resistance (Panke-Buisse *et al*., 2015, Timm *et al*., 2016).

The plant microbiome is compartmentalized into its rhizosphere, endosphere, phyllosphere and endophytic microbiota, with soil largely being the original source of the microbial diversity as observed in many plants. Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria are thought to make up the dominant bacterial communities in these compartments in a variety of plant species. (Bulgarelli, 2018). Working on 600 *Arabidopsis thaliana* inbred plants, Lundberg *et al*., (2012) described different bacterial communities in geochemically different soils. The difference in soil type was also reflected in the communities colonizing the rhizosphere and other endophytic compartments in the root. Glassner *et al*., (2015) identified endophytic bacteria from cucurbit fruits. In grapes, root colonizing plant growth promoting bacteria were significantly promoted by the plant (Zarraonaindia *et al*., 2015).

Bulgarelli *et al*., (2012), showed that the roots of *Arabidopsis thaliana*, grown in different natural soils under controlled conditions, are preferentially colonized by Proteobacteria, Bacteroidetes and Actinobacteria. Each of these is represented by a dominating class. Using high-throughput sequencing methods Agler *et al*., (2016) studied the phyllosphere microbiome of *Arabidopsis thaliana* where they focused on bacteria, fungi, and oomycetes. Field experiments were conducted to study the effect of abiotic factors and plant genotype on microbiome composition followed by experiments under controlled conditions to reveal microbe-microbe interactions. They identified a small number of taxa which they referred as microbial 'hubs' which exert a strong influence on community network. These studies suggest that plant host genotype acts on certain microbial species, which then transmit these effects on whole microbial community by modulating microbe- microbe interactions and in turn modulate host fitness (van der Heijden and Hartmann, 2016). These so called 'microbial hubs' might be responsible for sustaining disease

suppressive soils and mediating defence signals among plants. In their review on rhizosphere microbiome, Berendsen *et al*., (2012) suggested that plants that manage their microbiome in a way that is beneficial to their reproductive success will be favoured during evolutionary selection.

1.2.1.1 The role of microbiome in plant stress management

Biotic and abiotic stresses affecting the plant can modulate their metabolic processes. For example, drought stress induced production of specific metabolites (proline, valine, threonine, homoserine, myoinositol, gamma amino butyrate (GABA) and trigonelline (nicotinic acid betaine) in *Pisum sativum* leaves (Charlton *et al*., 2008). In maize, metabolic profile of xylem sap was altered due to drought stress (Alvarez *et al*., 2008) and increase in amino acid production due to heat or cold shock was observed in *Arabidopsis* (Kaplan *et al*., 2004). *Capsicum annuum* L., grown under water deficit conditions, exhibited enriched populations of plant growth promoting (PGP) bacteria in their rhizosphere capable of enhancing plant photosynthetic activity and biomass synthesis under drought stress (Marasco *et al*., 2012).

Plants in nature are continuously challenged by different phytophagous insects and diseases. Most of the plants are resistant to individual pest species through production of different defence related proteins and metabolites (Zhou *et al*., 2015). The induced defence system is a form of resource conservation that allows more energy to be directed toward growth and reproduction in the absence of insect herbivory. Some of the examples of induced defence are production of nicotine in tobacco (Baldwin *et al*., 1998), benzoxazinoids in maize (Oikawa *et al*., 2004) and glucosinolates in *Arabidopsis* (Mewis *et al*., 2005). In response to insect attack, certain plants resort to reduced photosynthetic activity, thus reducing the carbon source available for insect herbivores. On the other hand, Halitschke *et al*., 2011 observed that mirid bug (*Tupiocoris notatus*) attack on *Nicotiana attenuata* increased carbon dioxide fixation because of response to a component in mirid bug saliva. Root inoculation of tomato (*Solanum lycopersicum*) with *Bacillus subtilis* BEB- DN, a plant growth promoting bacteria, promoted plant growth and induced resistance against whitefly, *Bemicia tabaci* (Valenzuela- soto *et al*., 2010). Induced defences against microbial diseases and herbivorous insects are primarily

controlled by salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) and these substances affect defense-signaling pathways that are intricately linked, which can result in beneficial or harmful interactions (Koornneef *et al*., 2008). Sometimes the cross talk between these pathways result in systemic induced susceptibility (Lazebnik *et al*., 2014). For example, there are reports where an attack by bacterial pathogen increases the incidence of insect herbivory in plants (Groen *et al*., 2013). Conversely, certain bacteria create protective compounds that shield the plant from disease attack. For example, *Trichoderma harzianum* Rifai produce non-anionic acid in cacao plants that inhibits the growth of two cacao pathogens (Aneja *et al*., 2005). Phillips *et al*., (2003) suggests that these antimicrobials may be acting as plant hormones that helps the plants to respond to the microbes. These effects that benefit the plant is conserved through natural selection.

Soil gets its 'disease suppressive' property by the presence of antagonistic bacterial communities (Mendes *et al*., 2011). *Pseudomonas fluorescens* produces an antifungal compound diacetylphloroglucinol (DAPG) that decreases the incidence of take-all disease in wheat caused by the fungus *Gaeumannomyces graminis* (Raaijmakers and Weller, 1998). Extended monoculture of wheat in the soil decreases the disease incidence and this natural phenomenon is referred as Take-all decline (Hornby, 1983). Mendes *et al*., (2011), conducted soil transfer experiments in which they found that small amounts of *Rhizoctonia solani* suppressive soil mixed with conducive soil in the ratio 1:9 (w/w) could transfer disease suppressiveness in sugar beet. This disease suppressiveness was attributed to the presence of bacterial communities belonging to Oxalobacteraceae and Burkholderiaceae (Mendes *et al*., 2011 and Chapelle *et al*., 2016). The fungal pathogen (*Rhizoctonia solani)*, as any other microbe, must survive the competition in the rhizosphere for the available nutrients. Chapelle and his colleagues (2016) sequenced the metagenomic DNA and RNA of rhizosphere microbiome of sugarbeet grown in soil suppressive to *Rhizoctonia solani*. They postulate that the disease suppressiveness may be due to the stress response induced by pathogen in the rhizosphere that activates stress related genes in bacterial families and helps in their survival over the pathogen.

In the world where the demand for food is continually increasing, it is important that crop plants grow unimpeded in the absence of pests and diseases to maximise yield. Chemical pesticides are commonly used to protect plants against pests and diseases. However, the use of chemical pesticides is under dispute as these agents can be harmful to humans and the environment. A possible organic alternative for the use of chemical pesticides is to exploit the plant microbiome. Microbiomes are very active players and interact with plants, influence their metabolism and hormonal pathways, providing them with new biosynthetic pathways. They can alter host development, physiology, and systemic defences (Goh *et al*., 2013; Pieterse *et al*., 2014), enable toxin production and disease resistance (Gerardo and Parker, 2014) and increase host tolerance to stress and drought (Bresson *et al*., 2013). The microbiome of plants play an important role in their interactions with competitors, predators, and pathogens.

1.2.1.2 The role of microbiome in plant nutrition

In natural ecosystems, nutrients such as nitrogen (N), phosphorus (P) and sulphur (S) are bound in organic molecules which limit their availability to plants. Plants rely on soil microbes like bacteria and fungi to depolymerize and mineralize organic forms of N, P, and S. Nutrient uptake by plants occurs in the rhizosphere and the activity of rhizosphere microbial community is of great importance for plant growth. Rhizosphere microorganisms release extracellular enzymes for the degradation of polymers like cellulose and chitin and mineralize them to mineral N, P and S (Nannipieri *et al*., 1996). Among bacteria, *Pseudomonas, Bacillus, Azotobacter*, and *Bradyrhizobium* are all capable of solubilizing phosphorus metal complex to release bioavailable P (Bargaz *et al*., 2018). Zak *et al*., (2003) found that changes in microbial community composition and function influence rates of soil carbon and nitrogen cycling. In a study using different genotypes of *Pinus pinaster* (pine trees), Pérez-Izquierdo *et al*., (2019) found that the shifts in the phylogenetic structure of ectomycorrhizal and bacterial communities affected the potential enzyme activities associated with nutrient cycling.

Soil enzymes are the biological catalysts of innumerable chemical reactions necessary for life processes of microorganisms in soils, decomposition of organic residues, cycling of nutrients, and formation of organic matter and soil structure (Dick, 1994). Thus, information on enzyme activity provides insight into biochemical processes in soils (Frankenberger and Dick, 1983), and enzymes are the potential indicators of soil quality because of their relationship to soil biology, ease of measurement, and their rapid response to changes in soil management (Dick, 1994; Dick *et al*., 1997). They provide a useful tool to long-term monitoring of the changes in soil health and quality. Any changes in management practices are reflected in the microbial biomass and soil enzymes in a short period of time; long before measurable changes in soil chemical properties occur (Powlson *et al*., 1987, Dick, 1994). Therefore, enzyme activities have been suggested as early indicators of changes in soil properties (Powlson *et al*., 1987).

The overall enzyme activity of the rhizosphere depends on enzymes localized in root cells, root remains, microbial cells, microbial cell debris, microfaunal cells and related cell debris, free extracellular enzymes or enzymes adsorbed onto or occluded into the soil colloids (Nannipieri *et al*., 2003). These enzymes include amylase, arylsulphatases, beta-glucosidase, cellulase, chitinase, dehydrogenase, phosphatase, protease, urease, and others, derived from plant, animal, or microbial origins (Dick and Tabatabai 1984). Plant roots are also considered as a source of extracellular enzymes. Juma and Tabatabai (1988) reported the presence of acid phosphatase in the roots of maize (*Zea mays*) and soybean (*Glycine max*). Enzymes are involved in transfer of energy and mineralisation of organic carbon (C), nitrogen (N), sulphur (S), and phosphorus (P) within terrestrial ecosystems. For example, phosphatase controls the mineralisation of soil esters of P (Nannipieri *et al*., 2011), and arylsulphatase mineralises organic esters of S (Castellano and Dick 1991), to produce inorganic phosphate and sulphate, respectively. Enzymes, glutaminase and urease are regulators of the N cycle in microbial cells (Dick and Tabatabai 1984); and produce mineral N during the decomposition of aliphatic and aromatic N compounds in soil organic matter.

There have been studies which have investigated the changes in soil enzymatic activities following N fertilization. Sinsabaugh *et al*., (2008) found that the activity of the N acquiring enzyme N-acetyl beta glucosaminidase (NAG) increased significantly with N fertilization. Legumes can regulate soil enzymatic activities more than mineral fertilizers (Solangi *et al*., 2019). Legumes like cowpeas (*Vigna unguiculata*)

were found to increase phosphatase enzyme activities in the rhizosphere (Makoi *et al*., 2010). Hydrolytic enzymes like beta glucosidase, N-acetyl beta glucosaminidase and phosphatase, which are related to C, N, and P cycles in soil were found to be responsible for soil properties like pH and organic matter content (Solangi *et al*., 2019) and these are valuable indicators of biological soil modification.

N- acetyl beta-glucosaminidase (NAG) is the enzyme that catalyzes the hydrolysis of N-acetyl-beta-D-glucosamine residue from the terminal non-reducing ends of chitooligosaccharides (Webb, 1989). Chitin, which is made up of N-acetyl-beta-D-glucosamine residues, is a primary structural component in insects and is found in the cell wall, structural membranes, and skeletal component of fungal mycelia, where it serves a similar structural role to cellulose in higher plants (Stevenson, 1982). This is considered as an important pool of organic C and N in soils. Thus, understanding the environmental controls on the activity of this enzyme in soils is important for better understanding the N cycling process.

Phosphatases are large group of enzymes that catalyse the hydrolysis of esters and anhydrides of phosphoric acid (Tabatabai, 1994). This enzyme plays a major role in the mineralization of soil organic P. Phosphatases are classified as acid and alkaline phosphatases, because they show optimum activities in acid and alkaline ranges, respectively. Several studies have shown that alkaline phosphatase activity in soils is totally derived from microorganisms (Dick *et al*., 1983). A study by Eivazi and Tabatabai (1977) suggested that acid phosphatase was predominant in acid soils, and they found that the distribution of acid and alkaline phosphatase is correlated to soil pH.

The collective genome of the rhizosphere microbiome is much larger than that of the plant and therefore referred to as the plant's second genome or pan-genome (Berendsen *et al*., 2012; Turner *et al*., 2013). Moreover, the genomic potential of the plant associated microbiome could conceivably dwarf the genomic abilities of plants and thus represents a vast largely untapped reservoir for improved host function. For these reasons, integrating beneficial microbiomes into agricultural systems offers the potential to greatly improve the efficiency of crop production (Mueller and Sachs, 2015, Nogales *et al*., 2016).

1.2.2 Factors affecting the soil microbiome

1.2.2.1 Soil type and land management

Soils of different types vary in their taxonomic composition of microbial communities. Soils vary in their physico-chemical properties like pH, structure, texture, organic matter content, microaggregate stability and the availability of nutrients. These properties influence and recruit specific microbes that can make use of the most efficient use of plant root exudates available. A study using different soil types in Eastern European region revealed that most of the microbial taxa were soil-type specific (Pershina *et al*., 2018). The authors attributed the difference in community composition to the variation in pH values and exchangeable potassium content. In another study conducted to understand the effect of soil type on rhizosphere bacterial communities in lettuce (*Lactuca sativa*), a significant effect of soil type on rhizosphere community was observed which again depended on plant growth stage (Schreiter *et al*., 2014). In the same investigation, the scientists discovered that soil type influenced the responsiveness of different taxonomic groups of bacteria following inoculation with the biocontrol agent *Pseudomonas jessenii* RU47. Toljander *et al*., (2008) highlighted that the change in community composition of arbuscular mycorrhizal fungi and bacteria were significantly correlated with changes in pH due to change in fertilizer regime. Rousk *et al*., (2010) collected soil samples across a long-term liming experiment in which the soils differed only in pH (varied from 4.0-8.3) and found a strong correlation between soil pH and the diversity and composition of bacterial communities across biomes. Both crop pathogenic bacteria as well as beneficial microbes are affected by soil properties (Latour *et al*., 1996). In their study of the genotypic and phenotypic diversity of populations of fluorescent pseudomonads from the roots of two cultivated plant species and from two uncultivated soils, Latour et al. (1996) discovered that the populations linked to the roots of a plant species vary from one soil to another. They linked the variation in plant exudation in the examined soil types to the variation in soil texture. Girvan *et al*., (2003) identified unique bacterial and fungal communities associated with soils of varying texture.

The effects of agricultural management on the soil microbiome are complex and diverse. The microbial communities in agricultural soils play a significant role in nutrient uptake and recycling. Many agricultural practices like crop rotation and no till methods are directed at maintaining nutrients and thus reducing the input of inorganic fertilizers. Tillage cause changes in pore space in soil which in turn affects the nutrient network and interaction between microbial species (Young and Ritz, 2000). Buckling *et al*., (2000) conducted an experiment to study the effect of disturbance on diversity of microcosms (artificial ecosystem) and found that disturbance produced 30% more diversity than static microcosm. So, it can be said that tillage practices produce species that are better dispersers which can tolerate disturbance, at the expense of species better in nutrient competition. No-till fields tend to have higher nutrient levels and distinct microbial communities (Smith *et al*., 2016).

It was found that long term no-tillage increased the organic carbon content in the soil which in turn improved microbial activity (De Sanctis *et al*., 2012). Soil enzyme activity is a good indicator of microbial activity and soil quality. Conservation tillage practices (reduced or no- tillage) increased these enzyme activities in soil (Acosta- Martinez *et al*., 2003). There is also an argument supporting conventional tillage (includes soil tillage like ploughing and harrowing, preparation of seed bed) that it stimulates microbial growth by uniformly distributing residues and increasing the oxygen supply to soil microbes (Janušauskaite *et al*., 2013). The authors also found that with no- tillage only 5- 7.5 cm of the surface soil had increased microbial activity, and this explains the less pronounced enzyme activity with depth in these soils. In Mediterranean ecosystems, tillage practiced without cover crops lead to fast growing microbial competitors (capable of rapid resource utilization) and no- till produced stress tolerators (capable of persisting under resource limiting conditions) (Schmidt., *et al*. 2018). It is clear from these studies that farming practices have a great influence of microbial communities. Homogenization of soil by tillage decreases the microenvironment necessary for some microbes leading to the growth of competitors that can respond quickly to the nutrients. Use of cover crops maintains the nutrient environment even with tillage and results in more diverse microbial community. To improve the productivity from our existing farming systems it is important to understand how different

farming practices influence the microbial communities and how these communities can be manipulated to suit our needs.

1.2.2.2 Effect of fertilizers on soil microbiome

Rhizosphere soil bacterial diversity is particularly vulnerable to environmental changes, such as those caused by anthropogenic disturbances, including long-term fertiliser inputs (Ai et al., 2013). Long-term fertiliser inputs invariably change the pH of the soil, which is thought to be a crucial factor in determining bacterial diversity and community structure. According to earlier research, fertilisation alters the variety, community structure, and activity of soil microorganisms (Chen et al., 2019). Surplus N- application in soil was found to limit the availability of other nutrients like phosphate and the plants responded to the elevated N input by recruiting plant growth promoting bacterias through secretion of organic acids (Chen et al., 2019). Ren et al. (2020) studied the impact of long-term continuous N fertiliser on the diversity and composition of soil bacteria and discovered that varying N application rates resulted in a decrease in the number of bacterial operational taxonomic units (OTUs). Raised organic fertiliser and decreased chemical fertiliser for two years significantly increased relative abundances of *Nitrospira*, *Pseudomonas*, *Arthrobacter*, and *Bacillus* in grapes (Wu *et al*., 2020).

1.2.2.3 Interaction of plants through root exudates

The interaction between plants and their surroundings is a dynamic process in which plants monitor their environment and react to changes. Given the history of evolution, it can be said that plants evolved into a microbial world. The roots of the plants altered the physical structure of the soil, extracted nutrients and water, competing with microbes. Their detritus led to the accumulation of organic carbon, which was then processed by heterotrophic microbes, leading to the formation of soil organic matter (Contrufo *et al*., 2013). The plants altered the physical and chemical environment in the rhizosphere through the release of different substrates through their roots. These affected the fitness of different microbial groups and their interactions leading to the evolution of new microbes better suited for the rhizosphere (Lambers *et al*., 2009, Bais *et al*., 2006).

The plants respond to the chemical signals released by microorganisms through the release of chemical compounds in the form of root exudates. Roots exude a variety of compounds to soil by diffusion, ion channels and vesicular transport (Bertin *et al*., 2003). The diverse compounds released by plants as root exudates include sugars, amino acids, flavonoids, aliphatic acids, proteins, and fatty acids (Badri *et al*., 2009). Fig. 1.1 below provides an overview of interactions in the rhizosphere (Adapted from Berendsen *et al*., 2012*).*

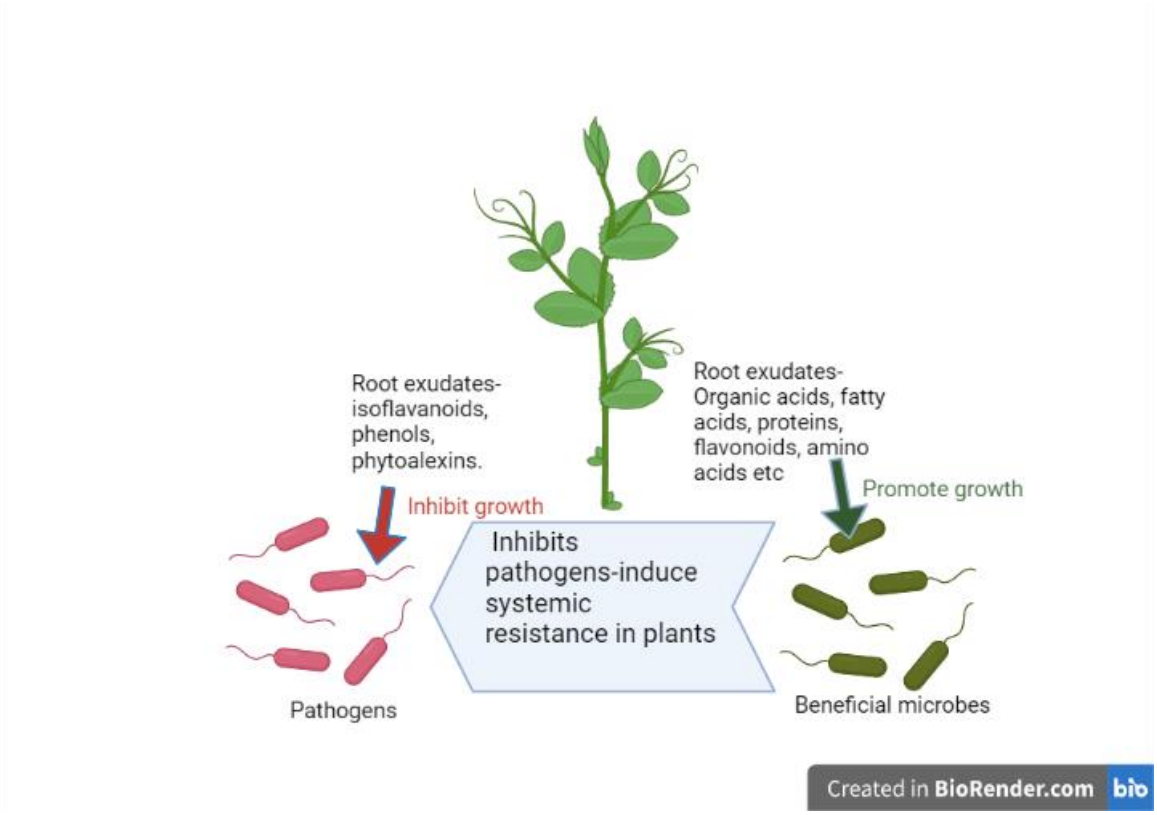


Fig 1.1 : **Rhizosphere and interactions between plant and microbes**: Plants can influence the composition and activation of their rhizosphere microbiome through exudation of compounds that stimulate (blue arrows) or inhibit (red arrows). Pathogens and beneficial microbes are in competition with each other for nutrients and space, beneficial microbes will induce systemic resistance in plants and thus limits the growth of pathogens

Many studies have investigated the colonisation of internal plant tissues by endophytes. In a recent study, Santoyo *et al*., (2016) reviewed available data about various entry points, the region in the rhizosphere was an important point of entry into the plant because of its nutrient rich nature, supplied by the root exudates, and hence its high concentration of microbes (Badri and Vivanco, 2009). The rhizosphere community was found to be a subset of bulk soil community which indicated that microbial assembly in the rhizosphere is because of the selection power of the plant and other environmental factors (Mendes *et al*., 2014). In tomato (*Lycopersicum esculentum*), cucumber (*Cucumis sativus*) and pepper (*Capsicum annuum*), citric acid, malic acid and succinic acid were the predominant components in the root exudates (Kamilova *et al*., 2006). In the same experiment the authors were able to correlate the ability of rhizobial strains to grow in vitro on citric acid to their root colonizing activity. Studies on different crops revealed the existence of a genotype dependent variation in rhizosphere community in addition to the soil dependent variation (Peiffer *et al*., 2013, Lundberg *et al*., 2012). The ability of plants to manage their root exudates vary with species and genotype. This is also dependant on their age, nutritional status, and exposure to stress.

Plant-soil feedback is an important process wherein plants modulate their soil environment by altering the growth conditions, stimulating the growth of specific soil communities, which in turn affects the growth of its offspring and other plant species (Hu *et al*., 2018). This is an important phenomenon in agriculture. Repeatedly growing the same crop depletes the soil nutrients and promotes growth of soil borne pathogens (McDonald and Stukenbrock, 2016). Plant soil feedback is utilized in agricultural systems in the form of crop rotation (Mariotte *et al*., 2017). Besides providing carbon and nitrogen for the microbes through root exudates, plants also secrete various signalling molecules, inhibitors and stimulants that have effect on microbial growth (Baetz and Martinoia, 2014). In *Arabidopsis thaliana*, the malic acid secreted by roots was found to signal and recruit beneficial rhizobacterium *Bacillus subtilis* FB17 (Rudrappa *et al*., 2008). The foliar infection by *Pseudomonas syringae* pv tomato triggered the production of malic acid in these plants. Under iron deprived conditions, plants recruit microbes that can provide them with iron in accessible forms (Fe3+ to Fe2+) (Romheld, 1987).

Calcicole plants deposit di- and tricarboxylic acids that can recruit microbes which produce siderophores that can form complexes with phosphates and ferric ions. This helps the plants to survive in alkaline conditions (Hartmann *et al*., 2009). *Hibiscus cannabinus* was found to have an enriched endosphere of Rhizobiaceae, Pseudomonadaceae, and Enterobacteriaceae when grown in metal polluted soil (Chen *et al*., 2018). They hypothesised that some metal-tolerant and plant growth promoting bacterial species were probably gradually enriched by directional selection of *H. cannabinus* during their prolonged cooperation under metal stress. Thus, it is increasingly becoming clear that plants can control the composition of their microbiome and this selective pressure has given rise to many favourable interactions in many plants.

1.2.2.4 Domestication of plants influence plant microbiota

Varying plants have different capacities for stress toleration, and these capacities can be explained by the differences in the microbiomes of the various plants. (Santoyo *et al*., 2017). Sequence analysis of different microbiome structures have confirmed that more distant the species, the greater the difference in microbiome (Turner *et al*., 2013). For example, when comparing the accessions or varieties of the same species, the effect of genotype on microbiome structure was observed among *Arabidopsis*, *Zea mays* and barley (Bulgarelli *et al*., 2015, Peiffer *et al*., 2013). The environment and soil factors also contribute to this variation. Domestication and development of different cropping systems began to alter this natural selection process. The selection of plants under high fertilization regime and selection for traits like seed size and reduced bitterness lead to the decoupling of soil microbiome from plant fitness (Schmidt *et al*., 2016, Wallenstein, 2017). The fact that genotypic and phenotypic variations in plants select for different microbiomes suggests the ability of a plant to support beneficial microbiome as a trait under selection (Wallenstein, 2017). Plants' volatile emissions were reduced because of domestication, which harmed some plants' ability to defend themselves against herbivorous insects. (Chen *et al*., 2015). Legumes when inoculated with a mixture of rhizobial strains, nodule formation with the effective strain was not uniform across legume genotypes (Kiers *et*

al., 2007). Perez-Jaramillo *et al*., (2016) found differences in the composition of rhizobacterial communities between wild and modern bean accessions, indicating a genetic foundation for the rhizosphere microbiome. He suggested that domestication of plants has given rise to a loss of genetic diversity among crop cultivars which in turn has affected their ability to establish beneficial rhizosphere microbes. Therefore, it will be of great interest to try and re-build these lost abilities of plants, including the potential to control pests.

1.2.2.5 Legume control on microbiomes

Boussingault in 1838 presented first evidence for nitrogen fixation in legumes (Bargaz *et al*., 2018). Prior to this discovery, farmers used to transfer productive soils from one field to another as they noticed beneficial effect in crop productivity (Penna *et al*., 2011). Signalling compounds exuded by rhizobia induce symbiosis in legumes. The remarkable ability of legumes to establish a symbiotic relationship with nitrogen (N)- fixing bacteria gives them a critical role in sustainable agriculture. This reduces the application of external N-fertilizer as well as providing us with a protein enriched crop product (Wang *et al*., 2012). N is one of the principal elements limiting growth and development of crops, particularly in agricultural soils. Legume nodulation is a result of a two- way signal recognition. The flavonoids secreted by legume roots are perceived by bacteria which then initiate the production of signal factor (Nod). The nod factors can bind with lysin motif (LysM) receptor kinases (Radutoiu *et al*., 2007) on the host which induce nodule formation. A study using a rhizobial amino acid transport-defective mutant revealed that in pea nodules, the amino acid needed by rhizobia was supplied by legumes thus persuading the rhizobial community to continue their symbiotic association with legumes (Prell *et al*., 2009). This symbiotic relationship allows legumes to thrive in conditions with limited nitrogen availability (Peoples *et al*., 2009). The beneficial effect of symbiosis (soil enriched with N) can be transferred to non-leguminous plants through subsequent planting. The efficient use of this rhizobium inoculum in soil is important for sustainable agricultural ecosystems (Zgadzaj *et al.,* 2016).

The pea rhizosphere microbiome was strikingly distinct from that of oat and wheat, suggesting a strong effect of legume on rhizosphere population (Turner[b] *et al*., 2013). The *Trifolium pratense* (red clover) microbiome was rich in growth- promoting and disease resistant microbial communities (Hartmann *et al*., 2017). Legumes produce many secondary metabolites which serve as defence compounds (Wink, 2013) and this include alkaloids, amines, peptides, flavonoids, phenols, terpenoids etc. Alkaloids are said to be neurotoxins and can affect the signal transduction pathway of many herbivores (Wink *et al*., 2018). Isoflavanoids commonly found in legumes have insecticidal properties (Mazid and Mohammad, 2011). Manipulating the microbiome of legumes thus provides us with a feasible strategy for many agricultural problems in a sustainable way. Many of the microbiome engineering studies were conducted using model plants like *Arabidopsis*. Studying the behaviour of microorganisms in N rich atmosphere and how they adjust to the root exudates will be an important factor that can be studied using legumes.

1.2.2.6 Soybean as model crop

Soybean is one of the major cultivated legume crops worldwide as a source of protein for humans and as a high-quality animal feed (Goldsmith, 2008, Coleman *et al*., 2021). Soybean is also the most cultivated oilseed crop worldwide, with over 300 million tonnes produced globally (Sugiyama, 2019). The seed of soybean are considered as excellent source of proteins and lipids (Sugiyama, 2019). Total soya consumption in the UK is estimated to be 3.8 million tonnes, including soya beans and meal (efeca, 2018). Direct consumption of soybean by humans is estimated to rise due to shifts towards more plant-based diets. Soybean crop is also one of the common crops used in crop rotation due to its capacity to fix atmospheric nitrogen with the help of rhizobia. The Nitrogen fixing capacity of legumes associated rhizobia is a major source of nitrogen in soils and is an important ecological process to reduce plant dependence on industrial N fertilizers. Weeds and pests are becoming more resistant to chemical management in the contemporary agricultural environment and there aren't enough active chemicals to combat these problems due to strict regulations (Tilman *et al*., 2002). In

this context, it has become imperative to consider a more sustainable approach like crop rotation which helps in interrupting the life cycles of pests and diseases and reduce crop specific weeds (Venter *et al*., 2016). Due to these facts, including a spring sown protein crop such as soybean is of increasing agronomic interest to UK farming. Hooper *et al*., (2000) reported that crop rotation practices increase the above ground biodiversity which results in corresponding increase in diversity belowground. Studies on soybean rhizosphere have contributed to our understanding of various metabolites and microbes in the soybean rhizosphere (Sugiyama *et al*., 2014, Sugiyama, 2019, Liu *et al*., 2019). Liu *et al*., (2019) pointed the importance of combining soil effect and plant genetic effect of soybean in assembling its rhizosphere microbiome and suggested the use of host traits in assembling beneficial rhizosphere microbiome.

As previously discussed, the difference in microbiome can be attributed to the difference in root exudate chemistry. The phytobiome is defined as the complex network that includes plants, their environment and different microscopic and macroscopic organisms that influence plant health and productivity (Leach *et al*., 2017). This network is maintained by various mechanisms involving nutrient cycling, signalling, competition, and chemicals. Most of the studies have focused on exploring the above and below ground interactions using a simplified system involving single microbial species and herbivores. There is a need to study the plant- microbe interaction in a more complex system that represents the systems in nature (Pangesti *et al*., 2013). Since many of the beneficial effects for the plant are due to the consortium of microbes, it has become necessary that we broaden our research to include wider microbial diversity for studying microbe- mediated plant protection studies (Busby *et al*., 2017).

1.3 Engineering of the plant microbiome

Soil microbiomes can be manipulated, as has been done for centuries through agricultural practices such as crop rotation or the use of soil amendments (adding compost, manure etc. to soil to improve its physical and biological properties). Plant-soil and plant-plant interactions can be subjected to

ecosystem-level selection (Swenson *et al*., 2000). Vanderkoornhuyse, (2015), suggested that plant microbiome is the powerhouse of adjustment to local conditions. Any disturbance in microbiome can cause extensive changes in host, affecting their physiology and fitness. Microbiome engineering is defined as strategies used in experiments to select microbial communities with particular effects on host fitness, hence enhancing host performance (Mueller and Sachs, 2015).

1.3.1 Ecological principles of microbiome engineering

Microbiome engineering alter the microbiomes through ecological and evolutionary processes. The ecological processes include changes in diversity of microbes, relative abundance of species and the interaction between microbes (Christian *et al*., 2015). The evolutionary processes include changes in community composition of microbial types, changes in allele frequencies, mutation and horizontal gene transfer that restructure the microbial genome (Theis *et al*., 2016). The host behaviour plays an important role in microbiome engineering. Host selectively alters the microbial community through resistance and immunity mechanisms (Sachs *et al*., 2004). The traits of plants and animals that have evolved over time selectively recruit beneficial microbes and exclude ineffective symbionts (Schweitzer *et al*., 2008). The selection occurs by sanction mechanisms where the host regulates proliferation of beneficial microbes and disfavours ineffective ones including pathogens. Once the microbes colonize the host, some of them are transmitted to their offspring by vertical transmission through seeds (Shahzad *et al*., 2018). This vertical transmission and host control tie the microbes to their hosts and guarantee the transfer of this microbiome to the next generation. Using microbiome engineering, improved microbiome functions can be selected without any knowledge on the microbiome composition. The important feature of this microbiome trait is that it affects a host phenotype and selection can focus on this phenotype (Foster and Wenseleers, 2006). The host is used as a probe to evaluate microbiome functions that impact plant fitness (Mueller and Sachs, 2015). The microbiomes are not measured directly, but their performance is evaluated indirectly by measuring the host performance. The two different ways that microbes can mediate plant functional traits are

through the synthesis of new biologically active compounds which can support plants and by altering the existing physiological pathways in plants through production of phytohormones (Friesen *et al*., 2011). Microbes are found to be important factors determining many of the plant functional traits like nitrogen content, leaf longevity, root: shoot ratio, specific root length and abiotic stress tolerance (Cornelissen *et al*., 2003).

The plant microbiome is a tool which can be selected along with the plant genome to direct development of new generations of plants (Gopal and Gupta, 2016). The plant microbiome with a desired characteristic like disease suppression can be used as initial inoculum to develop into a new microbiome to produce next generation crops (Mendes *et al*., 2011). Superior plant hosts can mediate best microbiome assembly and thus host mediated microbiome selection leverages host traits that have evolved to select for favourable microbiomes that enhances host fitness (Schweitzer *et al*., 2008). In microbiome engineering community coalescence plays a major role (Rillig *et al*., 2016) and is important in the engineering process where different communities are mixed. A successful network of microbes will be established based on the communication between them and their ability to adjust to the new abiotic condition formed. Panke- Buisse *et al*., (2015) used a mixture of soils from agricultural, forest and grassland sites as starter microbiome. This mixing of microbiomes is a coalescent event where different microbial communities encounter each other. This coalescent event might affect physiological functions in *plantae*. It is therefore important to integrate physiological experiments with study of the microbiome, as the plant microbiome responds to environmental changes much faster than the plant and they influence the hormonal activity in plant and thereby its physiology (Berg *et al*., 2014).

1.3.2 Different methods of microbiome engineering

The functions of the plant microbiome (disease resistance, salinity and drought tolerance, for example) are performed by a small number of microbial species and their synergistic or additive

effects between strains (Timm *et al*., 2016). Transferring these species between hosts enhances the defence system in other host plants. This can be achieved in different ways, host mediated and multigeneration microbiome selection, inoculation into bulk soil, rhizosphere, seeds or atomisation into tissue or direct injection into tissues or wounds (Orozco-Mosqueda *et al*., 2018). Chihaoui *et al*., (2015) analysed the effect of inoculation of *Agrobacterium* sp. 10C2 on growth and rhizosphere microbiome of *Phaseolus vulgaris*. The inoculation of this strain in the rhizosphere recruited plant growth promoting bacteria from four separate phyla (Firmicutes, Actinobacteria, Bacteroidetes and Proteobacteria) and beans that were produced showed a significant increase in the contents of phosphorus, polyphenols, and flavonoids, and total antioxidant capacity. Similarly, in orchids, inoculation of bacterial species such as *Pseudomonas fluorescens* and *Klebsiella oxytoca* into *Dendrobium nobile* Lindl. increased the germination, and growth capacity of orchids (Pavlova *et al*., 2017). Mitter *et al*., (2017) employed a new technique to introduce the endophyte bacterium *Paraburkholderia phytofirmans* PsJN by atomisation into the flowers of mono- and dicotyledonous plants and found that seeds that inherited the PsJN strain showed significant differences in plant development with respect to the controls (plants with an unmodified microbiome). Wicaksono *et al*., (2018) successfully transferred the biocontrol effect of bacterial endophytes from *Leptospermum scoparium* (a medicinal shrub) to *Actinidia deliciosa* (kiwi fruit) by wound inoculation. The bacterial endophytes inhibited colonization by *Pseudomonas syringae* pv. *actinidiae* (Psa) and reduced disease severity in two different commercial cultivars.

In methods where species are introduced to soil or seeds, only culturable or easily transferable microbial species can be used whereas, in host mediated artificial selection both culturable and non-culturable microbes can be engineered (Mueller and Sachs, 2015). This new research horizon in medicine and agriculture aims to improve animal and plant performance by altering their microbiomes (Gopal *et al*., 2013). The new approach employs artificial selection on a host microbial association to engineer the microbiome. Host-mediated artificial selection can generate diverse microbial communities that can affect

the host performance in a significant way (Swenson *et al*., 2000, Panke-Buisse *et al*., 2015, Williams and Lenton, 2007).

1.3.3 Host- mediated microbiome engineering studies

Swenson *et al*., (2000) conducted an experiment to select a microbial ecosystem for degradation of 3-chloroanilline. The experiment was carried out in test tubes containing 3-chloroanilline and inoculated with planktonic microorganisms in 1ml of unsterilized pond water from a single source. The selection was carried out for 30 generations and after the incubation period it was found that, the test tubes, each representing an ecosystem, differed in their species composition and thus in their 3- chloroanilline degradation character. Williams and Lenton (2007), further investigated on this selection mechanism conducting a similar experiment using a 'flask model'. The ecosystem is simulated in a flask with a microbial suspension in liquid matrix. Some of the chemicals added to the matrix acted as nutrients and non-consumable (chemicals that were not used my microbes) part of the chemicals formed the abiotic environment. They tried to address the question of how artificial selection produces a response in the selected ecosystem. The individual level selection pressure on metabolic requirement and abiotic factors like temperature and pH result in changes in species composition which manifests as phenotypic variation at higher level. Due to the interaction, mutation occurs at individual levels and artificial selection removes unfavourable ones. This proves that a significant fraction of artificially selected ecosystem response cannot be accounted for by a single species but due to community interactions. A multigeneration experimental system using *Arabidopsis thaliana* Col was designed to select for microbiomes that induce earlier or late flowering in hosts (Panke-Buisse *et al*., 2015). The selected microbiome from the tenth generation was used to grow different hosts. 16S rRNA sequencing revealed distinct soil microbiota associated with flowering treatment. In a study, where soybean rhizosphere was used to explain community assembly processes, a clear selection process was found at both taxonomic and functional levels operating in soybean rhizosphere. The rhizosphere community was found to be a subset of bulk soil community which indicated that microbial assembly in the rhizosphere is due to the selection power of the plant and other environmental factors (Mendes *et al*., 2014). Several examples where the plant microbiome, particularly of the root and endophytic compartments, has been used to suppress diseases of field and horticultural

crops (Mendes *et al*., 2011; Spence *et al*., 2014; Cha *et al*., 2016), improve drought resistance in desert crops (Lau and Lennon, 2012; Marasco *et al*., 2012) and grapevine (Rolli *et al*., 2015) and alter above-ground herbivory (Hol *et al*., 2010) have unequivocally proved that the host microbiome indeed impacts the fitness of plants. Niu *et al*., (2017), through host-mediated selection, obtained a simplified synthetic bacterial community in maize rhizosphere. It consisted of seven bacterial strains and these strains when inoculated on maize plants offered protection against *Fusarium verticillioides*, the causal agent of maize blight. Individual strain inoculation offered less protection to plant against *F. verticillioides.* Jochum *et al*., (2019) demonstrated the feasibility of employing the host phenotype as a selective marker to construct microbiomes that influence changes in the rhizosphere environment and increase wheat plant tolerance to drought stress.

1.4 Challenges in microbiome research

Microbiome research has improved our perception of the complexity and structure of microbial communities. In studying the human microbiome, the importance of analysing microbial interactions, separately for everyone has resulted in novel therapies (Human Microbiome Project Consortium, 2012). To exploit the full potential of microbiomes, it is important to have new analytical strategies to distinguish the functional capability of microbial communities (Bashiardes *et al*., 2018). In crop production, there is an essential need to maintain a diverse and balanced microbiome at the plant – soil interface (Syed Ab Rahman *et al*., 2018).

Advances in sequencing techniques has facilitated the study of rhizosphere- related microbiome studies (Fierer, 2017). A popular technique for examining the phylogeny and taxonomy of bacteria is microbial profiling utilising 16S ribosomal RNA (rRNA) sequencing mainly because it comprises both highly conserved and hypervariable sections. Amplicon sequencing techniques use PCR products that have been generated by selectively binding universal primer pairs to highly conserved sections of the genomes of certain microbiome members of interest (Fricker *et al*., 2019). Fierer (2017) points out that finding precise connections between soil taxa and the soil microbiome's functional abilities is

frequently challenging. The focus is given to genes with known functions which results in ignoring the genetic information that has the potential to perform novel functions (Sergaki *et al*., 2018). Even closely related species vary in lifestyle (either as pathogen or mutualist) depending on the environment (Hacquard *et al*., 2016). This variability can lead to changes in microbial phenotypic traits. As a result, more sensitive microbiome characterization approaches beyond the genus level are necessary. In addition to improving our understanding of multitrophic interactions using plant and microbiology- based approaches, there is a need to develop new ecological systems with growing complexity.

Host mediated microbiome engineering studies involve different selection lines and this is very time consuming. A minimum of six independent selection lines is typically recommended, six selection lines all responding in the same direction compared to controls will meet the significance criteria (Mueller and Sachs, 2015). The experimental set up requires space as well as an aseptic environment to carry out inoculum transfer between generations. Mueller *et al*., (2016), to engineer microbiome to confer salt tolerance to plants made a custom- made flow hood to harvest and propagate microbiome across generations.

Ultimately, it is necessary to test microbial community function in field studies, which are highly complex and diverse systems, to bridge the gap between lab and the farm (Sergaki *et al*., 2018). The main gap that limits the extension of lab study to field is that lab studies generally do not capture the complexity of interactions in the natural setting. Any benefits mediated by microbes under controlled conditions should ultimately need to be operative in field. So, studies aimed at engineering microbiomes should consider different soil conditions and management practices that are important in sustainable land management. The current study aims to address some of the factors influencing microbiome study like influence of soil management practices and cropping history, but whether this manipulation emulates exact field conditions will remain a question to address and a challenge to overcome.

1.5 Outstanding questions

Microbiome research has advanced significantly in the recent decade. Our understanding of the complexity and structure of microbial communities has changed. The intricacy of microbial interactions and the significance of examining them uniquely for everyone has already resulted in novel therapeutics in the study of the human microbiome and these `personalised` microbiome approaches will increase the efficiency of treatment (Human Microbiome Project Consortium, 2012). Similar efforts are required in understanding the functional capabilities of plant microbial communities. In crop productivity, it is critical to maintain a diversified and well-balanced microbiome at the plant–soil interface and microbiome applications should be focused on improving key factors like nutrient availability, soil, and plant health (Syed Ab Rahman *et al*., 2018). Validating stable synthetic communities require understanding the underlying inter-microbial processes that drive community formations and research should focus on deciphering this complex microbial and plant-microbial interactions (Sergaki *et al*., 2018).

Land management has a big impact on the structure and stability of soil and root microbial communities, and thus on microbiome-related functions (Hartman *et al*., 2017). More research is needed to understand how different management strategies affect the soil microbiota and whether both cropping practises and microbiome engineering can contribute to long-term sustainable agriculture. Host mediated microbiome engineering is a valuable tool for manipulating and understanding microbiomes (Mueller and Sachs, 2015). Panke- Buisse *et al*., (2015) suggested that new forms of interactions between plants and microbes controlling plant development can be discovered when a fast-growing plant is investigated in conjunction with its microbiome throughout numerous generations. Earlier Swenson *et al*., (2000) reported that the change in plant biomass across multigeneration selection might be due to the changes in soil nutrient variables. The difference in soil nutrients can be attributed to the difference in soil extracellular enzyme activities (Sinsabaugh, 2010). Only a few research have taken an experimental-evolutionary strategy to change microbiomes or

figure out how they work (Mueller and Sachs, 2015). The changes in soil enzyme activity, root exudation in plants and the changes in microbial population can be studied using multigeneration selection. The current study will use multigenerational microbiome selection to try to answer some of these problems.

1.6 Thesis outline

This thesis aims to improve our understanding of the changes in soil enzymes, rhizosphere bacterial population and root exudates taking place during microbiome selection. Soils differing in their management practices and cropping history will be used as microbiome inoculum for this study. Through multigeneration selection, the study will aim to select for a microbiome that can render better plant growth under nutrient limiting conditions. The study is divided into following sections.

1. Study the difference in physical (soil texture and water holding capacity), chemical properties (pH, soil moisture and nutrient content) and extracellular enzyme (N-acetyl beta glucosaminidase (NAG) and phosphatase (PHOS) enzyme) activities of soil samples collected from field sites differing in cultivation practices (untilled, tilled and legume grown soils). These soils will be used as microbiome inoculum in the microbiome selection study using soybean plants (Chapter 3).
2. In this study, we were investigating changes in soil enzyme activity happening in the rhizosphere of soybean plants during microbiome selection based on plant height. The soil enzyme activity is related to nutrient cycling happening in the rhizosphere which will be reflected in plant growth (Chapter 4)
3. This study focuses on the difference in exudation of carbohydrates, phenols, and amino acids by soybean varieties during microbiome selection in two generations of plant growth in different soil types. (Chapter 5)

4. This study uses next generation sequencing to characterise bacterial communities in the soil samples collected from field and to study how these community composition changed during microbiome selection. (Chapter 6).

Chapter 2. Materials and methods

2.1 Soil sampling for experimental work

The purpose was to collect soil samples from farmed areas under different cultivation practices. Soil samples were collected from three locations in Sonning farm (Fig. 2.1); marked as S1, untilled/uncultivated soil (Lat 51.47843; Long -0.89845); S2, tilled/cultivated soil (Lat 51.4789; -0.89627) and S3, legume/red clover grown soil (Lat 51.48188; Long -0.8973). The sites were sampled on 29/11/2018, following a standard operating procedure (Tóth *et al*., 2013). Soil was collected from the top 20cm of the soil profile using a soil corer (10cm diameter) after removal of the overlying litter and residue by hand. The soil sample from the legume site (S3) was sampled near the roots of clover plants (4 plants at one sampling point). The sampling at each field was done in a 'W' pattern collecting soil from 20 sampling points per field. Each sampling point was 1m apart. The soil corer was wiped clean using ethanol (70%) before sampling each field. The collected soil was immediately transferred into sealed sterile boxes (separate boxes for each soil type, soil from the individual sampling points within fields combined). The samples were homogenized by hand, sieved (4mm sieve, Endecotts Ltd, London) and stored in boxes at 4˚C until further analysis (5 months). Gloves were worn while handling soil and changed frequently between soil samples. The sieves were wiped clean with ethanol (70%) after each use. A random sample (stored in 15ml sterile falcon tubes) was collected from each soil type and stored at -80^{o}C to allow for downstream microbial community analysis.

2.1.1 A brief history of the sampled plots

Untilled (S1)

The untilled plot had been left untilled for many years (more than 5 years) prior to the sampling. Grasses on the plot were machine cut every year and left on the surface.

Tilled (S2)

The field from which the tilled soil samples were obtained was covered with grass from November 2013 until November 2017. The grass was then cut by mechanical means and left on the surface. The field was ploughed afterwards to grow barley (*Hordeum vulgare*) from November 2017 to August 2018. No fertilizer

was applied. The plot was ploughed again in August 2018, approximately 3 months prior to the soil sampling.

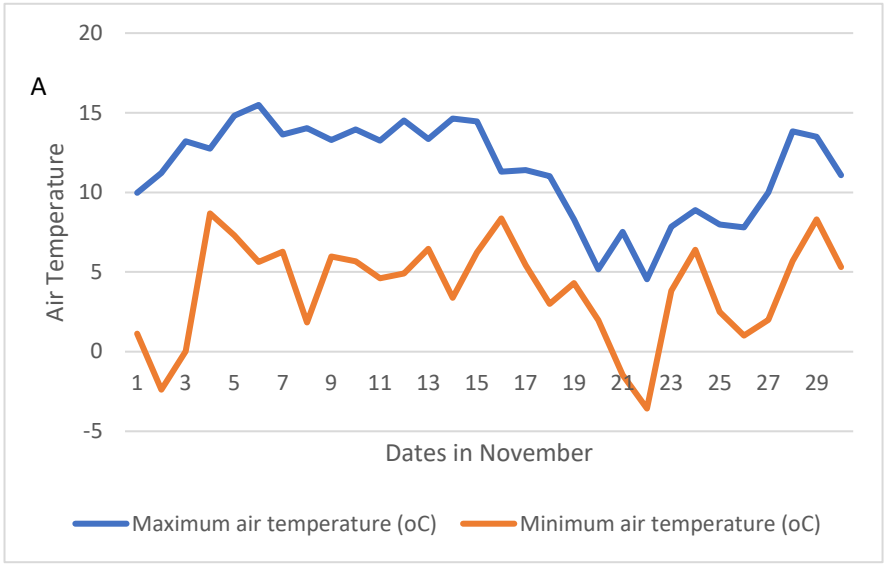
Soil with legumes (S3)

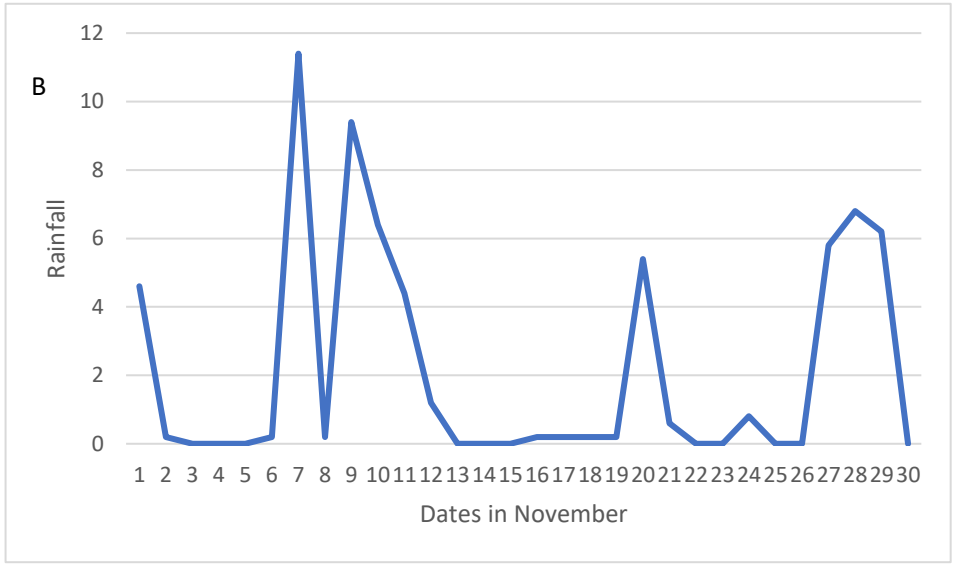
The field from which the soil with legumes was sampled had been ploughed and planted with legumes (red clover, *Trifoleum pratense*) interspaced with grass in August 2017. The grass was machine cut and left on the ground. Before 2017, the field was used as experimental plot fertilised with Nitrogen and Sulphur during the growing season. In September 2015, it had a base dressing of 100kg Potassium oxide fertilizer.

Fig 2 1 **(A) Map of the UK showing location of Sonning Farm (coloured dot). (B) Aerial photograph of Sonning farm with each field marked and labelled as S1 (Untilled), S2 (Tilled) and S3 (Legume)**. The blue boxes indicate areas of the farm from where the soil samples were taken. Samples were collected in a `W` pattern across each field from points approximately 1m apart. Photo taken from Google Maps

2.1.2 Weather parameters

The three sampling sites had experienced similar weather conditions prior to sampling which was done on 29th of November,2018. The air temperature varied between -3.5 to 14.8^{o}C. The variation in soil temperature was between 4.8 to 10.1^{o}C. The Fig 2.2 below shows the measurements of rainfall, air temperature and soil temperature in the month of November 2018.





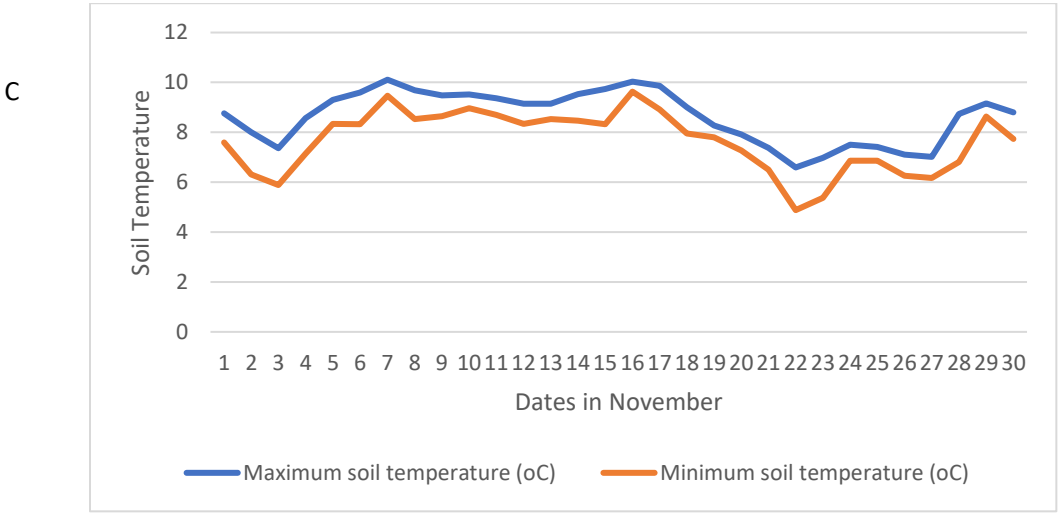


Fig. 2.2 **Weather parameters, A. Air temperature (°C), B. Rainfall (mm) and C. Soil temperature (°C) during the month of sampling (November 2018)**; data from Sonning farm meteorological records

2.2 Soil mixing

A fourth soil type was obtained by mixing the soil collected from the three field sites (S1, S2 and S3).

The three original soils were weighed into a sterile autoclave bag in equal amount (200 g each), mixed thoroughly by hand (with gloves) and stored for one week at 4^{o}C before conducting any analysis. The resulting soil mix will, from now on, be referred to as S4 or 'mixed soil'.

2.3 Growth medium for microbiome study

A potting medium with low level of available nutrients provide a strong filter to impose microbiome effects on soil nutrient mineralization (Panke-Buisse *et al*.,2015; Mueller and Sachs, 2015). Coir (Melcourt coir [coconut coir, particle size, 0-6mm], Melcourt Industries Limited, Gloucestershire) and sand (Jubilee Building supplies, Bracknell) were mixed in 1:1 volume/volume ratio (60 litres of coir was added to 60 litres of sand and mixed homogenously). Coir: sand mixture was used as control soil/plant growth substrate for all microbiome inoculations in the microbiome study (see below). The low nutrient content in this growth media makes it ideal for microbiome propagation which is important in this study as the plants were grown and selected under nutrient deficit conditions. Properties of the coir: sand mixture, including pH, water holding capacity and nutrient contents (Appendix Table B.1) were assessed prior to the start of the study (methods given in Chapter 3).

The coir: sand mixture was divided into different autoclave bags for sterilizing. The mixture was autoclaved twice for 90 min at 121°C. The two periods were separated by a 24 hr cooling period at room temperature (this helps in killing any bacterial spores in the next autoclave cycle; Wagner *et al*., 2014). 1 g of sterilized mixture was mixed with 10 ml sterile water and serial dilutions (10^{-1}, 10^{-2},10^{-3}) were prepared from this mixture using sterile water and plated on Luria Bertani media (LB) with agar (10 g Bacto-Tryptone [Oxoid], 5 g Bacto-yeast extract [Oxoid; Oxoid Limited, Hampshire, U.K], 5 g NaCl [BDH; BDH laboratory supplies, Dorset, U.K], 15 g Agar) on Petri dishes (90mm diameter) and incubated at 27^{o}C for 5 days to check for bacterial growth.

2.4 Microbiome inoculum preparation and plant growth procedures

The phenotype (plant height) of the host was used as a probe to select the soil microbiome that affect the host fitness. The host plant used was soybean (*Glycine max*), a legume crop known for its high protein content and its ability to recruit nitrogen fixing bacteria. The four soil types described above (Untilled, tilled, legume and mixed) were used to provide starter inoculum for the study. Sterilized coir:sand mixture was inoculated with field soil suspension prior to growing soy bean plants. Selection of microbiome was based on the plant height, the soil suspension from the rhizosphere of the best-performing replicates was used to inoculate the next generation of the respective line. The plants were grown in pots in the glasshouse for 2 generations. The preparation of inoculum for both generations of plant growth, pot preparation, plant growth conditions and sample collection are explained in detail in each section. The flow chart explaining the treatments is given in Fig. 2.7.

2.4.1 Inoculum preparation, inoculation, and planting- 1st generation

2.4.1.1 Preparation of microbiome inoculum

Soil samples were removed from 4°C storage and left at room temperature overnight. Specific soil mixes for the microbiome studies were prepared following previously published methods (Panke-Buisse *et al*., (2015) and Yergeau *et al*., (2015)). 200g of each of the four soil types was weighed into separate 2L sterile conical flasks (Thermo Fisher). 1800ml of sterile water was added to the soil sample and the suspended soil sample was shaken for an hour in an orbital shaker (Rotatest Major Luckham) at speed 5. The resulting soil suspension was used as the inoculum and 100ml of the suspension was mixed with autoclaved coir/sand mix in each pot (Section 2.4.1.3). The use of soil suspension minimises the transfer of soil derived nutrients and forces the transferred microbial communities to rely on the uptake of nutrients derived from root exudates (Tkacz *et al*., 2015).

2.4.1.2 Preparation of pots

The pots used were terracotta troughs (Injection moulded from durable polypropylene in plain terracotta, 20 cm wide x 44 cm length x 14 cm depth; PLNT728T; LBS, Lancashire). The pots were wiped clean with ethanol (70%) prior to use. Each pot was filled with 12 litres of autoclaved coir:sand mix. All the pots were watered using tap water immediately after filling and excess water was allowed to drain out before inoculating with microbiome. This ensured equal volume of water in all pots prior to the inoculation.

2.4.1.3 Microbiome inoculation

The microbiome inoculum was prepared from the field soils as detailed in section 2.4.1.1. The pots were placed on a glass house bench (metallic bench) in five sections according to treatment (type of inoculum). There were 5 treatments: inoculum from untilled soil (S1), inoculum from tilled soil (S2), inoculum from legume grown soil (S3), inoculum from mixed soil (S4) and no inoculum (i.e. control; C). For each treatment type, 15 pots were prepared, 6 pots for each of two soybean varieties (Kenchen and Siverka) and three pots into which no soybeans were planted (i.e., soil mix alone). The pots without plants are referred to as 'fallow' pots and represent the bulk soil in fields which is not under the influence of plant root exudates. The arrangement of pots on the bench is shown below in Fig. 2.3. Each pot except control pots, received 100ml of the soil suspension made from the respective field soil sample. Control pots received 100ml of sterile water. The soil inside the pots was mixed thoroughly by gloved hand to ensure uniform distribution of the soil suspension and suspended microbes. Separate pairs of gloves were used for each pot to prevent transfer of microbiome between pots.

2.4.1.4 Preparation of seeds

Different host genotypes may recruit different kinds of microbes into their rhizosphere. To study potential differences in the soil microbiome associated with different host types, two soybean varieties, Kenchen and Siverka, were used (Fig. 2.4). These are the most popular soybean varieties in UK and they differ each other in their morphology, Kenchen being a trailing variety and Siverka a non-trailing type. Seeds were kindly supplied by David McNaughton from Soy-UK (Longways House, Burnetts Lane, West End, Southampton). All seeds used for planting in both the generations were obtained from same batch of

inbred. It was important to ensure that plants were derived from the same genetic stock, to maintain consistent genetic make-up across generations so that any observed changes in plant characteristics were due to changes in the microbiome (Panke-Buisse *et al*., 2015, Mueller *et al*., 2016).

Uniform sized seeds, weighing 0.1- 0.15g were chosen for the study. Seeds were surface sterilized by vortexing with 70% ethanol for 10 seconds followed by 5 rinses in sterile water. A few of the surface sterilized seeds (3 seeds per plate and 3 plates as replicates) were placed on LB media plates and incubated at 27^{o}C for 7 days to check for any contamination by bacteria. No contamination was observed. Sterilized seed surfaces may not necessarily indicate sterile seeds as found in study by Robinson *et al*., (2016) in wheat (*Triticum aestivum*). Sterilized seeds were germinated in autoclaved coir:sand (sown in seed trays at depth of 2.5cm) before being transplanted to pots with treatments 10 days after germination (at unifoliate leaf stage).

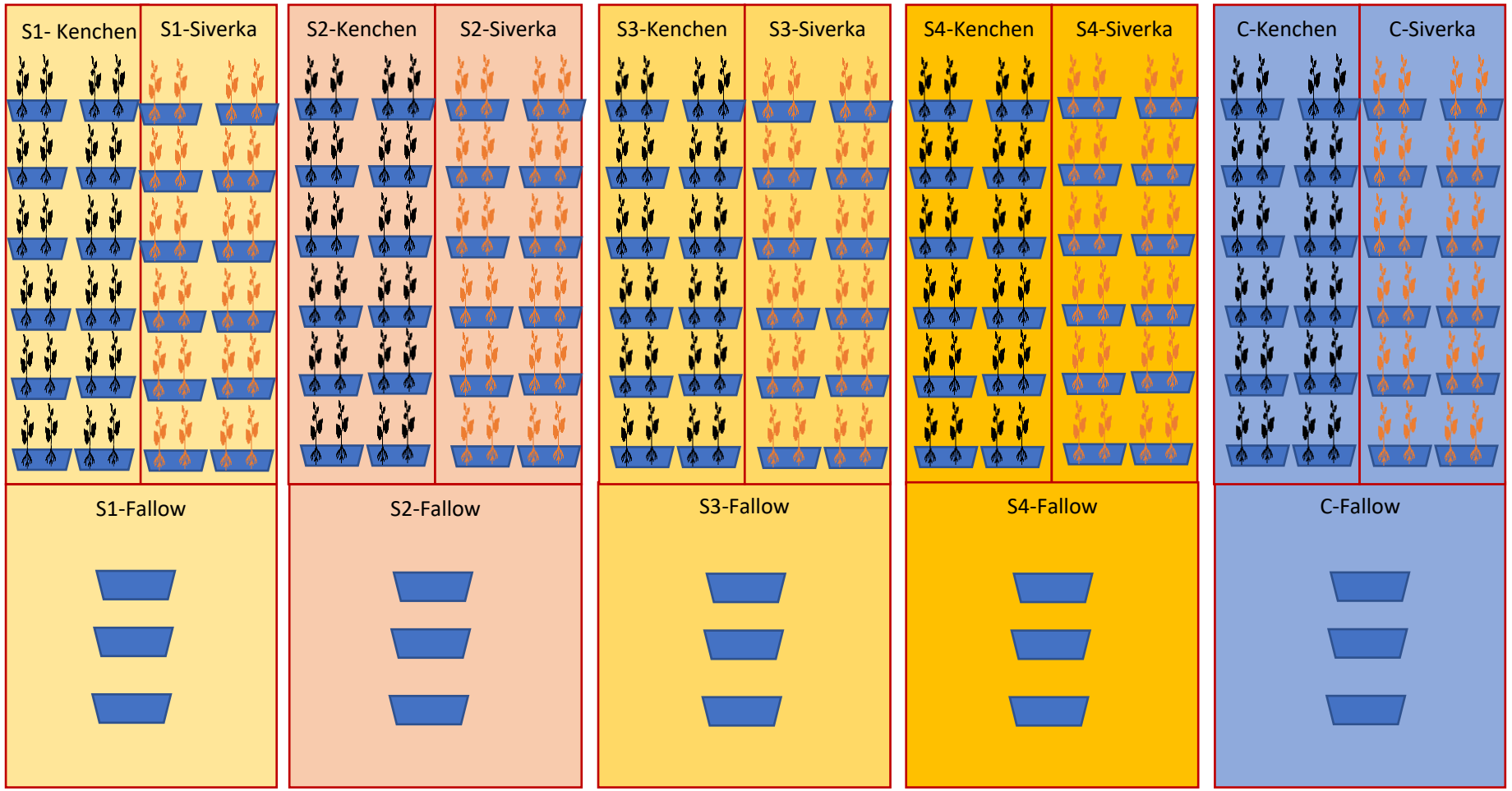


Fig. 2.3 **Diagram showing treatment positions in the greenhouse;** All pots were filled with autoclaved coir:-sand mix. All pots except control pots inoculated with soil suspension (microbiome) from respective field soils; S1- Untilled soil; S2-Tilled soil; S3-Legume grown soil; S4- Mix of S1,S2,S3; C- Control(sterile coir: sand without field soil suspension); Each soil type has 6 pots with variety Kenchen and 6 pots with variety Siverka and each pot had 2 plants; Fallow pots- No plants grown, all pots except for control have microbiome from respective field soils

Fig. 2.4 **The two studied soybean varieties differ in their morphology**. A. Variety: Siverka, hairy and non-trailing variety; B. Variety Kenchen, non-hairy and trailing type

2.4.1.5 Planting

To give plants sufficient time to respond to the field microbiome in their pot and ensure that the root encounters soil microbial communities in the early stages of the plant growth (Mueller *et al*., 2016), the plants were transplanted to inoculated pots at unifoliate stage (10 days after sowing). Two seedlings were planted per pot at a 15cm distance from each other (corresponding to the typical spacing for soybean in field). Fallow pots were left unplanted.

The plants received no additional water for the first 2 days after transplanting as the soil was very wet. Thereafter, the plants were watered using drip irrigation system (Netafim, UK; drip capacity of 2L per hour) method (Fig. 2.5) to standardise watering regime. The drips were wiped clean using 70% ethanol before pot installation. Tap water was used in the irrigation regime. Watering regime was adjusted dependent on plant growth stage. Each plant received 50 to 100ml of water depending on growth stage. Fallow pots also received the same amount of water each time as control and treatment pots. No fertilizers were added to pots and plants were allowed to grow with available nutrients in soil.

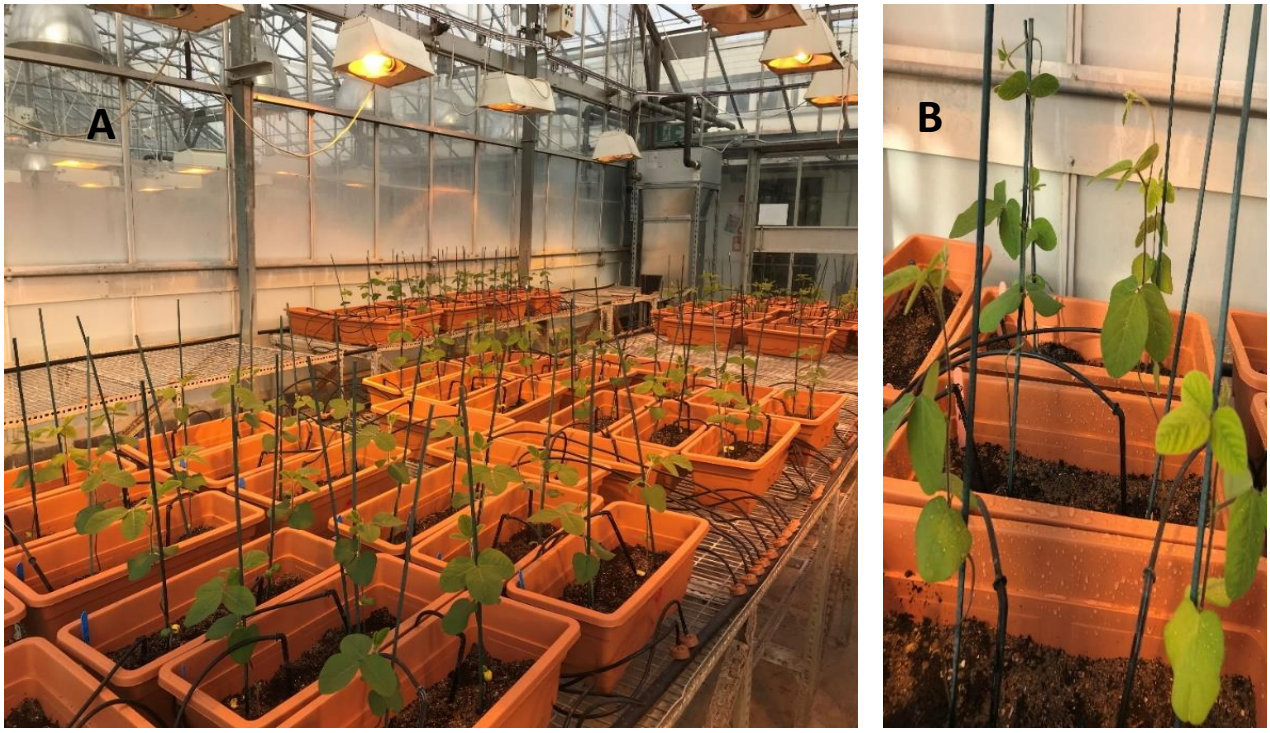


Fig. 2.5 **Experimental set up in glass house: A. Pots arranged in sections based on soil types**, 12 pots per soil type and 6 pots per variety in each soil type; 2 plants per pot; **B: Drip given to each plant for uniform water application**

2.4.1.6 Plant growth conditions

The plants were grown (12 h photoperiod under fluorescent light, 25-27°C day/ 15- 20°C night temperature and with a relative humidity of 60-80%) in a specially designed glasshouse on University of Reading grounds.

2.4.2 Host mediated artificial selection of microbiome

Microbiome engineering aims to improve a host trait by artificial selection on the. In this study, plant height was chosen as the trait for selection of microbiome. This study hypothesises that a microbial community with more advantageous (or more intense) characteristics that support plant growth is associated with an increase in height. In soybean, plant height is positively correlated with yield (Li *et al*., 2019). The tallest plants were chosen for the harvest of microbiome for the next generation of crop.

2.4.3 Selection and grading of plants based on plant height

The plants were allowed to grow for 38 days in the treatment pots. At this point, flowering of plants had started in most of the treatments. To select the best growing plants, plants were graded based on plant height. Plant heights (in cm) were recorded for all plants before harvest. Plant height was measured from above soil level to the apical tip of the plant.

2.4.4 Microbiome harvest

The plants were graded as high growing (High) and low growing (Low) plants based on plant height. The six tallest plants of each variety (Kenchen and Siverka) and in each treatment (2 variety × 4 treatment combinations), regardless of which pots they grew in, were named as High growing plants. The remaining plants were named as Low growing plants. Rhizosphere soil from each plant (both High growing and Low growing) was harvested separately. The plants were uprooted by hand from the pot along with any associated soil material. Loosely adhered soil was removed by tapping on the sides of the pot. The volume of soil completely colonized by roots (Fig. 2.6) is taken as rhizosphere soil (Bobille *et al*., 2016). This rhizospheric area is marked by microbial abundance and activity due to the presence of plant exudates. 5g of soil adhering to the root was collected and stored immediately at -80^{o}C for microbial DNA analysis. 10g of the rhizosphere soil was collected in sterile falcon tubes (50 ml, Thermofisher) for soil enzyme analysis and stored at 4^{o}C until analysis (one week after sampling). The remaining rhizosphere soil was used for the analysis of soil pH, nutrients, enzyme activity and microbiome preparation. The whole root system (keeping the above ground plant part intact) was stirred in 100 ml of sterile water in sterile cylindrical flasks to remove rest of the rhizosphere soil from the roots. This root wash was used while preparing microbiome inoculum for the next generation of plants. Soil samples were collected from fallow pots at 20cm depth and stored for analysis.

Fig. 2.6 **Rhizosphere soil- The plants were uprooted from pots and the loosely adhered soil removed by tapping on the sides of the pot**. The volume of the soil shown in the picture is completely covered by roots and therefore under the influence of root exudates and this soil is selected as rhizosphere soil. This soil was used for the estimation of pH, nutrient content, enzymatic activity, and microbial population.

2.4.5 Inoculum preparation, inoculation, and planting- 2nd generation

2.4.5.1 Preparation of microbiome inoculum

The microbiome inoculum for the 2nd generation of plants was prepared from the rhizosphere soil of the selected (section 2.5) high growing plants (High) from the first generation. The rhizosphere soil from the 6 tallest (High) plants within each treatment × soybean variety (4x2) combination was pooled and made into a homogenous mixture. 200 g of this mixture was used to prepare soil suspension with 1700 ml of sterile water + 100ml root wash obtained earlier by washing roots (section 2.4.4) after harvesting the microbiome from 1st generation. Separate inoculums were prepared for each soybean variety in each soil type.

2.4.5.2 Microbiome inoculation and planting

The procedures as in the first generation were followed. There were separate fallow pots for each variety (3 each). The bench and drip systems were wiped clean with 70% ethanol before the start of the 2nd generation planting. Preparation of soil inoculum and planting is shown in a flowchart (Fig. 2.7). Three unseeded pots containing a sterilised coir: sand mix were included for each soybean variety.

Following the transplanting of seedlings into the inoculated pots in 2nd generation, there was mice infestation on the crops and all the plants need to be uprooted 5 days after planting. New seeds were sown at the rate of 2 seeds per pot. Surface sterilized seeds were used for sowing. In second generation, seeds were sown directly to pots with microbiome. This is different from the first generation planting where 10 days old seedlings were transplanted to the pots. To make the harvesting time uniform as in 1st generation, the 2nd generation of plants were harvested 45 days after sowing. Most of the plants were in flowering stage. Early pod development was noted in some of the treatment groups.

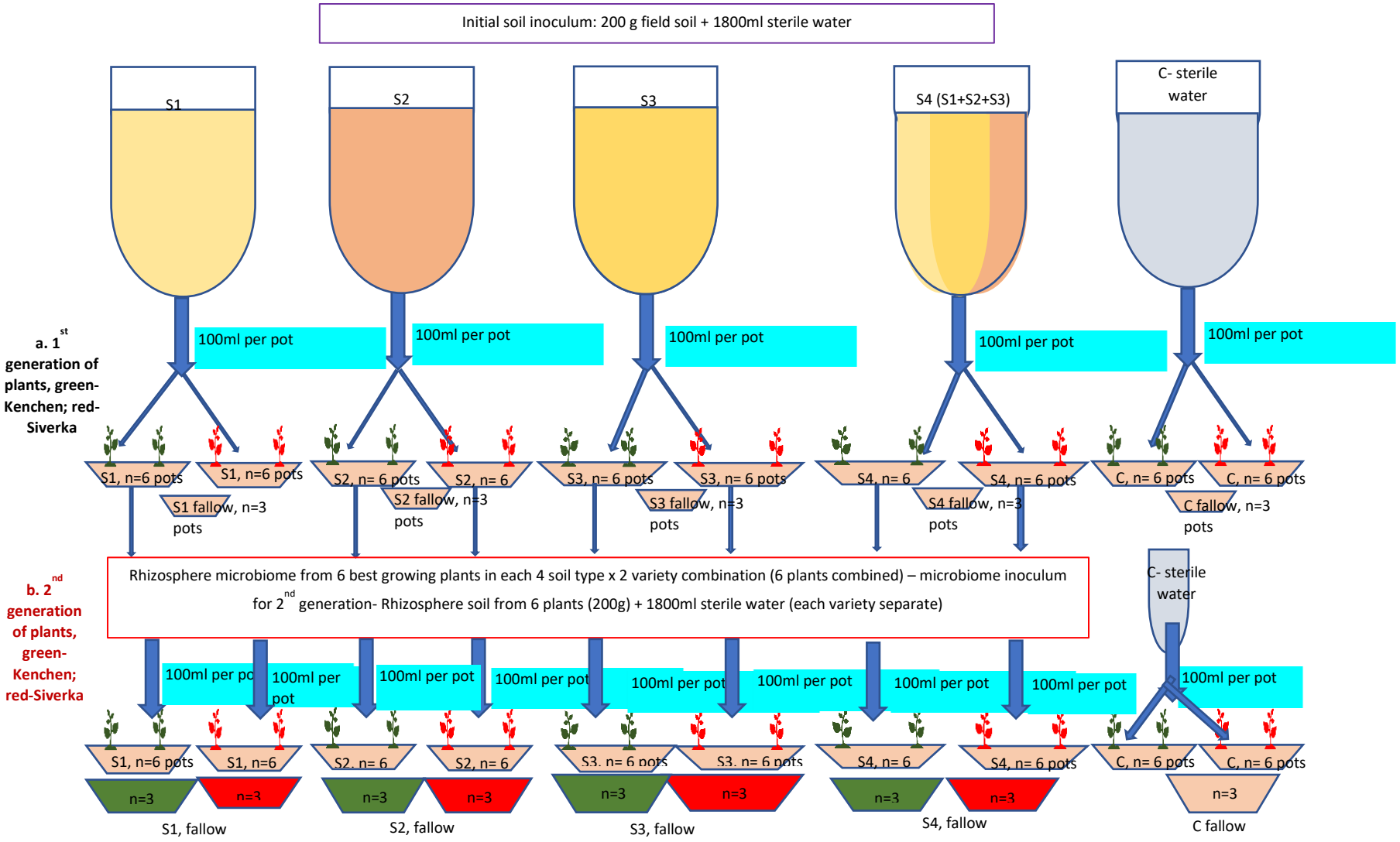


Fig. 2.7 **Flowchart showing preparation of microbiome inoculum and treatments in two generations**: a: inoculum prepared from each field soil, S1(Untilled), S2(Tilled), S3(Legume), S4(Mixed); Each pot received 100ml of soil suspension; each soil type had 12 pots, 6 pots per variety (Kenchen/Siverka), 2 plants per pot; b: Microbiome inoculum for the 2nd generation: prepared from the rhizosphere soil of 1st generation plants selected based on height.

2.5 Collection of root exudate (1st and 2nd generation)

Root exudates were collected from each plant immediately after harvest. The washed roots along with the whole plant was immersed in 35 ml of sterile water in 50 ml falcon tubes and kept on a shaker (Fig. 2.8) for 3 hr to collect root exudates (Kawasaki *et al*., 2018). The tubes were covered using aluminium foil to avoid light affecting the roots. The root exudates were collected in the same glass house condition. Collected root exudates were filter sterilised by passing through 0.22 µm filters (sterile modified acrylic polyethersulfone membrane, Millex®, Sigma, UK) and stored in 50 ml falcon tubes at -20°C until analysis.

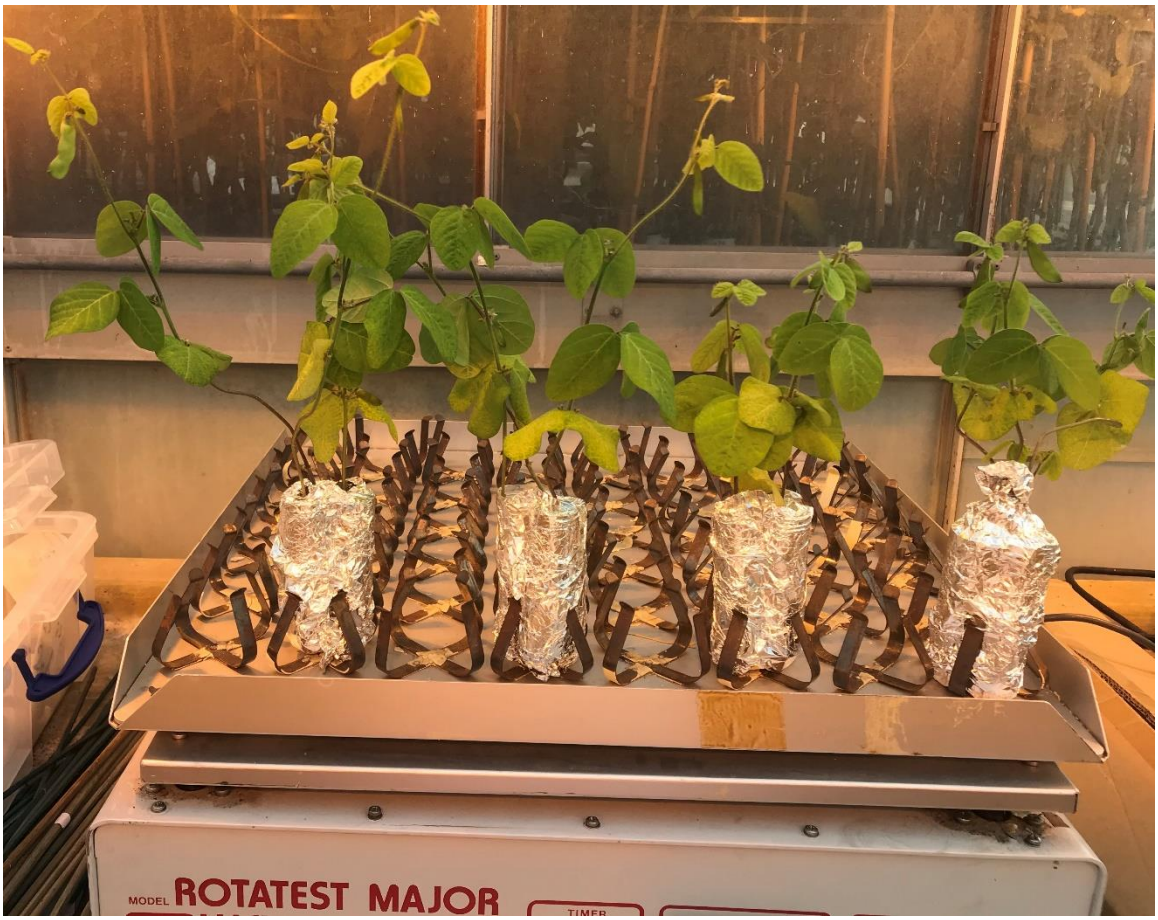


Fig. 2.8 **Root exudate collection**; plants uprooted from soil, after removing rhizosphere soil, washed in sterile water. These plants transferred to 35 ml sterile water in 50 ml sterile falcon tubes, tubes covered with aluminium foil to exclude light; shaken for 2 hr at glass house conditions.

2.6 Plant dry weight (1st and 2nd generation)

Following suspension filtration and exudate collection, roots were blot dried. The roots were separated from stem using a sterile scalpel, placed in 50 ml falcon tubes, and stored at -20°C. The fresh weight of the shoots was measured. The shoots of the plants were then dried separately in oven proof paper bags (7x7 inch) at 70°C for one week and dry weights of the samples were recorded.

2.7 Soil nutrient analysis (1st and 2nd generation)

10 g of rhizosphere soil was mixed with 100 ml distilled water to form a soil suspension and shaken (Gallencamp™) for an hour at 100 rpm. The nutrient contents of rhizosphere soil were measured from the supernatant collected from soil suspension. The supernatant was filtered through Whatman no.1 filter paper to remove soil particles. The filtrate was then passed through 0.45 µm syringe filter (sterile modified acrylic polyethersulfone membrane , Millex®, Sigma, UK). 20 ml of this filtrate samples were transferred to centrifuge tubes and frozen for estimation of total carbon and total organic carbon in soils using TOC analyzer (Shimadzu TOC-L, Global analytical and measuring instruments). The samples were analysed in TOC-L by Anne Dudley in University of Reading, SAGES division. 10 ml of the 0.45 µm filtered samples were stored at 4°C prior to analysis of potassium using Flame photometer (Corning 410). The measurement of the intensity of the light (760 nm wavelength for potassium) that is emitted when a metal is added to the flame is the basis of the flame photometer's working principle. Reagent grade potassium chloride (Sigma Aldrich) solution was used as standard and the concentration of potassium present in samples were calculated using calibration curve prepared from standards. 5 ml of samples were stored in 15 ml falcon tubes for N and P estimation using a flow injection analyser (Skalar San SA1050 autosampler). The samples were analysed using Skalar injection analyser at University of Reading, SAGES division by Marta O'Brien. Skalar's automated method for phosphate analysis was based on the following procedure. The samples were analysed following manufaturer's instructions. The antimony-phospho-molybdate complex was created when the ammonium hepta molybdate and potassium antimony (III) oxide tartrate reacted in an acidic medium with diluted solutions of phosphate. This complex was then reduced by L (+) ascorbic acid to an intensely blue compound that was detected spectrophotometrically at 660 nm. To determine nitrate,

a copperized cadmium column was used to convert nitrate to nitrite, and the nitrite was subsequently diazotized with sulfanilamide followed by coupling with N-(1-naphthyl) ethlyenediamine dihydrochloride and the product's absorbance was measured at 520 nm. The concentration of ammonia was evaluated by heating salicylate and hypochlorite in an alkaline phosphate buffer, and measuring the reaction product's absorbance at 660 nm, which was proportionate to the original ammonia concentration.

2.8 Soil enzyme analysis (1st and 2nd generation)

Soil enzyme analysis was carried out for each rhizosphere soil sample collected as mentioned in the sampling section. The soil microbial activity was assessed by measuring the activity of two extracellular enzymes in the soil. In soil, N-acetyl beta glucosaminidase (NAG) is involved in the recycling of nitrogen (Muruganandam *et al*., 2009) and Phosphatase (PHOS) helps in the recycling of phosphorus (Margalef *et al*., 2017). Both enzymes were measured by fluorometric quantification using 4-methylumbelliferone as a substrate (modified from Saiya-Cork *et al*., 2002). The soil slurries were prepared by weighing 2.75g of moist soil into a Waring blender with 91ml of 50mM sodium acetate buffer solution. The contents were blended at high speed for 1 minute and poured into a glass bowl with a magnetic stir bar and placed on a stir plate at low speed. Stirring gently helps keep the soil particles suspended and minimizes variation between each well. 800ul of the soil slurry was pipetted into each well of a 96 well plate (1.5ml Nunc™ 96-Well Polypropylene DeepWell™ Storage Plates, Thermo Scientific™, Thermo Fisher) using a multi-channel pipette. Soils were organised by columns and each soil type was pipetted into a separate column. Two separate wells were prepared for the measurement of a standard curve and enzyme activity. 200ul of 200uM substrate of 4-Methylumbelliferone (MUB)-N-acetyl- beta-glucosaminidase (NAG substrate) and 4-MUB phosphate (PHOS substrate) were added to assay wells for enzyme activity measurement. Blanks were prepared using 200ul enzyme substrate and 800ul sodium acetate buffer. The standard plates were prepared by pipetting 200ul of different concentrations (0, 2.5, 5, 10, 25, 50 and 100 uM) of 4-MUB standard to soil slurries. The plates were sealed with lids and inverted carefully to mix the contents. Both standard and sample plates were incubated at 25^{o}C for 3 hr. After the incubation period the plates were centrifuged at 1500 rpm for 3 minutes. 250ul of the supernatant was transferred to flat bottomed black 96

well plates (Thermo Scientific™ Nunc™ F96 MicroWell™). Fluorescence was measured using a microplate reader (TECAN SPARK®) at excitation and emission wavelengths of 365 and 450nm, respectively. The standard and sample plates were read at the same gain as per the instructions for TECAN SPARK (difference in gain changes the readings). The concentration of enzymes in the sample was calculated from the standard curve prepared from fluorescence readings from standard plate. The analysis was repeated just before the growth of plants

2.9 Analysis of root exudates (1st and 2nd generation)

Frozen root exudates were lyophilised using a freeze drier (Heto PowerDry Thermo scientific PL3000) until the volume was reduced to 10ml. The pre-frozen samples were transferred to the freeze dryer which consists of a condenser operating at -40°C to -50°C and an acrylic chamber in which the samples were placed and which was then evacuated using a pump to create a vacuum which was necessary for the freeze drying process. This sample was used for the estimation of phenols, sugars, and amino acids. All the estimations were done in triplicates.

2.9.1 Analysis of Phenols

Phenols are aromatic secondary metabolites found in the plant root exudates. Phenols in root exudates were analysed based on the method described in Chantigny *et al*., (2007).

0.4 ml samples of root exudates were placed in 2ml microcentrifuge tubes and volumes made up to 1.4 ml using deionized water. 100 µl of Folin-Ciocalteu's reagent (F9252-100 ml, Sigma-Aldrich) was added to this sample, mixed, and allowed to stand at room temperature for 3 min. 200 µl of saturated sodium carbonate (Na_2CO_3, 216 g in 1 L of deionized water, Sigma-Aldrich) and 300 µl of deionized water were added, and the contents of the tubes were mixed and allowed to stand for 20 min at room temperature. Development of a blue colour strongly indicates the presence of phenols in the sample. The mixture was transferred to 1 cm path length quartz cuvettes and absorbance read at 725 nm using a spectrophotometer (Jenway UV-spectrophotometer, Model 7315). Standard used was 2-hydroxybenzoic acid (salicylic acid, Sigma-Aldrich analytical grade,100 mg L^{-1}) in deionized water. Working standards (0, 2.5, 10, 20, 30, 40 mg L^{-1}) were

prepared from this. Standards were prepared in the same way as samples and absorbance measured at different concentrations. A calibration curve was plotted using readings from standard and phenol concentration in samples were calculated using this. The values are expressed in mg L^{-1} 2 hydroxybenzoic acid equivalent.

2.9.2 Analysis of Sugars

Carbohydrates/ sugars are the primary metabolites secreted by plants through root exudates. Total carbohydrate in root exudate samples was determined by a sulfuric acid method based on Albalasmeh *et al*., (2013).

800 µl of root exudates were pipetted into glass test tubes in racks and volumes made up to 1 ml by adding deionized water. The tubes were moved to a fume cupboard and 3 ml of concentrated sulfuric acid was added to each tube. The samples were vortexed carefully for 30 sec. Each sample was processed separately. Heat proof gloves and safety glasses were used while handling samples. After mixing, the samples were immediately placed in polystyrene boxes containing ice for 2 min and then allowed to equilibrate at room temperature for 30 min. The change in colour of samples to brown indicates the presence of sugars with colour intensity proportional to the sugar concentration. The absorbance was read at 315 nm using a UV spectrophotometer. Standards were prepared using a glucose stock solution (1000 µg ml^{-1}). Standards were treated in the same way as samples and absorbance read at different concentrations (0,20,40,60,80,200 µg ml^{-1}). A plotted calibration curve was used to calculate total carbohydrate concentration in samples and expressed as µg ml^{-1} glucose equivalent. Blank reactions were prepared using 1 ml of soil extract and 3 ml distilled water and used to correct for background UV absorbance.

2.9.3 Estimation of amino acids using gas chromatography- mass spectrometry (GCMS)

2.9.3.1 Sample preparation (EZ:faast method)

Amino acids in root exudate samples were estimated by gas chromatography-mass spectrometry (GCMS) method. The samples were prepared using an EZ:faast (Phenomenex®, USA) kit using the supplied protocol. The EZ: faast amino acid analysis procedure includes solid phase extraction (SPE) of samples followed by

derivatization and liquid/liquid extraction. The derivatized samples were analysed immediately by GC-MS. In SPE, samples were passed through a sorbent tip that binds to amino acids while allowing the interfering compounds to flow through. These amino acids on sorbent were then extruded into sample vials provided in the kit and derivatized. Derivatized amino acids that migrate to organic layer were removed, solvent evaporated and re-suspended in solvent provided and analysed in GC-MS.

100 µl of the sample was pipetted into sample vials (Fig. 2.9, B) provided in the kit. To this equal amount of Reagent 1 (internal standard) was added. In the test run, the concentration of amino acids in sample was found low. So, internal standard was diluted by a factor of 10 (1 part to 10 parts of 0.01M HCl) before adding with sample. A sorbent tip (Fig. 2.9, A) was attached to 1.5 ml syringe and the sample was allowed to pass through the sorbent tip slowly. The syringe was pulled slowly so that it takes a minute for the whole sample to pass through the sorbent tip. Once the whole sample was pulled through the sorbent tip, 200 µl of Reagent 2 was added to the same sample vial and syringe pulled back to let the whole solution pass through the sorbent slowly as before. The accumulated liquid in the syringe was discarded into waste bottles. 200 µl of freshly prepared eluting medium (Reagent 3) was pipetted into the same sample vial. A 0.6 ml syringe, with piston pulled back halfway up the barrel was attached to the sorbent tip used. This was then placed in the sample vial with eluting media. The liquid was allowed to rise slowly through the sorbent particles till it reached the filter plug in the sorbent tip. The liquid along with the sorbent particles was then ejected into the sample vial. Using Drummond dialamatic microdispenser provided in the kit, 50 µl of Reagent 4 was added to the sample vial containing eluted sorbent particles. This is vortexed for 10 sec and allowed to stand for 1 min. To this, 100 µl of Reagent 5 was added carefully using microdispenser, vortexed for 10 seconds and allowed to stand for 1 min. Two layers were formed in the vial, the upper part containing the organic layer was transferred to an autosampler vial with insert using Pasteur pipette. These are then analysed in GC-MS.

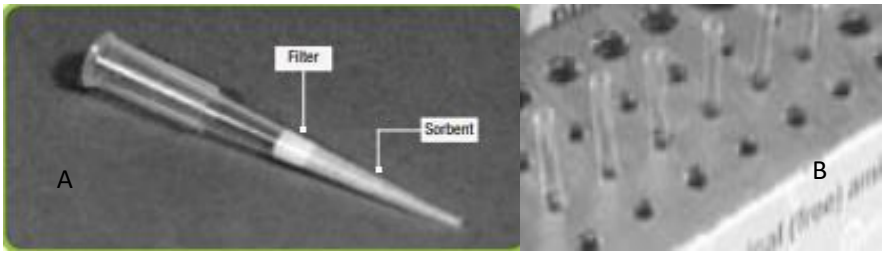


Fig 2.9 **A. Ez: faast sorbent tip; B. sample vials**; these come with the EZ: faast kit.

For quantification, mixture of amino acid standards was prepared in the same way as samples. The EZ: faast kit was provided with 2 standard mixtures, SD1 (23 amino acids, 200 nmoles ml^{-1}) and SD2 (3 amino acids, 200 nmoles ml^{-1}). The given standards were diluted as the concentrations in samples were found very low in test run. On the day of the analysis, diluted multi-standards were prepared as explained below (Table 2.1). Standard solutions required to prepare a calibration curve were prepared from these multi-standards.

Table 2.1 **Preparation of amino acid Multi standard solutions for GC-MS analysis**, these were prepared from the original standards provided in the kit. This was done to get diluted stock of standard solutions.

Standard	Concentration	Preparation method
Multi-standard 2	2 nmol/ 100 µl SD1 +SD2	0.1 ml SD1 + 0.1ml SD2 + 0.8ml 0.01 M HCl
Multi-standard 0.2	0.2 nmol/100 µl SD1 +SD2	0.1 ml multi-standard 2 + 0.9 ml 0.01 M HCl

Combinations of multi-standard solutions required to prepare a calibration curve for all amino acids is given in Table 2.2. Amino acid concentrations in samples are calculated from the calibration curve and values expressed in n moles /100 µl.

Table 2.2 **Combinations of amino acid multi-standard solutions required to prepare calibration curve** for GC-MS analysis; These volumes of multi-standards were added to the vials for solid phase extraction and derivatization as samples.

Standard concentration (nmol)	**Multi-standard 0.2 (volume in µl)**	**Multi-standard 2 (volume in µl)**
0.05	25	
0.1	50	
0.2	100	
0.5		25
1		50
2		100

2.9.3.2 Gas chromatography- mass spectrometry (GC-MS)

A split mode Agilent 7890A GC system (Agilent Technologies, 5975C insert XL EC/CI MSD with triple axis detector) was used to examine 1 µl aliquots. The details of the column, instrument settings and oven conditions are given in the table (Table 2.3) below. The GC- MS run was done by Dr. Stephen Elmore, Department of Food and Nutritional Sciences, University of Reading.

Table 2.3 **Gas Chromatograph- mass spectrometry**: Instrument settings; The table gives the conditions of Gas Chromatography -Mass Spectrometry during the sample run

Column	Capillary column (Zebron- ZB-AAA GC column)	Nominal length: 10.0 m Nominal diameter: 250.0 µm Nominal film thickness: 0.25 µm
Injection	Split ratio 1:10 @ 250°C, 1 µl sample volume with autosampler	
Carrier gas	Helium, 1.1 mL/ minute constant flow	
Oven program	30°C/ minute from 110°C to 310°C	
MS information		
MS source	230°C, maximum 250°C	
MS Quad	150°C, maximum 200°C	
Auxiliary	320°C	
Scan range	Low mass: 40m/z High mass: 380m/z	

2.10 Molecular biology techniques

2.10.1 DNA extraction

Total DNA from soil samples was extracted using a DNeasy PowerSoil Pro kit (QIAGEN) following the manufacturer's protocol (Fig. 10). To increase the DNA yield and quality, step 3 (Fig. 2.10) in the protocol was repeated twice. The extraction was done using 0.3 g of each soil sample after thawing the frozen samples. The soil sample was added to power bead pro tube provided in the kit and homogenised in a Fast Prep (MPBiomedicals) bead-beating system (5.5 m/s for 30 sec twice). The extracted DNA was eluted in 50 µl of sterile nano pure water. DNA concentration was measured using a spectrophotometer/fluorometer (DeNovix® DS-11 series). The extraction was repeated 2 times and the extracted DNA were pooled and stored in 2ml eppendorf tubes at -20^{o}C.

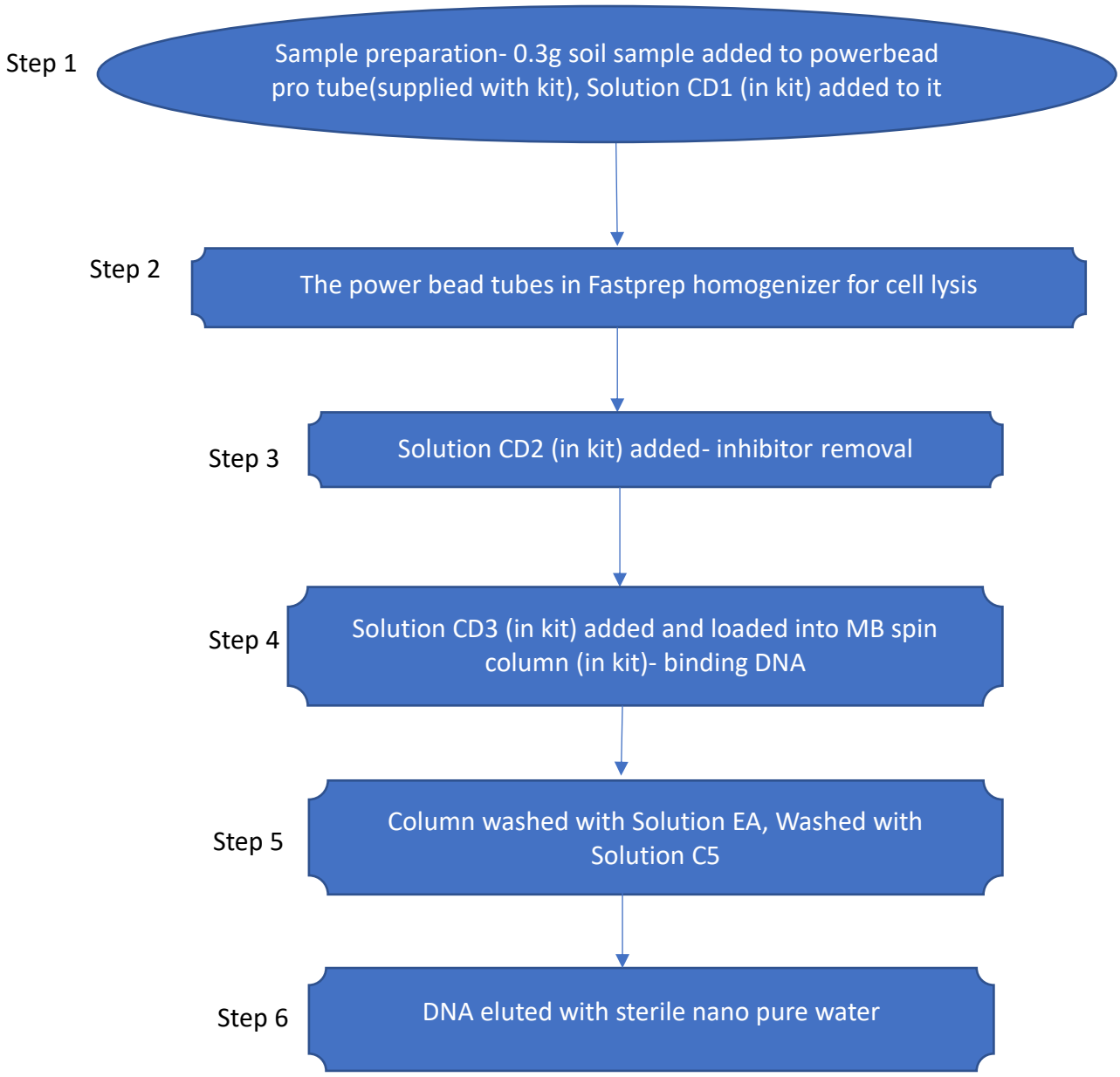


Fig 2.10 **Dneasy PowerSoil Pro kit Protocol, the method used for the extraction of microbial DNA from soils**.

2.10.2 Polymerase chain reaction (PCR) conditions

The polymerase chain reaction (PCR) was used to amplify specific fragments of DNA for use in sequencing reactions. PCRs were carried out in a table-top thermal cycler (Axygen Maxygene II). Standard PCR reactions were performed using Go Taq® Green Master Mix (Promega®). The amplification was carried out using 10 µM of working solution of 16s primers 5F (5'-CCTACGGGAGGCAGCAG-3') and 518R (5'-

ATTACCGCGGCTGCTGG-3'). Conditions for PCR reactions were as follows: initial denaturation of 94°C for 5 minutes followed by 30 cycles of 94°C for 1 minute, 52°C for 1 minute, 72°C for 1 minute; and followed by incubation at 72 °C for ten minutes.

2.10.3 Agarose gel electrophoresis

PCR products were separated by agarose gel electrophoresis. Gels were self-cast using Bioline® Molecular Grade Agarose powder. Dependent on required final concentration (0.4 - 1.5% w/v) agarose powder was dissolved in 0.5X Ambion® TBE buffer (10X solution contains 0.89M Tris, 0.89M Borate, 0.02M EDTA). Biotium Gel Red™ (X10000 in water) was added to a final concentration of 0.1 mg ml-1. PCR samples were loaded and run in a Biorad® gel tank at 40 - 120 mV for the desired amount of time (usually 45 – 90 min). BIOLINE HyperLadder™ 1 was most often run-in tandem with the samples as a DNA band size marker. On completion of the run, DNA bands were visualized under UV-light using G box (Syngene). Once the bands were confirmed, the DNA samples in the eppendorf tubes were sealed and packed in dry ice. These were sent to NOVOGENE, Cambridge, UK for Amplicon Metagenomics Sequencing.

2.10.4 Statistical analysis

All the statistical analyses were carried out using R software (Version 4.1.2, R Core Team, 2021). Data were analysed using linear models. Continuous variables were tested for normality before model fitting, transformations were applied as necessary (Table 4.1). Analysis of variance (ANOVA) was carried out to test for differences in plant height, dry weight, soil nutrients, pH, and enzyme activities between soil types and plant varieties and to study the effect of growth and plant generations on soil enzymes and soil properties. All graphs were produced using `ggpubr` package (version 0.4.0, Kassambara, 2020) and `ggplot2` package (version 3.3.5, Wickham (2016)). A correlation matrix to study the relationship between growth and soil enzymes was based on Spearman correlation coefficients. Principle component analysis (PCA) was used to identify directions (or principal components) along which the variation in the soils is maximal. PCA was done using R package `FactoMineR` (version 2.4, Sebastien et al., 2008) and package ` factoextra`(version 1.0.7, Kassambara and Mundt, 2020).

Table 2.4 Table showing transformed variables used for ANOVA based on linear model

Response variable	Transformation
Height	None
Above ground dry mass (Dry weight)	None
NAG	Cube root
PHOS	None
pH	None
Total carbon (TC)	Log
Available phosphorus (P)	Log
Available potassium (K)	None
Number of nodules	None

Chapter 3. Effect of field management practices on soil properties and enzyme activities

3.1 Introduction

The quality of soil, as a medium for the growth of plants, mainly depends on its physical structure and compaction (Janušauskaite *et al*., 2013). Agricultural practices such as tillage and crop rotation affect soil compaction and influence soil physico-chemical properties with consequences for associated microbial communities. In a field setting, different areas are typically subjected to different management practices. The structure of microbial communities also varies with this space according to management practices. These soil microbial communities can play a major role in nutrient recycling, stress mitigation and detoxification of soil pollutants and thus influence soil quality and in turn plant growth. Use of cover crops, such as legumes and grasses maintain the high nutrient environment even with tillage and results in more diverse microbial community. Crop rotations lead to greater abundance of plant litter (Zak *et al*., 2003) and this can support a greater number of microbial decomposers (Hooper *et al*., 2000). To improve the productivity from our existing farming systems, it is important to understand, how different farming practices influence the microbial communities and how these communities can be manipulated to suit our needs.

3.1.1 Importance of soil physico-chemical properties on plant growth and microbial activity

To understand the suitability of specific land for agricultural purposes, it is important to know the composition of soil at that site. The physical properties of soil such as texture, influence the amount of water, air, and nutrients available to plants grown in that soil. The microbial diversity in soil depends on the heterogeneity of soil particles and their structural arrangement (Or *et al*., 2007). This is because, the transport of metabolites for the microbes are controlled by diffusion through gas and liquid phases in soil. More heterogeneity in soil structure causes variations in the distribution of water and nutrients which impacts microbial activity and diversity (Curd *et al*., 2018). Seaton *et al*., 2020, found that soil physical

environment can influence the soil microbiota in different ways. Chemical properties of soil like pH, organic matter content and nutrient content influence the carbon storage capacity of soils and are associated with the activity of nutrient recycling enzymes and microbial activity (Li *et al*., 2020). These physical and chemical properties of the soil interact in a complex way to give the soil its quality, which is important to support the growth of plants. These parameters are thus used as indicators of soil quality, and they are influenced by land management practices.

3.1.2 Importance of cropping history and management practices on soil quality

Hiltner (1904) coined the term `Rhizosphere` to describe the narrow region of soil layer around the roots that is influenced by the chemicals released from plant roots and which is occupied by unique communities of microorganisms. Even within the same field, rhizosphere microbial community was found to be varying with different plant species or cultivars (Berg and Smalla, 2009). Again, cropping history and management practices were shown to influence this community (Costa *et al*., 2006). The main reasons for practicing crop rotation in agricultural settings is to improve soil fertility, disrupt pest cycles and maintain soil structure. For example, potato beetles lay eggs in soil and if this soil is used next year for planting potatoes, there will be a huge loss for the farmer. Crop rotation with any non-host grain crop like rye or wheat was found to significantly reduce the infection (Wright, 1984). Many of these functions are mediated by microbes. Leguminous crops are commonly used in crop rotation and their positive effects on soil health are mainly attributed to their ability to form associations with Nitrogen-fixing Rhizobium species in soil which increases the N content in the soil (Peoples *et al*., 2009).

Like crop rotation, practices like tillage also bring about significant changes in the soil environment. Tillage practices can alter the soil physical and chemical conditions that can result in shifts of soil microbial community structure. It was found that conventional tillage (exposes the soil's surface and loosens soil particles, leaving them open to erosion from wind and water) increases the abundance of aerobic soil microbial communities whereas conservative tillage tillage (a system of low tillage that leaves enough crop residue to cover the soil's surface) and no-tillage (soil is not turned over and the leftovers are left on top of the soil) increases microbial diversity and activity in soil (Mathew *et al*., 2012).

Soil enzymes play important roles in nutrient cycling and are considered as indicators of soil quality. These extracellular enzymes secreted by microorganisms into the soil matrix play a role in the degradation of polymers like cellulose, chitin, lignin, and proteins which release N, P and S. Soil enzymes are described as useful tools in predicting ecosystem functions. Apart from being easy to measure, soil enzymes are described as "biological fingerprints" of past soil management practices, and their activity is a consequence of varying soil tillage and structure (Bandick and Dick, 1999, Utobo and Tewari, 2015). In this chapter, the main objective is to understand how differences in cultural practices such as levels of tillage and field cropping history has affected the soil properties and extracellular enzyme activities. These soil samples collected will be used as microbial inoculum for the further studies explained in next three chapters.

In this chapter, I aim to

1. Study the difference in physical (soil texture and water holding capacity) and chemical properties (pH, soil moisture and nutrient content) of soil samples collected from field sites differing in cultivation practices (untilled, tilled and legume grown soils).
2. Study the difference in the activity of N-acetyl beta glucosaminidase (NAG) and phosphatase (PHOS) enzymes in soil samples collected from the same sites. In earlier field studies, it was found that simplified tillage system significantly stimulated the activity of the soil enzymes irrespective of the soil type. Based on this I hypothesised that both NAG and PHOS activity will be high in untilled soil.

3.2 Methodology

The soil sampling details are explained in the Chapter 2, section 2.1.

3.2.1 Soil characterization

The soil samples were analysed for their texture, pH, moisture content, water holding capacity, organic matter, carbon, nitrogen, phosphorus, and potassium content. All the tests were conducted using standard methodologies at Soil Research Division, University of Reading (SAGES). The analysis of nitrate and ammonium content was done on fresh soil, one month after soil sampling. Air drying was found to decrease nitrate nitrogen in soil and will not give reliable results (Li, 2012). The soil samples were air dried in oven at 40^{o}C and sieved through 2mm sieve for all other analyses. pH and water holding capacity were measured using 5 replicates per soil sample and rest of the analysis were performed using 9 replicates per soil sample.

3.2.1.1 pH

10g of air-dried 2mm sieved soil was mixed with 25ml deionised water in a 50ml falcon tube and mixed on a rotary shaker (Stuart SB3), 100rpm for 15 minutes. The pH meter (Jenway pH meter 3310) was calibrated using standard pH buffer solutions of pH 4 and 7 before taking the sample readings.

3.2.1.2 Soil texture

Air dried, 2mm sieved soils were used to estimate soil texture. The particle size was measured using laser beam diffraction on a soil sample suspended in water. A sample was prepared by gently disaggregating a small amount (2 g) of soil by hand, using a rubber tool and sodium hexametaphosphate solution (5% calgon) to break up the soil. This was then added to the water tank on the mastersizer and passed to the measurement cell. Two lasers (red and blue) were shone through the suspension and the diffraction measured on photocells surrounding the measurement cell. The measurement was done in SAGES lab using Mastersizer particle size analyser (Malvern instruments Ltd.).

3.2.1.3 Soil water holding capacity

50g of air dried, 2mm sieved soil was placed in a plastic cylinder with wire mesh on the bottom. The containers were placed in a dish of tapwater for approximately 12 hours, to allow for maximal soil saturation. Cylinders were removed from water and the tops sealed with cling film to reduce evaporation.

The containers were then suspended using retort stand to drain overnight. Approximately half of the wet soil from each container was placed in a pre-weighed aluminium tray. The weight of the wet soil + dish was noted, and the dishes were placed in an oven at 105°C for 24 hours. The weight of the dish with oven dry soil was measured after cooling in a desiccator. The water holding capacity is expressed as grams per 100g oven dry soil.

3.2.1.4 Soil moisture and loss on ignition for organic matter content

Analysis followed the procedure described by Davies (1974): clay crucibles of known weight containing 10 g soil were incubated at 105°C overnight and re-weighed to determine soil dry weight (grams). The moisture content is expressed as g water per 100g oven- dry soil. This soil was further subjected to overnight incubation at 500°C in a furnace to determine the soil organic matter content by loss on ignition (g g^{-1} dry weight soil). Samples were then placed in a desiccator with silica beads to cool for 20 minutes before weighing.

3.2.1.5 Available Nitrate and Ammonium

40g fresh, 5mm sieved soil was mixed with 200ml 1M potassium chloride solution in a 500ml shaking bottle and placed on a horizontal shaker (Gallencamp™) at 100rpm for 1 hour. The soil suspension was then passed through a GF/A 15cm filter paper to collect the extracts. The nitrate and ammonia in the extracts were measured colorimetrically using a Flow injection analyser (Skalar San SA1050 autosampler system, Skalar, Netherlands). Repeat analyses were conducted 3 months after sampling and just before the growth of plants (explained in chapter 2).

3.2.1.6 Available Phosphorus

Available P was quantified following the procedure described by Olsen *et al*., (1954): Sodium bicarbonate ($NaHCO_3$) (pH 8.5) was prepared by adding 420 g of analytical grade $NaHCO_3$ with 0.05% polyacrylamide solution and volume made upto 10 L using ultra pure water and pH adjusted to 8.5 using 50% sodium hydroxide solution. 100 ml Sodium bicarbonate ($NaHCO_3$) (pH 8.5) was added to 5 g of air-dried, 2mm sieved soil in 150 ml wide necked plastic shaking bottle and samples were loaded on a rotary shaker at 100 rpm for 30 min. The suspension was then filtered through Whatman no. 2 filter paper (pore size: 8 µm) and

the filtrate stored in 100 ml narrow necked bottles, overnight at 5°C prior to analysis for phosphate using an autoanalyzer (Skalar San SA1050 autosampler system, Skalar, Netherlands).

3.2.1.7 Available Potassium

10 g of air dried 2 mm sieved soil was placed in a 50 ml centrifuge tube and 25 ml of 1 mol litre^{-1} of ammonium nitrate (NH_4NO_3) was added to this. The tube was placed on a shaker for 2 hours and then centrifuged at 3600rpm for 10 minutes. The supernatant was filtered using Whatman no. 540 filter paper and the filtrate was analysed for potassium using a Perkin Elmer Inductively coupled plasma- Optical Emission Spectrometer (3000 ICP-OES Perkin Elmer Optima). The stock multi element standard solution IV (1000 mg L^{-1}) containing Ag, Al, B, Ba, Bi, Ca, Cd, Co, Cr, Cu, Fe, Ga, In, K, Li, Mg, Mn, Na, Ni, Pb, Sr, Tl and Zn was used to prepare the standards for calibration curve to find the concentration of K in samples.

3.2.1.8 Total Carbon and Nitrogen

Concentrations of carbon and nitrogen in the soil was estimated using a Thermo FLASH 2000 analyser. The oven dried soil was ground to a fine powder using a pestle and mortar. The sample tray (96 well plate) was prepared by weighing 10mg of soil into foil cups. The cups were folded using forceps making sure to remove any trapped air before placing in the sample tray. Aspartic acid standards (1mg and 3mg; 10.52% nitrogen ,36.09% Carbon) were also placed in aluminium foil cups in the tray. A quality control material (certified reference material, GBW 07412, soil with known % C and N; 0.33% nitrogen, 3.52 % carbon) was also analysed along with samples and standard. The foil cups were loaded into the autoanalyzer of the Thermo FLASH 2000 NC analyser. The % C and % N were determined from the standard graph. This value was then used to calculate the C:N ratio of each soil sample.

3.2.2 Soil enzyme estimation

The method is detailed in section 2.8.

3.2.3 Statistical analysis

The statistical analyses were performed using R software (Version 4.1.2, R Core Team, 2021). Differences in pH, water holding capacity, moisture content and nutrient content between soil types were analysed using One-way ANOVAs and the level of significance was measured using Tukey HSD test at 95% confidence

level. Linear regression models were used to test for relationships between soil enzymatic activity and soil carbon and organic matter content.

3.3 Results

The study was conducted to understand the effect of differences in cultural practices such as levels of tillage and field cropping history on soil properties and extracellular enzyme activities.

3.3.1 Soil characterization

The physical and chemical properties of the soils studied are summarised in the Table 3.1.

3.3.1.1 pH

The difference in pH were statistically significant between soils (Fig. 3.1A); $F(2) = 1722.1$, $p < 0.001$). The mean pH of untilled soil was 5.45, tilled soil was 6.03 and legume soil had a pH of 5.64.

3.3.1.2 Soil texture

The particle size was measured in phi units. The data was then grouped according to particle size as clay, sand, and silt (Fig. 3.1B) and each soil is classified into textural class based on USDA (USDA, 1987) classification. The details of the soil texture analysis are given in the table below (Table 3.2). Untilled and legume soil were of similar texture.

3.3.1.3 Soil Water holding capacity

The water holding capacities varied significantly ($F(2) = 38.20$, $p<0.001$) between three soils (Fig. 3.1C). The water holding capacity was significantly higher for untilled soil as compared to tilled and legume soils.

Table 3.1 Physical and chemical properties of three soil types, S1(Untilled), S2(Tilled) and S3(Legume), mean values with ± standard error (se).

Soil type	Soil texture	pH	Percentage moisture	Percentage water holding capacity	Percentage organic matter	Ammonium (mgkg-1)	Nitrate (mgkg-1)	Phosphorus (mgkg-1)	Potassium (mgkg-1)	Percentage total carbon
Untilled(S1)	Sandy loam	5.45±0.01	30.85±0.005	35.43±1.62	6.46±0.05	2.04±0.05	27.4±2.16	68.28±0.98	463.52±5.32	2.799±0.08
Tilled(S2)	Silty loam	6.03±0.01	32.55±0.005	34.31±1.62	5.23±0.05	1.01±0.05	12.4±2.16	47.83±0.98	76.63±5.32	2.154±0.08
Legume(S3)	Sandy loam	5.64±0.01	16.30±0.005	21.45±1.62	2.73±0.05	1.01±0.05	14.4±2.16	23.32±0.98	101.46±5.32	0.918±0.08

Table 3.2 Soil texture analysis showing percentage of sand, silt and clay in each soil type. S1- untilled soil, S2- tilled soil, S3- Legume soil

Sample	**Texture**	**Sand%**	**Silt%**	**Clay%**
Untilled (S1)	Sandy loam	50.13	49.66	0.22
Tilled (S2)	Silty loam	33.33	65.79	0.88
Legume (S3)	Sandy loam	51.36	47.49	1.15

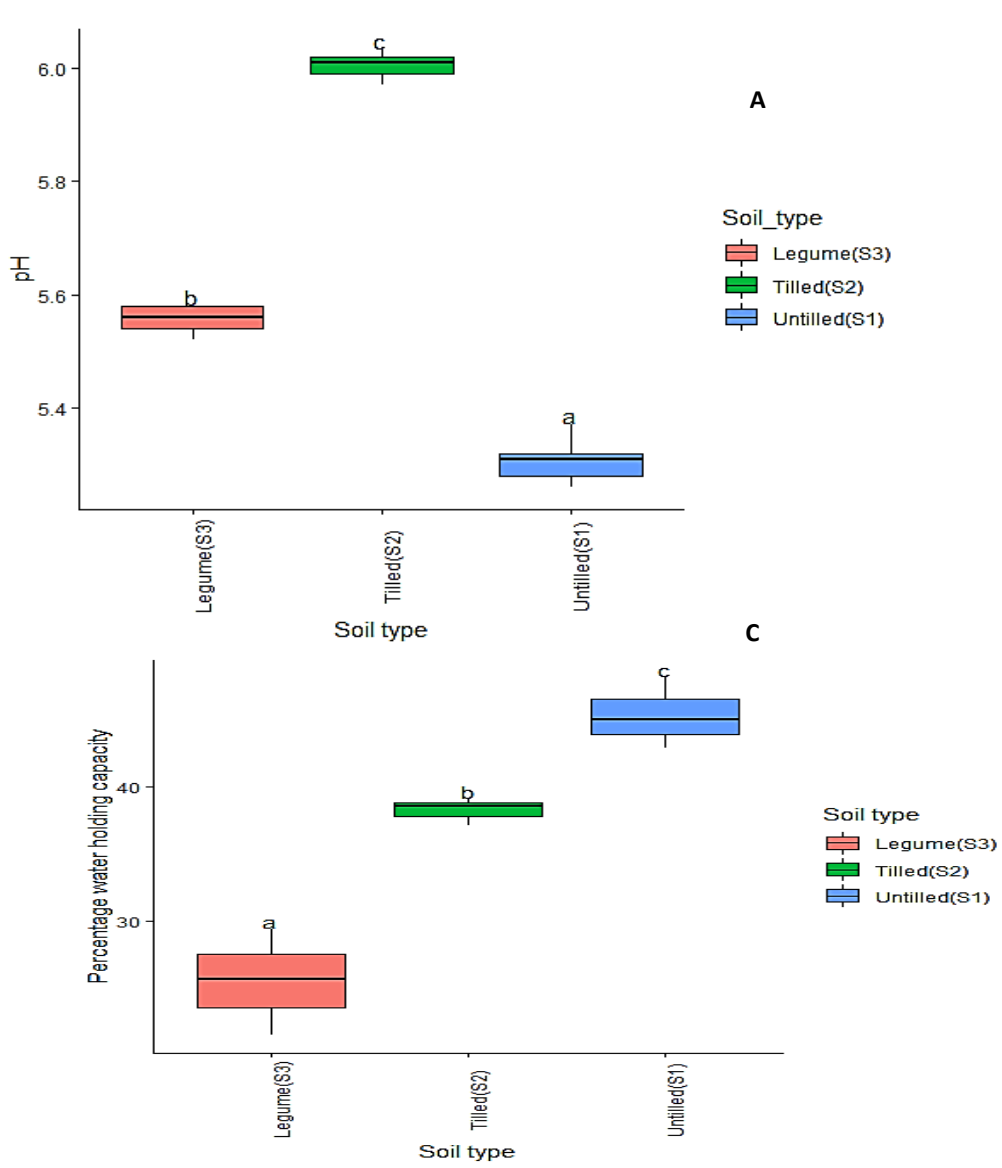


Fig. 3.1 **Physical characteristics of initial field soils; A. pH content of soils. B. Soil texture- percent volume of sand, silt and clay content in each soil; C. Percentage water holding capacity of soils**. S1-Untilled, S2- Tilled, S3- Legume grown. Letters, a,b,c= Tukey method for comparing a family of 3 estimates; significance level used: alpha = 0.05 , n=5

3.3.1.4 *Soil moisture and organic matter*

The percentage moisture was high for untilled soil as compared to other two, tilled and legume soil. The organic matter content was significantly higher for untilled soil as compared to other two soils (Fig. 3.2). All the three soils significantly ($F(2) = 1084.4$, $p < 0.001$) varied between each other in their percentage organic matter. The organic matter percentage measured in untilled soil was 6.46 as compared to tilled and legume soils.

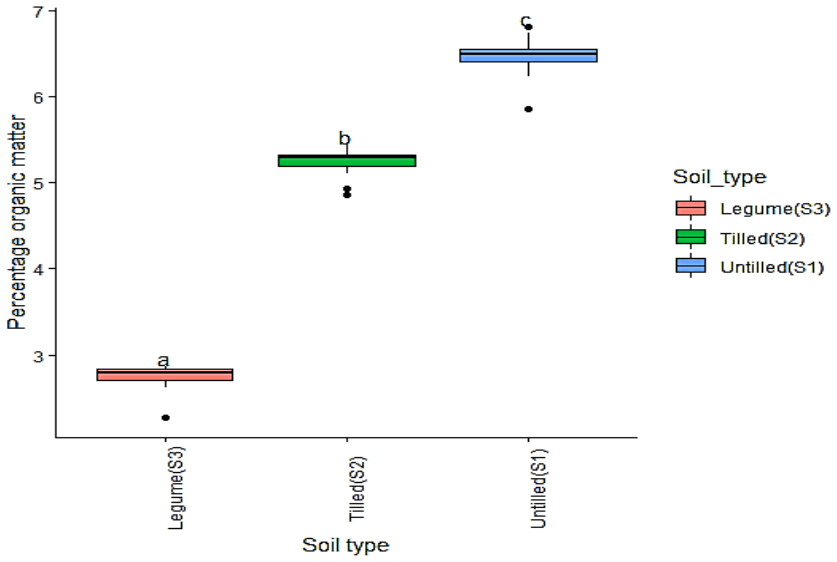


Fig 3.2 **Percentage organic matter in Initial field soils**. S1-Untilled soil, S2- Tilled soil, S3- Legume grown soil. a, b, c= Tukey method for comparing a family of 3 estimates; significance level used: alpha = 0.05, n= 9

3.3.1.5 *Available ammonium and nitrate content*

There was a significant influence of soil type on available ammonium ($F(2) = 29.99$, $p<0.001$) and nitrate ($F(2) = 83.96$, $p<0.001$) content in soils. The difference was significant for nitrate for all the 3 soils, whereas there was no significant difference ($P=0.97$) in means between legume soil and tilled soil in ammonium content. The untilled soil was high in both nitrate (Fig. 3.3A) and ammonium (Fig. 3.3B).

3.3.1.6 *Available Phosphorus*

Soil type had a significant effect ($F(2) = 522.47$, $p < 0.001$) on available phosphorus content in soil. The available phosphorus was high in untilled soils followed by tilled and legume soils (Fig. 3.3D).

3.3.1.7 Available Potassium

There was significant influence of soil on potassium content ($F(2) = 4898.7$, $p<0.001$). The highest available potassium content was recorded for untilled soil (Fig. 3.3C). The soil with legumes had significantly high potassium ($P=0.03$) than tilled soil.

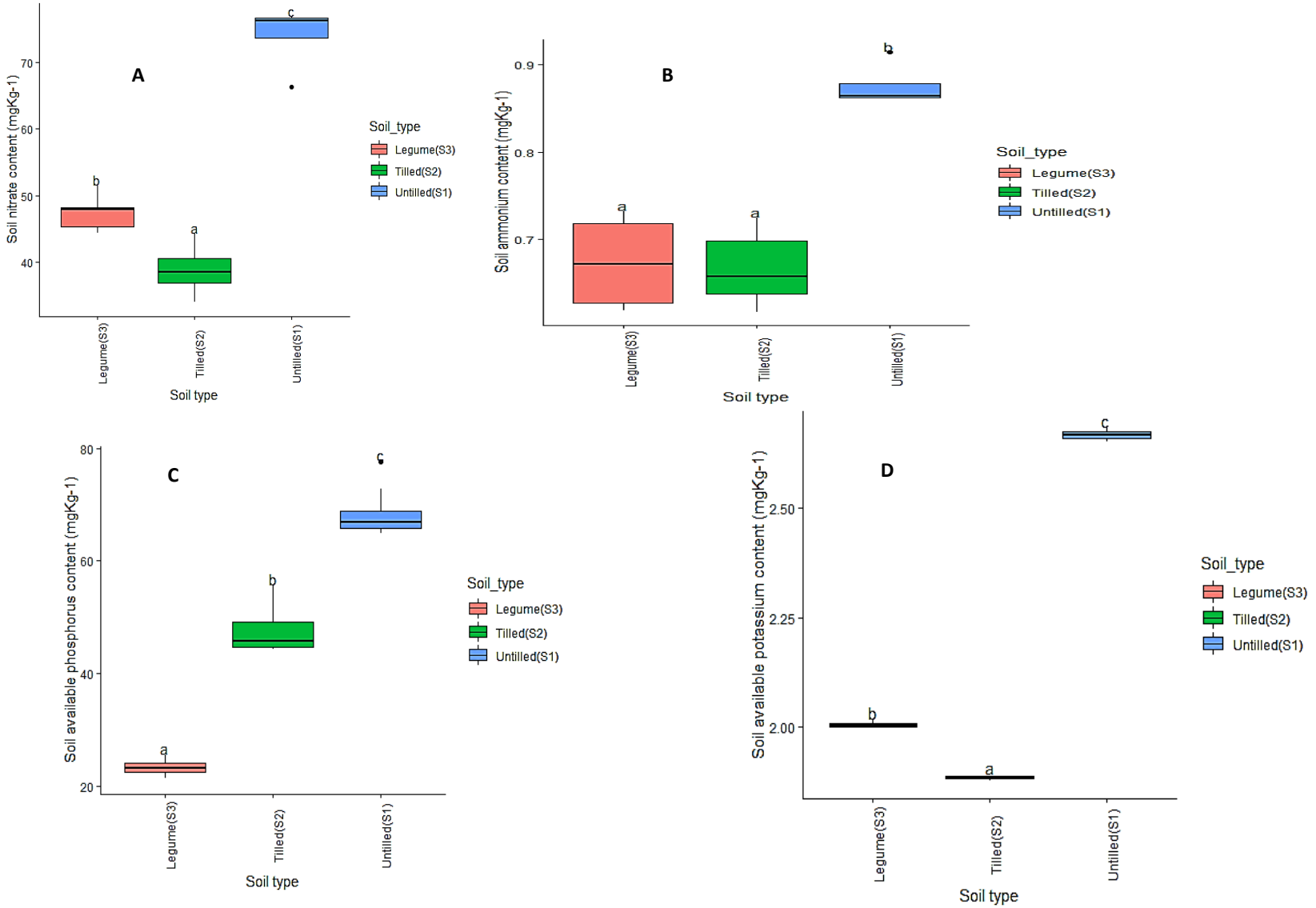


Fig. 3.3 **Soil nutrient content in Initial field soils**. **A. Soil nitrate content (mg per Kilo gram fresh soil); B. Soil ammonium content (mg per Kilo gram fresh soil); C. Available potassium content (mg per Kilo gram oven dried soil); D. Available phosphorus content (mg per Kilo gram oven dried soil);** S1-Untilled, S2-Tilled, S3-Legume soil. a, b, c= Tukey method for comparing a family of 3 estimates; significance level used: alpha = 0.05, n=9

3.3.1.8 Total Carbon (TC) and Nitrogen (TN)

The C:N ratio is the mass of carbon relative to the mass of nitrogen in a substance. This is an important factor affecting residue decomposition in soil (USDA, 2011). The TC ($F(2) = 125.05$, $p<0.001$) and TN ($F(2) = 112.65$, $p<0.001$) differed significantly for the soils. Both TC and TN was high in untilled soil followed by tilled and legume grown soils (Fig. 3.4). The C: N value ranges between 9:1 (Legume grown soil) and 11:1 (Untilled soil) for the soils.

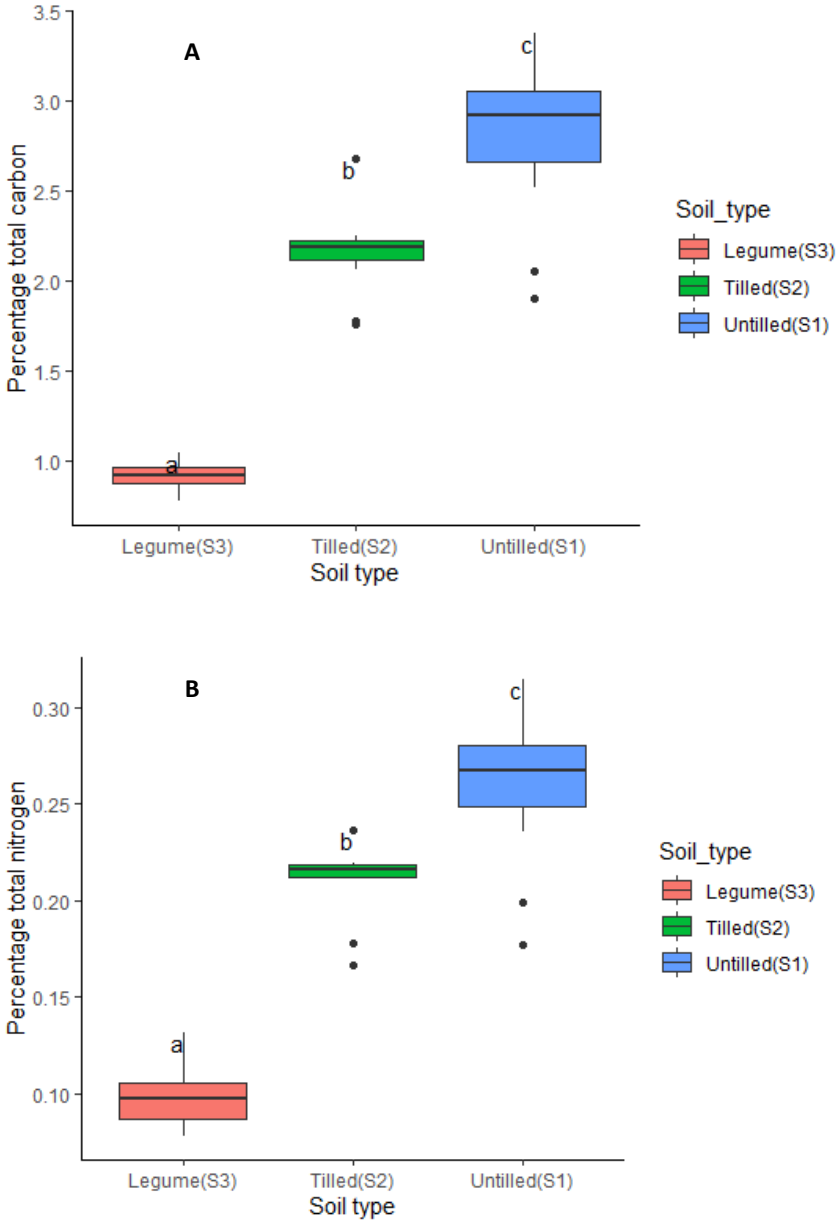


Fig. 3.4 **A. Total percentage carbon and B. Total percentage nitrogen content in Initial field soils (% in oven dried soil)** S1-Untilled, S2-Tilled, S3-Legume. S1-Untilled soil, S2- Tilled soil, S3- Legume grown soil. a, b, c= Tukey method for comparing a family of 3 estimates; significance level used: alpha = 0.05

3.3.2 Difference in Ammonium and nitrate content of soils during storage

Ammonium and nitrate content in soils are always analysed from fresh soils. The low temperature storage was found to stimulate soil mineralization and nitrogen forms in the refrigerated and frozen storage soil samples were found significantly higher than those in the fresh samples (Wu *et al*., 2018). The soils collected from field was stored at 4^{o}C until further use. So, it was necessary to understand the change in ammonium and nitrate content in soils during storage. The changes in the ammonium and nitrate content during storage is shown in Figures 3.5 and 3.6. Sampling time had a significant effect on ammonium and nitrate content in soils. The ammonium content tended to decline during storage in all the three soils. The decline was more prominent in untilled soil. In tilled soil, the decrease in ammonium content was significant in all the three sampling times.

The nitrate content increased over time upon storage in all the three soils. The increase was significant in all three soils.

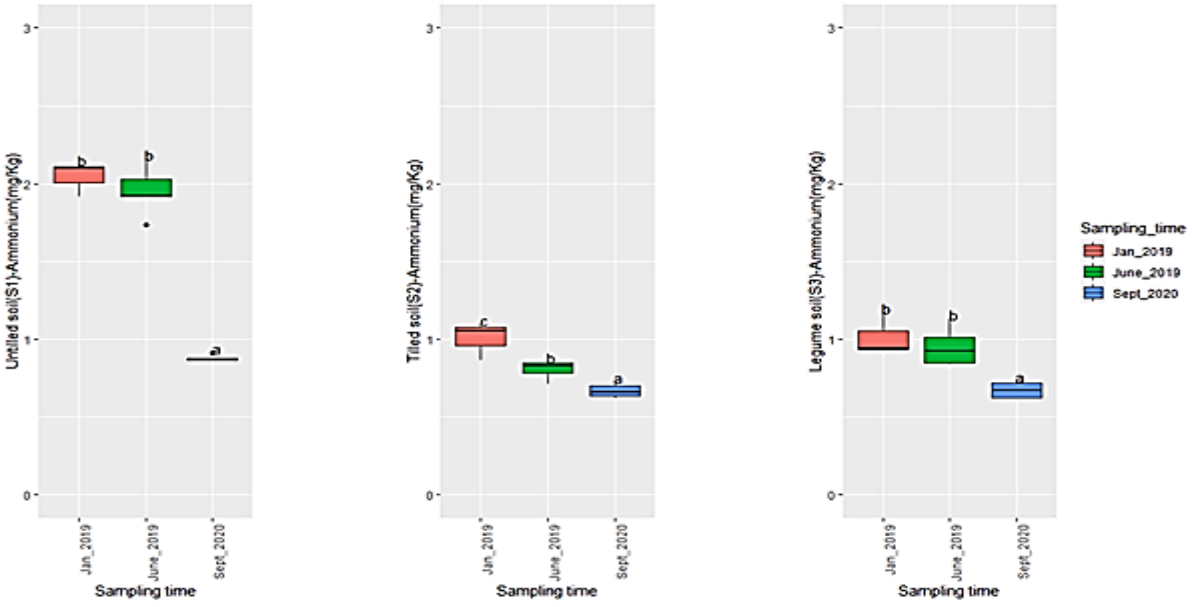


Fig. 3.5 - **Changes in available ammonium content of Initial field soils (mg per Kilo gram fresh soil) upon storage**. S1-Untilled soil, S2-Tilled soil, S3- Legume grown soil. a, b, c= Tukey method for comparing a family of 3 estimates; significance level used: alpha = 0.05; Sampling times: January 2019, June 2019 and September 2020

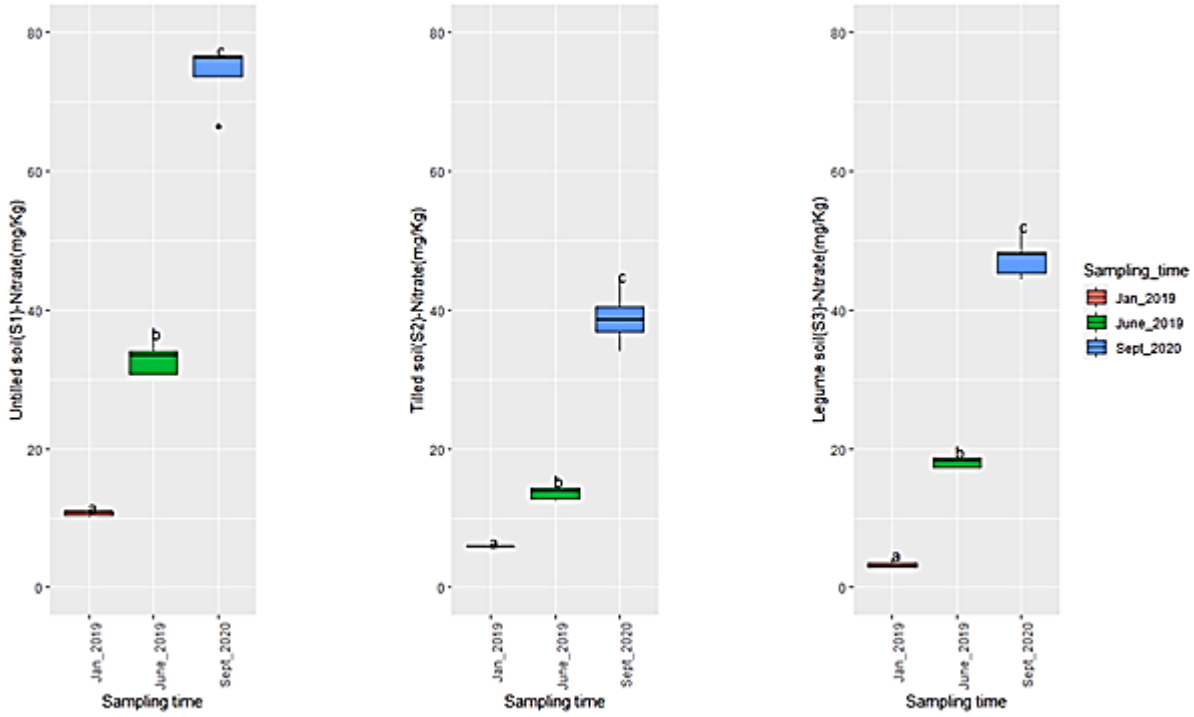


Fig. 3.6 **Changes in available nitrate content in Initial field soils (mg per Kilo gram fresh soil) upon storage**. S1-Untilled soil, S2- Tilled soil, S3- Legume grown soil. a, b, c= Tukey method for comparing a family of 3 estimates; significance level used: alpha = 0.05; Sampling times: January 2019, June 2019 and September 2020

3.3.3 Soil enzymes

The activity of N-acetyl beta glucosaminidase (NAG, F (2) = 21.47, p<0.001) was significantly different between soils. NAG was found significantly higher in untilled soil than in tilled and legume soils. Tilled and legume grown soil did not vary significantly in NAG activity (Fig. 3.7A). 47% of the variance in NAG activity is explained by organic matter content, total carbon content in soil and total nitogen in soil (Fig. 3.8). A decrease in NAG activity was noticed in all the three soils during storage (Fig. 3.10). The difference in activity was significant between 2 time points (6 months after soil sampling and 1 year after soil sampling).

The activity of phosphatase (PHOS) enzyme was significantly different between soils (F (2) = 151.8, p<0.001) with highest for untilled soil followed by tilled soil. The lowest activity of PHOS was measured in legume grown soil (Fig. 3.7B). Total carbon and organic matter content in soil could explain 93.7% of the variation

observed in phosphatase activity in this study (Fig. 3.9). Phosphatase activity decreased during storage for all the three soils (Fig. 3.11). The decline was significant between time points.

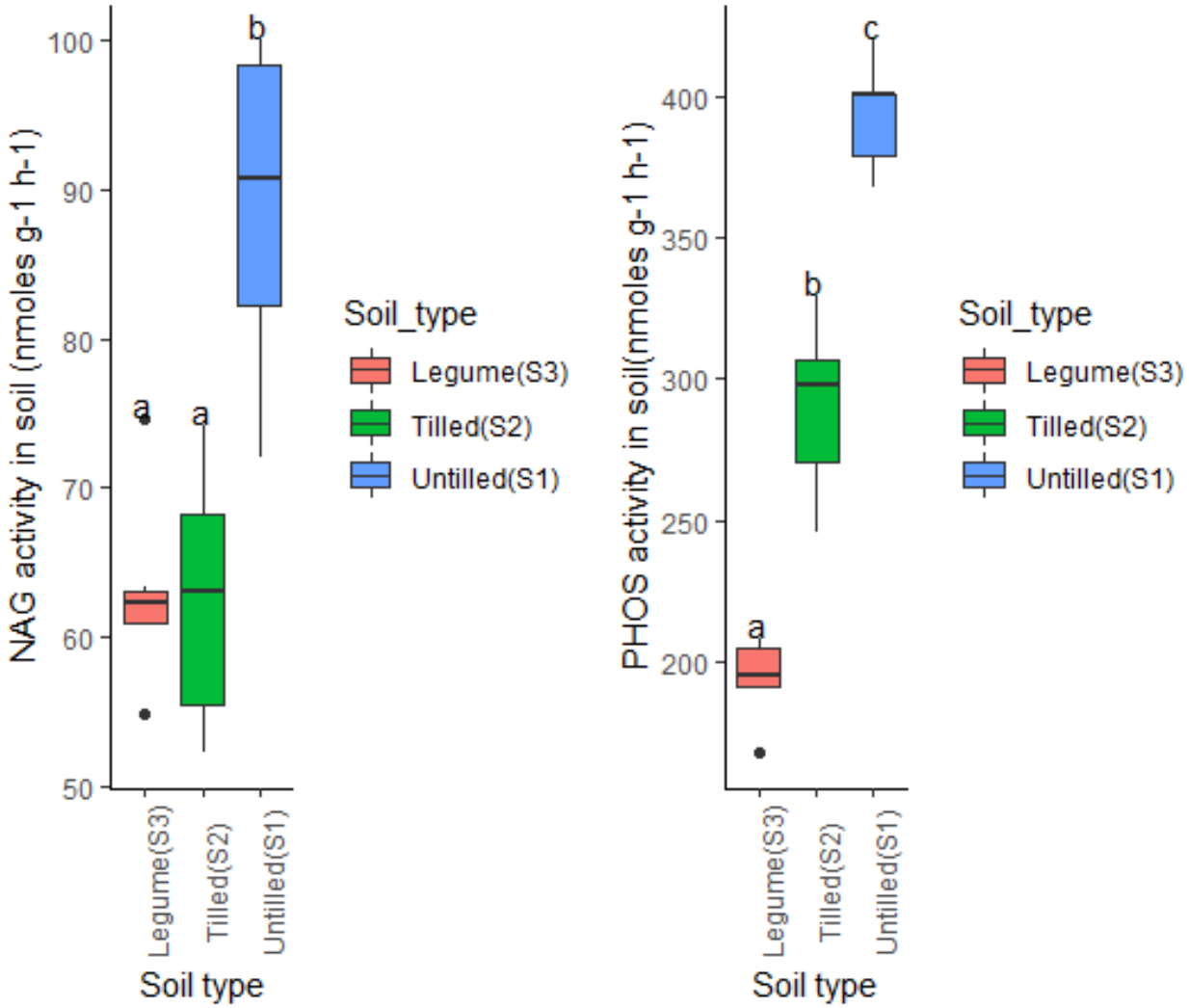


Fig. 3.7 **Soil enzyme activity in Initial field soils: A. NAG (N-acetyl beta glucosaminidase) and B. PHOS (Phosphatase) activity in soils (nmoles/gsoil/hour)** S1-Untilled, S2-Tilled, S3-Legume. a,b,c= Tukey method for comparing a family of 3 estimates; significance level used: alpha = 0.05 , n=9

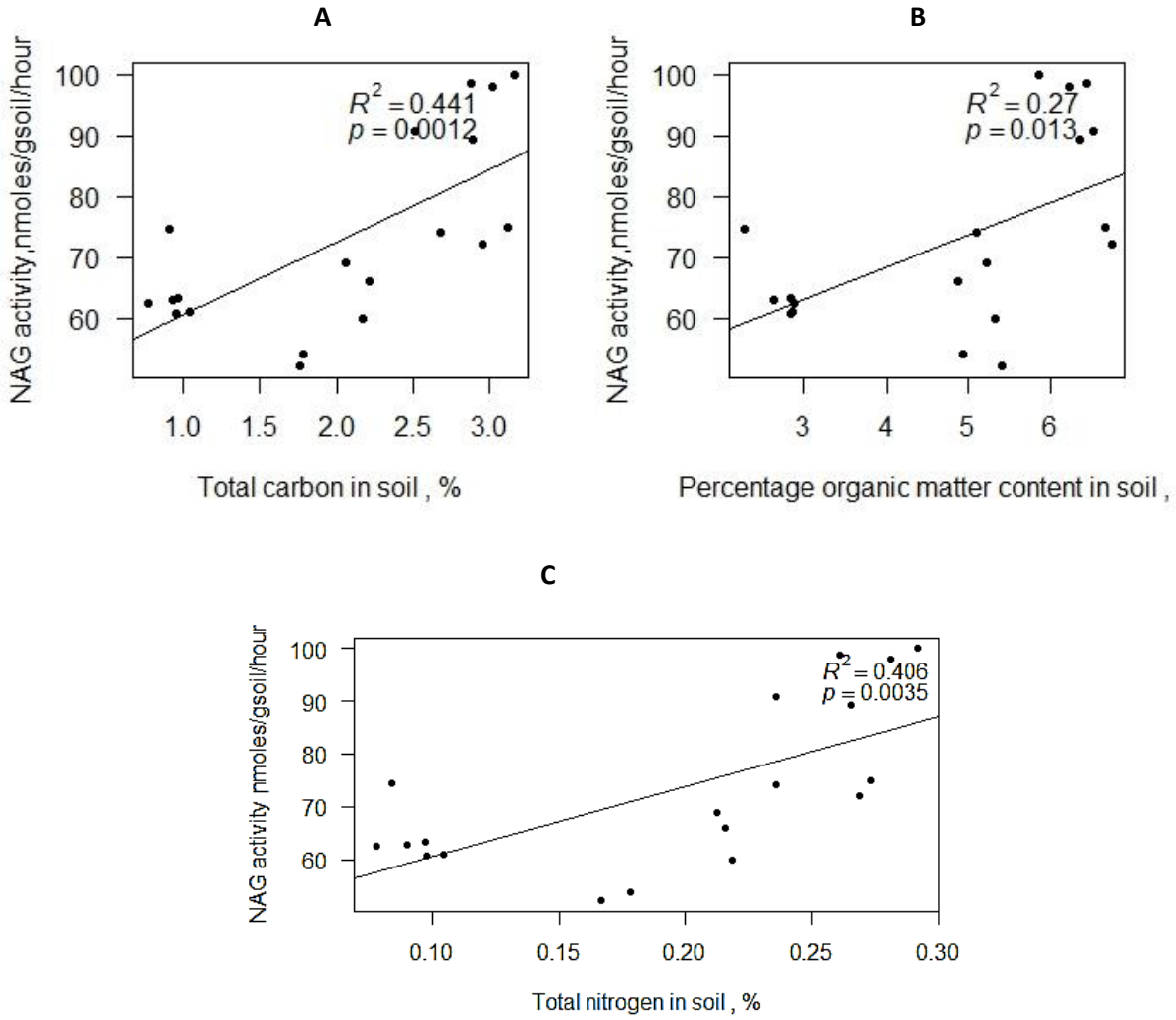


Fig. 3.8 **Simple linear regression plot showing linear relationship between soil enzyme, NAG (N-acetyl beta glucosaminidase) with (A) soil carbon (B) total organic matter in Initial field soil and (C) total nitrogen in soil.** P value indicates the level of significance. R^2 value indicates the percentage of variation explained by the variable. Low R^2 value indicates that the rest of the variation can be explained by adding other independent variables in multiple regression model.

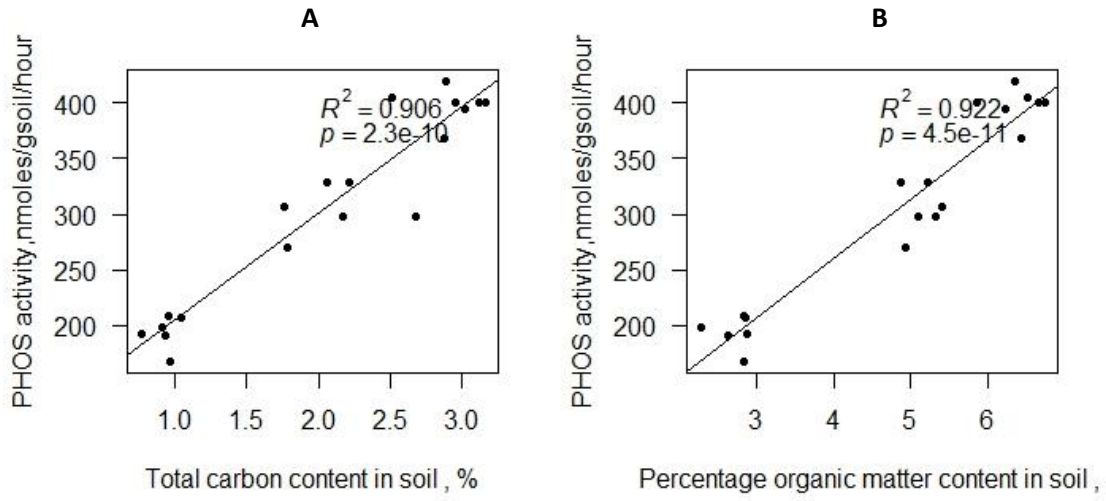


Fig. 3.9 **Simple linear regression plot showing linear relationship between soil enzyme, PHOS (Phosphatase) with (a) soil carbon and (b) total organic matter in Initial field soils.** P value indicates the level of significance. R^2 value indicates the percentage of variation explained by the variable. Low R^2 value indicates that the rest of the variation can be explained by adding other independent variables in multiple regression model.

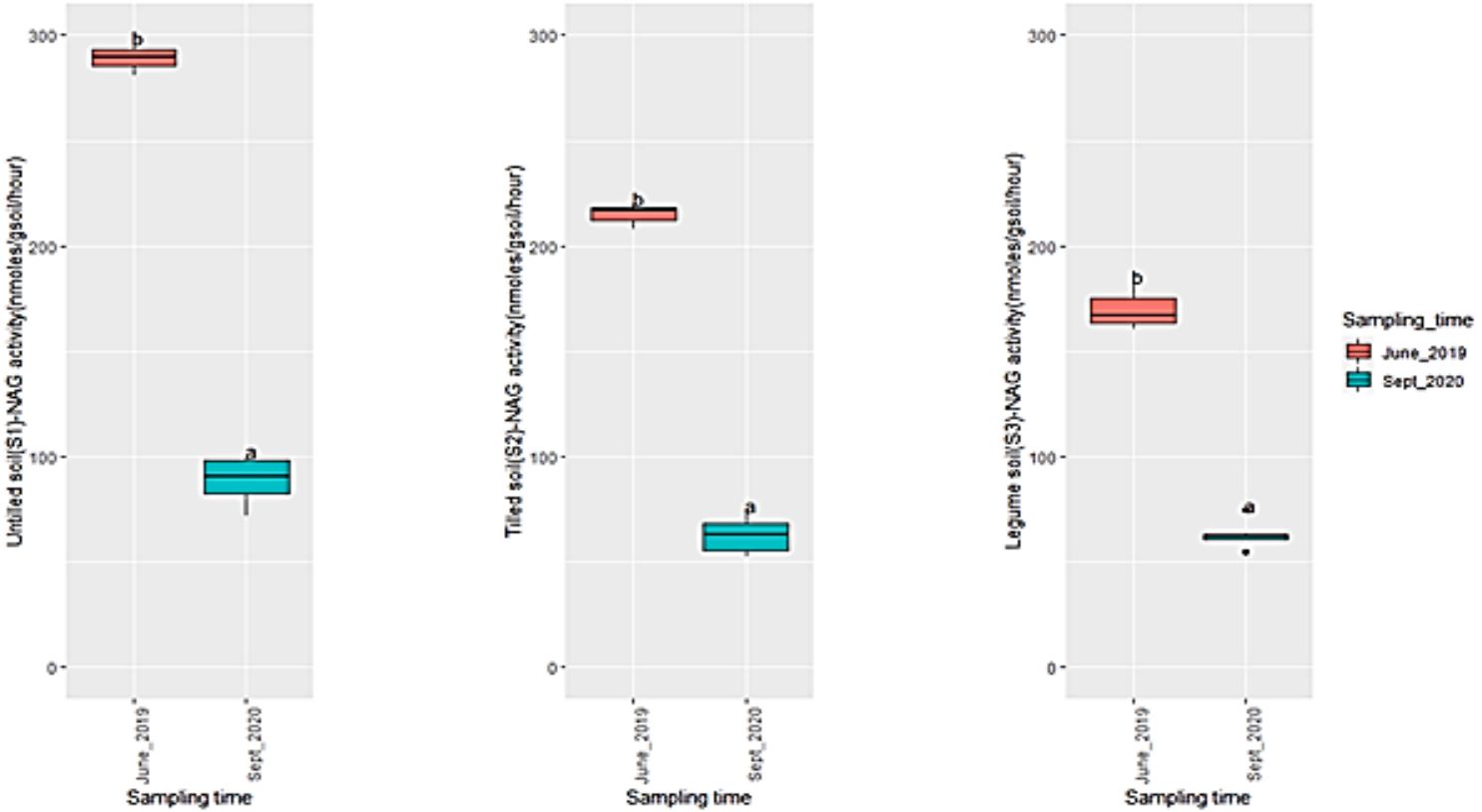


Fig. 3.10 **Change in Soil enzyme NAG (N-acetyl beta glucosaminidase) activity in Initial field soils upon storage (nmoles/gsoil/hour**; S1-Untilled, S2-Tilled, S3-Legume. a,b,c= Tukey method for comparing a family of 3 estimates; significance level used: alpha = 0.05 , n=9

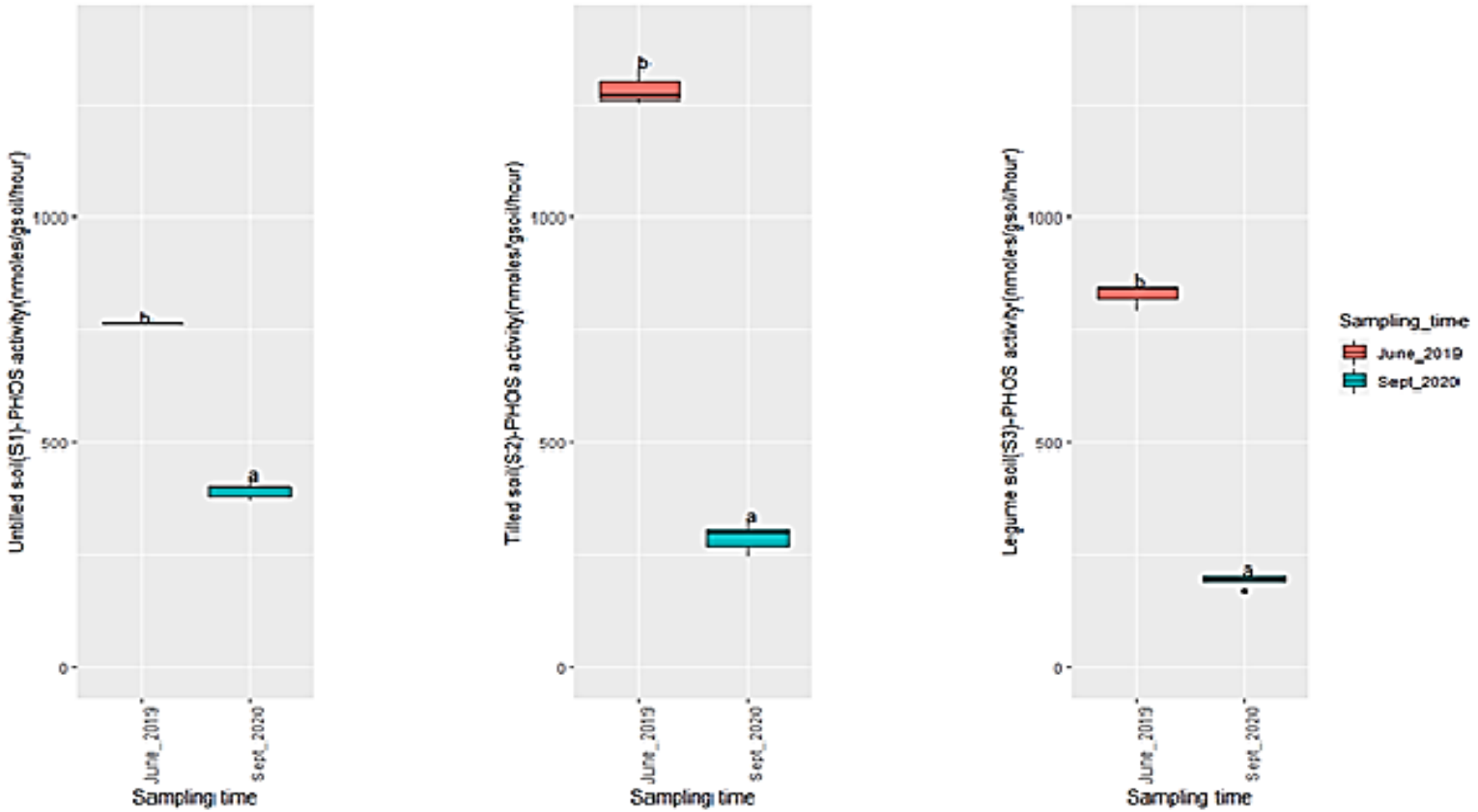


Fig. 3.11 **Change in Soil enzyme PHOS (Phosphatase) activity in Initial field soils upon storage (nmoles/gsoil/hour)**; S1-Untilled, S2-Tilled, S3-Legume. a,b,c= Tukey method for comparing a family of 3 estimates; significance level used: alpha = 0.05

3.4 Discussion

3.4.1 Soil physical and chemical properties

The soil samples for this study were collected from different areas of Sonning farm, Berkshire, UK and based on the cultural practices such as levels of tillage and field cropping history on these areas of the farm, the soils from these areas were classified as untilled (left untilled for more than 6 years), tilled (ploughed two times, last ploughing was three months prior to sampling), and legume (planted with legumes (red clover, *Trifoleum pratense*) interspaced with grass). The main aim of this study was to establish the initial physical and chemical differences between these soil types. In this study, the untilled soil showed high values for all the parameters measured and most of them were significantly higher than tilled and legume soils. All three soils were acidic in nature. The pH of a given soil is known to influence its bacterial community composition (Lauber *et al.*, 2009). There is a significant interaction between soil pH and nutrient availability and catabolic activities in soil (Wakelin *et al.*, 2008). Soil amendments have been shown to alter the pH which in turn altered the bacterial community composition in soil and this is primarily because most of the bacteria have a narrow pH optima for their biological activities (Qi *et al.*, 2018). The low pH in untilled soil indicates the role of organic matter in decreasing the pH by releasing H^+ ions from organic fractions (Ritchie and Dolling, 1985).

The long-term no-tillage resulted in high organic matter content in the untilled soil. In untilled soil, soil organic matter is protected from degradation by the formation and stabilization of microaggregates within macroaggregates (Muruganandam *et al.*, 2009). In our study site, the grass sward had been cut every year and left on the ground. This would have contributed to the increased organic matter in this soil. No tillage along with addition of organic matter has been reported to increase soil organic matter significantly (Alam *et al.*, 2014). The water holding capacity (WHC) was high for untilled soil. This agrees with the findings by Nyborg and Malhi (1989). No tillage enhances soil WHC by stimulating the production of water-stable aggregates in the soil and increasing porosity (Yu *et al.*, 2011)., The high soil organic matter content also explains the presence of high total carbon and nitrogen content in untilled soils. The high nitrate levels in

untilled soil can be attributed to the larger mineralization potential from accumulated organic N reserves in untilled soil (Stinner *et al*., 1983).

Soil microorganisms respond rapidly to the addition of plant and animal residues. These microbes are responsible for the breakdown of complex organic compounds, such as cellulose and lignin in plant residues, into simple organic compounds. The increase in soil organic matter results in greater soil biological activity and soil biodiversity. In this study, in untilled soils, due to the accumulation of crop residues, there is an abundance of soil organic matter which in turn increases the biological activity. This is reflected in their high NAG and PHOS activity which in turn resulted in high relative concentrations of carbon (C), nitrogen (N) and phosphorus (P) in untilled soils in this study. Alam *et al*., (2014) observed that organic matter content decreases with deep tillage following successive cropping cycles. In this study, the tillage practiced during two cropping cycles, might have resulted in the decrease in organic matter content suggesting lowered total C and N content in tilled soil as compared to untilled soil. The significant increase in available P and N content in untilled soil as compared to the tilled soil is consistent with the findings by other researchers (Thomas *et al*., 2007, Mathew *et al*., 2012).

The soil sample collected from the area with legumes showed low values in all the physical and chemical parameters measured. This was an experimental plot before 2017 and was fertilized with N and sulphur (S) fertilizers during the growing season. In 2017, the plot was ploughed and planted with legumes (red clover, *Trifoleum pratense*) interspaced with grass. The soil has been under cultivation which resulted in the depletion of organic matter and available nutrient status. Fertilization has been shown to decrease the soil organic matter content of soil due to their negative effect on microbial biomass (Klein *et al*., 1989). The dynamics of soil organic carbon and nitrogen in soil is a major driving force of microbial activity and nutrient cycles in agricultural soils. This has a great influence on physical and chemical properties of soil. The continuous cultivation and fertilizer application in soil might have led to the changes in microbial activity in soil which has affected the nutrient cycling and availability of nutrients. At the time of sampling, the legumes and grass were found to be growing in the plot and the growing plants will also take in much available nutrients in soil which also might be the reason for less nutrients recorded in this soil. Legumes

have been shown to lower the activity of ammonium oxidizers in soil, which reduces nitrification and the loss of available nitrogen through leaching (Paungfoo-Lonhienne et al., 2017).

3.4.1.1 Effect on storage

In the later studies explained in next few chapters, the field soil samples collected were used as microbiome inoculum. Different soil types, characterized by different physicochemical properties harbour different microbial communities (Schreiter *et al*., 2014). So, changes in nutrient content during storage will have an influence on microbial community in soil. Moreover, plant growth and crop production depend to a large extent on soil N-supplying capacity (Li *et al*., 2012). In microbiological soil studies, sample pre-treatments may have a strong influence on measurement results (Wallenius *et al*., 2010). Ammonium and nitrate content in soils are always analysed from fresh soils. The low temperature storage was found to stimulate soil mineralization and nitrogen formed in the refrigerated and frozen storage soil samples were found significantly higher than those in the fresh samples (Wu *et al*., 2018). The soils collected from field was stored at 4^{o}C until further use. So, it was necessary to understand the change in ammonium and nitrate content in soils during storage. The nitrate content increased during storage, and this can be attributed to the mineralization taking place during low temperatures (Wu *et al*., 2018). Harding and Ross (1964) found that storage at low temperature increased nitrate concentration as compared to fresh samples. They suggested that the period of storage might have affected the microbial population in soil.

3.4.2 Soil extracellular enzymes

Soil enzymes are considered as the mediators and catalysts of important soil functions that include: (1.) decomposition of organic inputs, (2.) transformation of native soil organic matter and (3.) release of inorganic nutrients for plant growth (Dick, 1997). The enzymes associated with N acquisition include β-1,4-N-acetylglucosaminidase (NAG), leucine aminopeptidase (LAP) and urease. Among this NAG plays an important role in chitin degradation (Koyama *et al*., 2013). Phosphatases (PHOS) catalyse the hydrolysis of organic phosphorus in soil and release free phosphate that becomes available for plants (Condron *et al*., 2005). In the current study, the activity of NAG and PHOS in soils are measured to assess the difference in biological activity in these soils. Among the soil types studied, NAG activity was found high for untilled soil.

Muruganandam *et al*., (2009) reported high NAG activity in soils under no-tillage. They found significant increase in NAG activity in no- till than in tilled system. They suggested that no-till management enhances activities of N mineralization enzymes by enhancing the proportion of fungal organisms in the soil microbial community. Organic matter and total carbon content in soils were found to significantly influence the enzyme activities in soil. The high organic matter and carbon content in untilled soil in this study resulted in the increased activity of soil enzymes. 50% of the variance in NAG activity is explained by organic matter content and total carbon content in soil. Similar results were obtained in other studies (Tian *et al*., 2010).

Organic phosphorus content is a better indicator of PHOS activity in soil (Margalef *et al*., 2017). Phosphatases can further be stabilized in soils on clay or other oxides. Due to tillage and cropping in the tilled and legume soils, these immobilized, yet active phosphatase fractions also contributed to the initial increased activity measured in these soils as compared to the untilled soils. But this condition was altered during storage. Total carbon and organic matter content in soil could explain 93.7% of the variation observed in phosphatase activity in this study. Since organic phosphorus is the main factor controlling the phosphatase activity in soil (Margalef *et al*., 2017), the increase in organic matter in soil might have resulted in increased availability of organic phosphorus in untilled soil which resulted in high phosphatase activity in these soils over a period. A study conducted in Olive orchards to find the influence of organic matter on soil phosphatase activity found that soils with high organic matter had high acid and alkaline phosphatase activities (Christopoulou *et al*., 2021). The low disturbance of soil under untilled conditions, resulted in high soil carbon levels which served as food source for microbes, and they release extracellular enzymes to facilitate the degradation (Bhavya *et al*., 2017).

3.4.2.1 Effect of storage

The storage of soil at 4^{o}C resulted in decrease of enzymatic activities. Management practices like tillage influences the organomineral complexes in soil (Bergstrom and Monreal, 2000). During storage these are expected to differentially influence soil enzyme stability in storage (Peoples and Koide, 2012). In their study the authors suggested the need to study the effects of storage to properly interpret treatment effects on soil enzyme activity. Since available analytical capacity rarely supports immediate processing of

large sample sets needed for representative data, sample storage is frequently a necessary step in environmental data gathering. In this study, a change in enzymatic activity was observed and the enzymatic activity was directly correlated to the organic matter content in the soil. Wallenius *et al.*, (2010) reported similar changes in enzyme activity during storage and the changes varied with soil type.

3.4.3 Conclusions

To summarise, the three plots selected for soil sampling has provided three distinct soil samples with different physical and chemical properties. Soil management practices had significant effect on both chemical and biological properties of the soil. The untilled soil had high nutrient content and high activity of nitrogen (NAG) and phosphorus (PHOS) mineralizing enzymes. These soils vary significantly in their pH suggesting differences between respective microbial communities. Our microbiome study will use these soil samples as initial inoculum. The extracellular enzyme activity will be a useful indicator in measuring the biological activity in the soils over the generation of crops. The changes in extracellular enzyme activity are correlated to the soil organic matter and carbon content. Even though, there are many studies suggesting the decrease in soil enzymatic activities during storage, further studies are essential to understand the reasons for these changes and how these changes are influenced by soil type.

Chapter 4: Influence of host-mediated microbiome selection on soil pH, nutrients and enzymatic activity in the rhizosphere of soybean plants

4.1 Introduction

The relationship between plants and their surroundings has been the focus of research for centuries. The plant, the soil, and the soil microbes all work together to contribute to plant health and productivity. Microorganisms can directly impact plant growth by the production of phytohormones and by improving nutrient acquisition, and indirectly by inhibiting plant pathogens (through the production of antibiotics) and activation of the plant immune response (Berendsen *et al.*, 2012, Song *et al.*, 2020). It is possible to observe new forms of interactions between plants and microorganisms that shape plant development by studying a plant in conjunction with its microbiome across multiple generations (Panke-Buisse *et al.*, 2015). Through multiple generations it is possible to progressively enrich microbiota associated with a specific plant trait facilitating the use of more complex communities instead of a single microbial strain (Mazzola, 2004). Host mediated microbiome engineering indirectly selects microbiome based on host phenotype. In this study, by directly selecting for increased plant height, the host phenotype (increased plant height under nutrient deficit conditions) is used to indirectly select for beneficial microbiome-host-interactions over two generations using the same host species.

4.1.1 Microbial diversity influenced by soil properties

The environment of the soil influences the rhizosphere microbial community. Soils are highly diverse (in terms of texture, soil properties) with equally diverse communities of microorganisms with potentially as many as 2000 - 5000 species of microbes in 0.5g of soil (Schloss and Handelsman, 2006). Soil properties including pH and nutrient content influence the formation of bacterial communities at the roots (Lundberg *et al.*, 2012). The portion of the soil adjacent to the roots, which has different physical and chemical properties from the bulk soil due to the influence of roots, is referred to as the rhizosphere, and the surface of the roots is referred to as the rhizoplane (Fageria and Moreina, 2011). This soil, rhizosphere, rhizoplane

is considered as integral parts of rhizosphere continuum and the type of soil influences the microbial composition in both rhizosphere and rhizoplane ultimately influencing the microbial composition inside the root (root endosphere) (Berg *et al*., 2014). Here, the bulk soil acts as a reservoir of bacteria that can be recruited by plants to colonise the roots and it is assumed that this rhizosphere community shaped by the plant is based on the reservoir present in the soil (Schreiter *et al*., 2014).

4.1.2 Soil enzymes as indicators of soil quality

Soil enzymes play a major role in decomposition of plant residues and subsequent release of plant available nutrients. Living and dead microbes, plant roots and residues are the sources of soil enzymes. The importance of soil enzymes is explained by Dick *et al*., (1992) as "soil enzymes are useful in describing and making predictions about an ecosystem's function, quality and the interactions among subsystems". Soil enzymes are used as soil quality indicators due to their close relationship with soil biology, rapid response to changes in management practices and their ease of measurement (Bandick and Dick, 1999). Soil enzymes play significant roles in decomposition and transformation of soil organic matter, nutrient release from plants, detoxification of chemicals, nitrogen (N) fixation and denitrification processes. In a study to investigate the response of enzyme activities on soil amendment with organic compounds, a significant effect was seen on urease, catalase, alkaline phosphatase and β- glucosidase activities (Yu *et al*., 2016). The application of microbial inoculants increased the urease and sucrose activities in paddy soil over the growing season. Panke Buisse *et al*., (2015) found that in *Brassica rapa*, the increase in biomass was correlated with an increased activity of N- mineralization enzyme. All these studies indicate that soil enzymatic activity is a good indicator to measure the microbial activity in soil which can be correlated with phenotypic traits. Thus, it forms an important tool to study the effects of microbiome in the plant rhizosphere.

4.1.3 Understanding the interactions between plants, microbiome, and soil

It is reasonable to conclude that the plant-associated microbiome varies with soil type and biochemical processes like enzyme activity (mediated by microbes and plants) play major roles in the mineralization of nutrients in soil which in turn affects the plant growth and development. Thus, the beneficial effects of microbiome mediated functions are dependent on the genotype of plants, microbial interactions, soil types, management practices and the interaction among these factors (Schmidt *et al*., 2018). Schmidt, *et al* found that plants recruit management-system-specific taxa and shift N-cycling pathways in the rhizosphere which differentiates rhizosphere from bulk soil. In agriculture, microbial inoculation of single strains with beneficial effects has been used for disease management and improving yield (Chapparo *et al*., 2014; Berg and Smalla, 2009). Applications under field conditions have always been a challenge due to adverse environmental conditions, which are limiting factors for the successful colonization of the inoculated strain in soil (Sessitsch *et al*., 2019). Inoculated microorganisms must compete with a highly diverse endogenous microflora. The microbial community around a plant depends on the feedback between plant and its environment (Bever *et al*., 2012) and this reciprocal nature of interactions between plant and its environment is poorly understood.

Host mediated microbiome selection is based on the hypothesis that plants have evolved to selectively recruit beneficial microorganisms which are subsequently transmitted to the next generation of plants (Mueller and Sachs, 2015). This is a potential platform for exploring plant–microbiome interactions for improving the sustainability and productivity of global agriculture. In the current study, the focus is made on understanding the changes in soil enzyme activities taking place in the rhizosphere of soybean plants during microbiome selection. As explained in Chapter 1, there have been relatively few studies on host mediated microbiome selection. Among the few existing studies, Swenson *et al*., (2000), observed changes in plant biomass due to soil nutrient variables over generations of plant selection. Panke-Buisse *et al*., (2015) hypothesised that such a change in nutrient variables is due to the changes in extracellular enzyme activities in soil. None of these studies measured the enzyme activities between selection cycles. The below ground changes occurring during microbiome selection, in terms of nutrients and enzyme activities involved in nutrient cycling, are not addressed in any of these studies. With this gap in knowledge, it is thus

important to understand the involvement of various microbial taxa associated with nutrient recycling, which can then be manipulated to suit our needs.

In this study, the overall aim was to investigate the changes happening in the rhizosphere of soybean plants during microbiome selection, the impact of different varieties of soybean on rhizosphere soil enzyme activity, and how the microbiome inoculum from different soils affects the enzyme activity.

The specific aims were

1. To investigate potential changes in the soil enzyme activity between two generations of microbiome selection. Based on the studies by Swenson *et al*., 2000 and Panke-Buisse *et al*., 2015, I hypothesise that the changes in soil enzymatic activities will be reflected in the plant growth across generations of selection.
2. To study the interaction between soil, plant varieties, and plant growth on the soil enzyme activity. Several studies have found evidence that differences in above ground vegetation and soil properties influence the biochemical processes in soil. Here, I propose that the enzymatic activity will differ between different varieties of the same plant and the interaction between soil and plant growth will have significant impact on enzyme activities.
3. To investigate the relationships between enzyme activities in soil and soil properties including carbon content and pH. Here I hypothesise that the activity of the enzymes in soil with high carbon content will be high and changes in soil pH may induce significant changes to the soil environment and the associated enzyme systems.
4. To investigate the difference in enzymatic activity in the rhizosphere and fallow soils without plants. It can be hypothesised that the plant rhizosphere will have more enzymatic activity as compared to the fallow soils to meet the demand of nutrition by plants.

4.2 Methodology

4.2.1 Host mediated selection

Soybean crop (varieties Kenchen and Siverka) were grown in the Crop and Environment Laboratory (CEL) glasshouse, Whiteknights Campus, University of Reading, in plastic plant pots containing microbiome inoculum from different field soils (pot preparation, microbiome inoculation and planting explained in Section 2.4). The rhizosphere soils from the 1st and 2nd generation of plants were collected and analysed for soil pH, nutrients, and enzymatic activity (Section 2.7 and 2.8). In this study, we define fallow soil as that contained within pots and treated with our microbiome inoculum but left unseeded and without actively growing plants (Section 2.4). As controls, fallow soil pots without plants were treated with sterile water. During the second generation, fallow pots for each variety received a treatment of microbiome inoculum derived from the rhizosphere of the tallest plants of respective variety. Control fallow pots were treated with sterile water. To understand the difference in soil enzyme activities between fallow and cultivated soils, the soil from pots without plants and rhizosphere soil were analysed and the data was compared using ANOVA based on linear model. with NAG, and PHOS as response variables.

4.3 Results

Soybean plants were grown in the glasshouse under controlled conditions for two generations and rhizosphere soil from the tallest plants in the first generation was used as the microbiome inoculum for the second generation (Chapter 2). The results on changes in plant growth, soil enzyme activities and soil nutrients during microbiome selection is given below.

4.3.1 Plant growth and biomass

4.3.1.1 Plant height

There was a significant three-way interaction between soil type, variety, and generations on plant growth ($F(1) = 2.4908$, $p=0.0446$). In the first generation of plant growth, in variety Kenchen, the highest average plant height was recorded for mixed (S4) soil followed by legume (S3) soil. The lowest average plant height in Kenchen was recorded in untilled (S1) soil. In Siverka, first generation crop, the highest average plant height was in legume (S3) and Mixed (S4) and lowest in Control (C) soil (Table 4.2). Tukey test (Fig. 4.1A) revealed that, within the same soil type, the difference in plant height between varieties Kenchen and Siverka was significant in legume (S3) ($p<0.001$) and Mixed (S4) ($p<0.001$), whereas there was no significant difference in plant height for both the varieties in untilled (S1) ($p=0.887$), tilled (S2) ($p=0.142$) and control (C) ($p=0.09$). Plants in legume (S3) ($p=0.004$) and Mixed (S4) ($p=<0.001$) were significantly higher in average plant height compared to control (C) for variety Kenchen and the plant height was not significantly different between control (C) and other soils for variety Siverka.

In the second generation, variety Kenchen grew tallest in control (C) soils and was lowest in untilled (S1). In Siverka, the highest average plant height was recorded in legume (S3) and lowest in control (C). Tukey test (Fig. 4.1B) showed that there was no significant difference in plant height between varieties in legume (S3) ($p=0.99$), mixed (S4) ($p=0.37$), tilled (S2) ($p=0.86$) and untilled (S1) ($p=0.99$). A significant difference in plant height was recorded in control (C) ($p<0.001$) between two varieties. In variety Kenchen, plants in untilled (S1) ($p<0.001$), tilled (S2) ($p<0.001$), legume (S3) ($p=0.004$) and mixed (S4) ($p=0.017$) were significantly lower in average plant height than plants in control (C), whereas average plant height was not significantly different between control (C) and other soils for variety Siverka.

The plant height differed significantly between two generations for plants grown in untilled (S1) ($p=0.03$), tilled (S2) ($p=0.02$), legume (S3) ($p<0.001$), and mixed (S4) ($p<0.001$). There was no significant difference in plant height for plants in control (C) ($p=1$) between the two generations.

Table 4.1 Average plant height and above ground biomass of soybean plants (varieties- Kenchen, Siverka) in different soil types across two generations.

Soil type	Variety	Generation	df	Plant height (cm)	SE	Above ground biomass (g)	SE
Control(C)	Kenchen	First generation	192	35.13	2.638	0.81	0.11
Legume(S3)	Kenchen	First generation	192	48.36	2.250	1.43	0.09
Mixed(S4)	Kenchen	First generation	192	52.58	2.154	1.66	0.09
Tilled(S2)	Kenchen	First generation	192	37.42	2.154	1.27	0.09
Untilled(S1)	Kenchen	First generation	192	33.50	2.154	1.07	0.08
Control(C)	Siverka	First generation	192	21.14	2.820	0.74	0.11
Legume(S3)	Siverka	First generation	192	30.55	2.250	1.13	0.09
Mixed(S4)	Siverka	First generation	192	30.00	2.154	0.99	0.08
Tilled(S2)	Siverka	First generation	192	27.08	2.154	0.97	0.08
Untilled(S1)	Siverka	First generation	192	28.00	2.154	1.23	0.08
Control(C)	Kenchen	Second generation	192	36.50	2.638	1.00	0.10
Legume(S3)	Kenchen	Second generation	192	26.36	2.250	0.83	0.09
Mixed(S4)	Kenchen	Second generation	192	27.58	2.154	0.93	0.08
Tilled(S2)	Kenchen	Second generation	192	24.00	2.250	0.76	0.09
Untilled(S1)	Kenchen	Second generation	192	20.50	2.154	0.61	0.08
Control(C)	Siverka	Second generation	192	21.29	2.820	0.75	0.11
Legume(S3)	Siverka	Second generation	192	24.83	2.154	0.91	0.08
Mixed(S4)	Siverka	Second generation	192	22.27	2.250	0.83	0.09
Tilled(S2)	Siverka	Second generation	192	20.22	2.487	0.59	0.09
Untilled(S1)	Siverka	Second generation	192	22.30	2.359	0.61	0.09

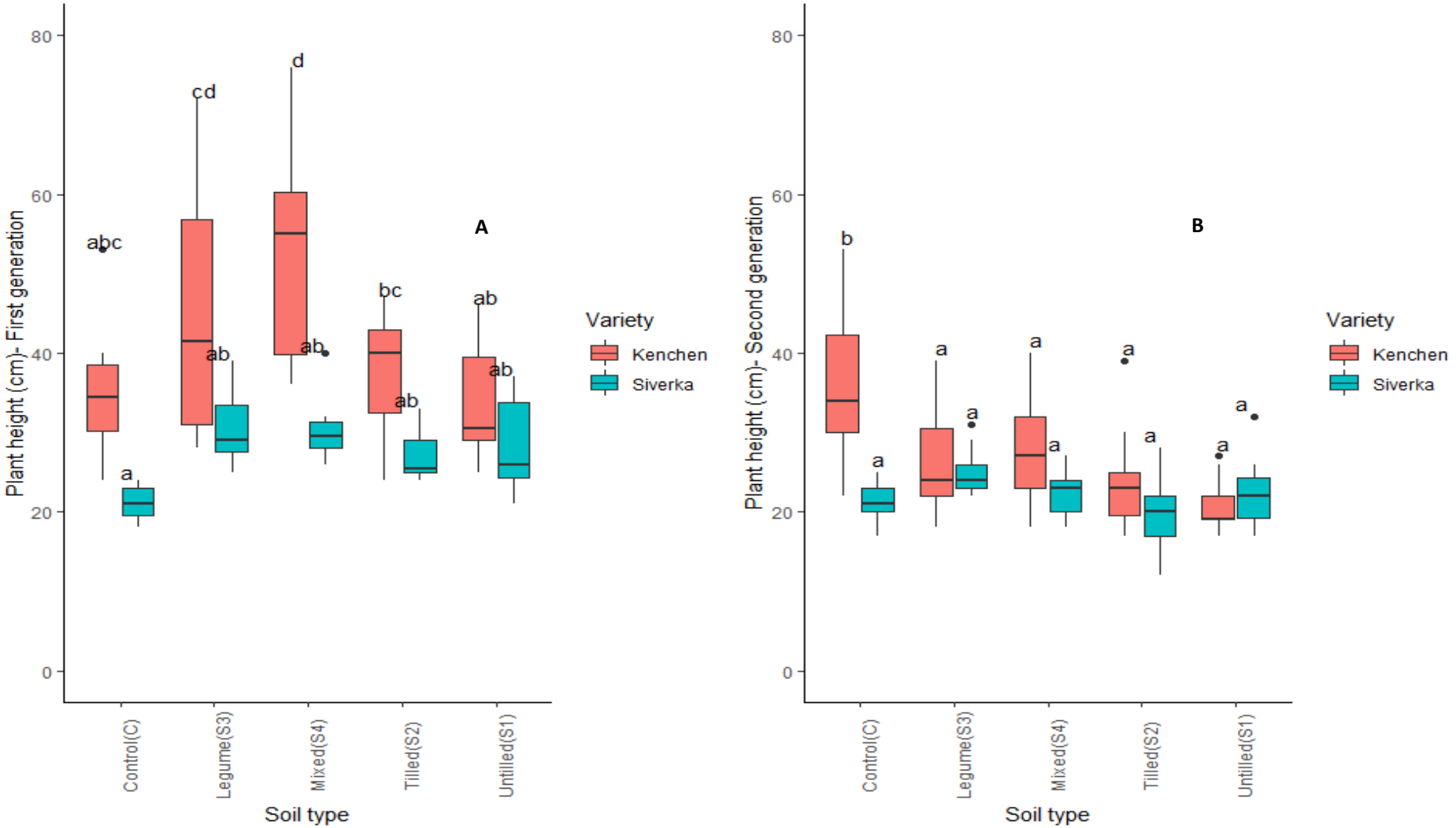


Fig. 4.1 **Difference in plant height (cm) between Control (C) and other treatments across two soybean varieties (Kenchen and Siverka) and across two generat**ions **A.** Tukey multiple comparisons of means at 95% family-wise confidence level for interaction between soil types and varieties in **first generation** of plant growth **B**. Tukey multiple comparisons of means at 95% family-wise confidence level for interaction between soil types and varieties in **second generation** of plant growth. Boxplots with the same letter were not significantly different from each other, `c` shows highest mean, obtained by Tukey method, significance level used: alpha = 0.05. n=12

4.3.1.2 Flowering

At time of harvest and rhizosphere soil sampling for both the generations plants were at the start of their reproductive stage of growth. In soybean, appearance of a flower at any node on the main stem is regarded as the `beginning of flowering (R1) ` and hence reproductive stage (Lee *et al*, 2011). Days taken for the first flower to appear was different in different soil types. A difference in flowering time was observed between control (35 days after transplanting) and other soil types (untilled (S1) (30 days after transplanting), tilled (S2) (28 days after transplanting), both legume (S3) and mixed (S4) (26 days after transplanting). In the 2nd generation of plant growth, early flowering was noticed in plants growing in untilled (S1) (41 days after sowing), tilled (S2) (36 days after sowing), legume (S3) (30 days after sowing) and mixed (S4) (30 days after sowing) soils. Flowering started 42 days after sowing in the control in 2nd generation plants. During the harvest of the 2nd generation, seed pods started developing in the legume (S3) and mixed (S4) treatments, whereas undeveloped pods were found in untilled (S1) and tilled (S2). It was not possible to do a statistical analysis on number of pods, due to their difference in pod development stage. Most plants with legume (S3) and mixed (S4) treatment had, on average, 2-3 pods per plant.

4.3.1.3 Above ground biomass

The interaction effect of soil, variety and generation was found to be significant ($F(4) = 3.4707$, $p=0.0092067$) on above ground plant biomass. The average above ground biomass was high in first generation for both the varieties. In variety Kenchen, first generation biomass average was highest for mixed (S4) and lowest was in control (C). In Siverka, average above ground biomass was highest in untilled (S1) and lowest in control (C). Tukey test for multiple comparisons (Fig. 4.2A) showed that the difference in average above ground biomass was significant between two varieties only in mixed (S4) ($p<0.001$).

In the second generation, the highest average above ground biomass in Kenchen was recorded in control (C) and lowest in untilled (S1) (Table 4.2). Siverka recorded highest above ground biomass in legume (S3) and lowest in untilled (S1). The difference in average above ground biomass between varieties was not significant (Fig. 4.2B) in all five soils.

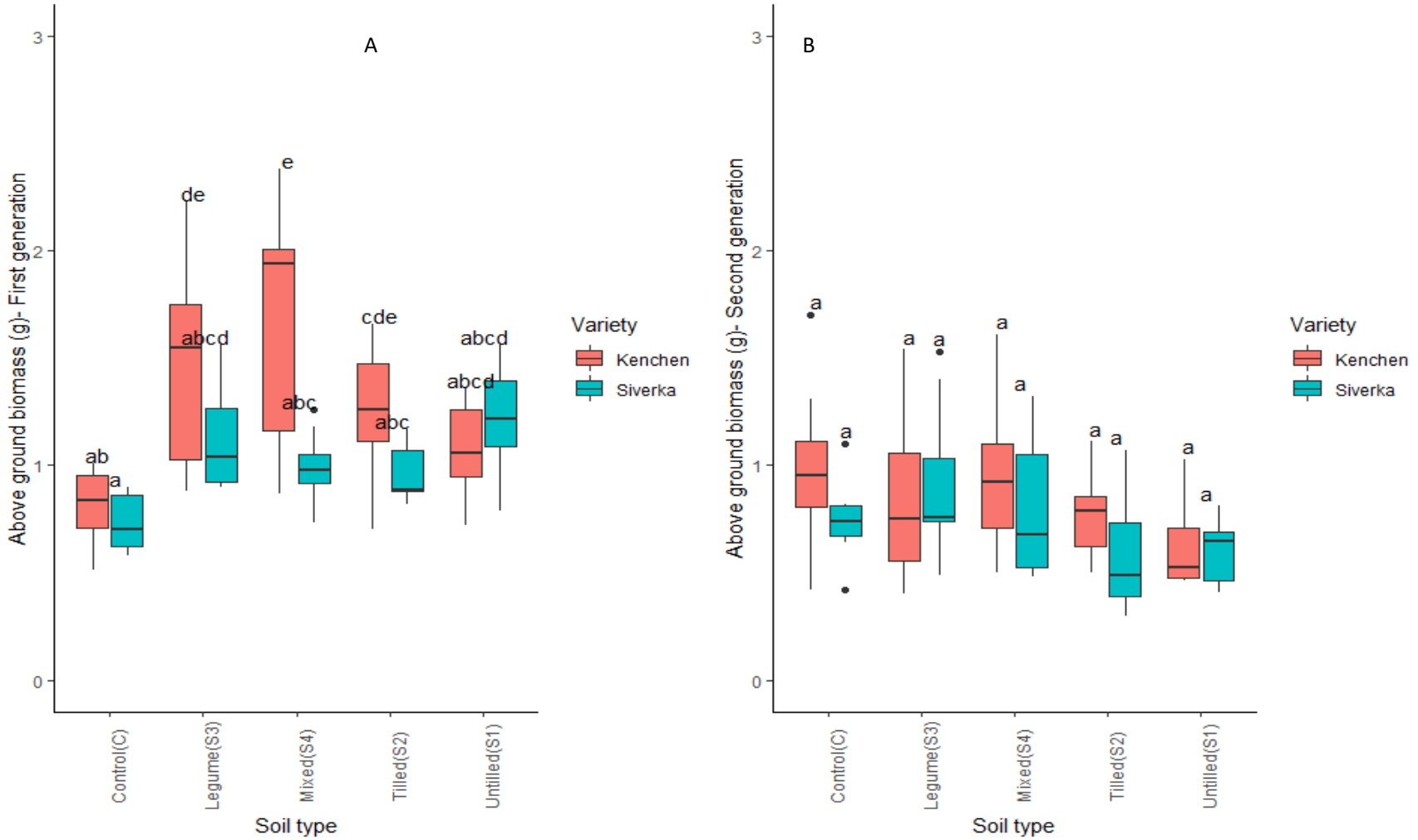


Fig 4.2 **Difference in above ground biomass (g) between Control (C) and other treatments in two soybean varieties (Kenchen and Siverka) across two generations A.** Tukey multiple comparisons of means at 95% family-wise confidence level for interaction between soil types and varieties in **first generation** of plant growth **B**. Tukey multiple comparisons of means at 95% family-wise confidence level for interaction between soil types and varieties in **second generation** of plant growth. Boxplots with the same letter were not significantly different from each other, `c` shows highest mean, obtained by Tukey method, significance level used: alpha = 0.05, n=12

4.3.1.4 Nodules

Nodulation was observed in plants grown in legume (S3) (Fig. 4.3 A,C) and mixed (S4) (Fig. 4.3 B,D) at both generations. There were no nodules in any of the plants grown in untilled (S1), tilled (S2) and control (C) at both generations. The data on number of nodules from legume (S3) and mixed (S4) were analysed separately to understand the difference in nodulation patterns in two soils. There was a significant difference in nodulation between generations in legume (S3) and mixed (S4) (Fig. 4.4). The average number of nodules in legume (S3) in the first generation were 7 and 14 for Kenchen and Siverka, respectively, whereas for mixed (S4) respective variety nodulation was 4 and 10. In the second generation, the number of nodules increased in both the varieties in legume (S3) and mixed (S4) soils. The average number of nodules in Kenchen was 25 in legume (S3) and 11 in mixed (S4) and Siverka had 10 nodules in legume (S3) and 14 in mixed (S4).

Fig. 4.3 **A**. Nodules in plants grown in legume soil (S3); **B.** Nodules in plants grown in mixed (S4) soil; **C**. Picture showing nodules in plants in S3 after washing and drying; **D**. Picture showing nodules in plants in S4 after washing and drying

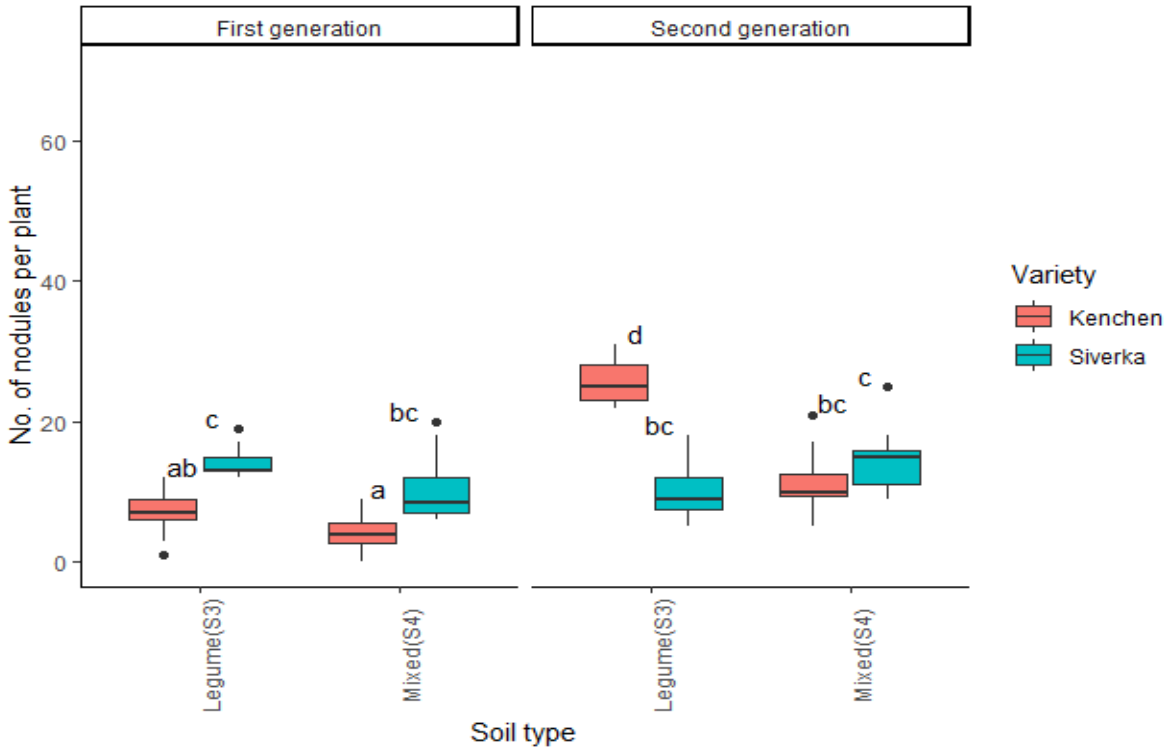


Fig. 4.4 **Graph showing difference in number of nodules per plant in legume (S3) and mixed (S4) soils in two generations of plant growth**. Boxplots with the same letter were not significantly different from each other, `c` shows highest mean, obtained by Tukey method, significance level used: alpha = 0.05.n=6

4.3.2 Soil enzyme activity in the rhizosphere of Soybean plants

4.3.2.1 N-acetyl beta glucosaminidase (NAG) activity

The interaction effect between soil type, growth, variety and generation was significant (F (4) =4.46, p=0.0019) on NAG activity in soils. The highest NAG activity was recorded in untilled (S1) after the first generation for both the varieties and lowest was recorded in tilled (S2) and legume (S3) (Fig. 4.5). Tukey test for multiple comparisons showed that the NAG activity in mixed (S4) (p=0.21) was not significantly different from that in C after the first generation. After the second generation, the highest NAG activity was recorded in legume (S3). The NAG activity in untilled (S1) and tilled (S2) decreased after second generation of plant growth.

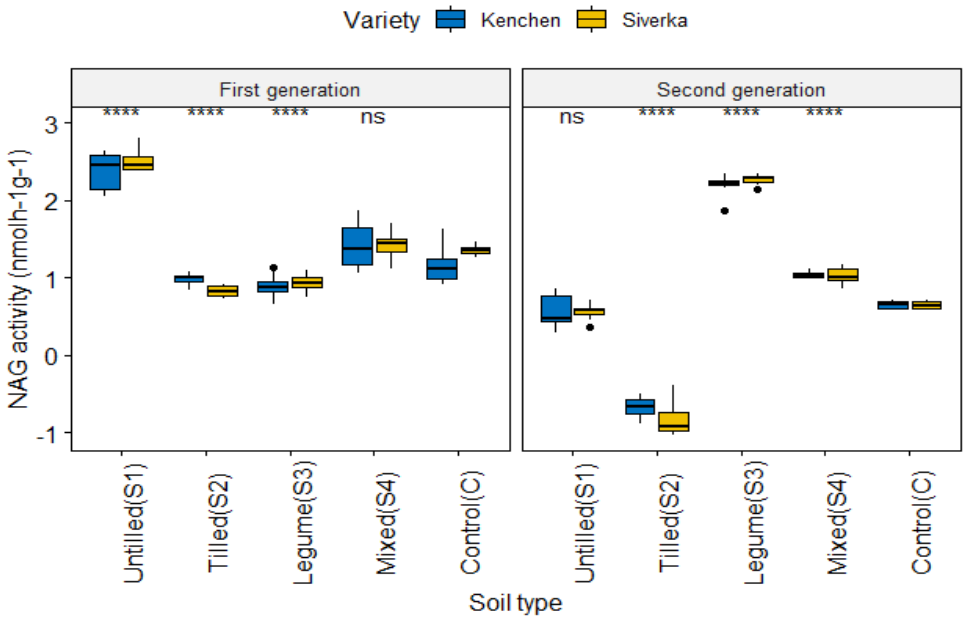


Fig. 4.5 **Difference in N-acetyl beta glucosaminidase (NAG) activity in the rhizosphere soils of plants in Control** (C) and other treatments across two generations of plant growth in soybean varieties Kenchen and Siverka, cube root values for NAG used; Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05, ns- not significant

Each soil type (untilled (S1) tilled (S2), legume (S3), mixed (S4), control (C)) was analysed separately to study the effect of host mediated selection based on plant growth (Fig. 4.6) on enzyme activities.

There was a significant interaction between growth, variety, and generation ($F(1) = 6.5905$, $p = 0.014$) on the NAG activity in untilled (S1) soil. Tukey test results indicated that within generations, the difference in NAG activity was significant between high and low growth plants only in variety Kenchen ($p=0.0001$) after the first generation of plant growth, whereas the difference in NAG activity between high and low growth plants was not significant in both the varieties after the second generation.

The interaction effect of growth, variety, and generation was not significant ($F(1) = 1.3193$, $p = 0.258$) in tilled (S2). In Tukey multiple comparisons, it was clear that the difference in NAG activity with growth was significant only after the first generation for both varieties Kenchen ($p=0.021$) and Siverka ($p=0.028$). As in untilled (S1), the NAG activity in the rhizosphere of both the varieties decreased significantly after the second generation.

In legume (S3) (Fig. 4.6C), the NAG activity was significantly different between generations ($F(1) = 1674.52$, $p < 2e\text{-}16$), but both growth and varieties did not have any significant effect on NAG activity in legume (S3) rhizosphere soil. There was no significant interaction effect noticed in this soil. Unlike in other two soils (untilled (S1) and tilled (S2)), NAG activity was higher in rhizosphere soils of legume (S3) after the second generation.

In mixed (S4) (Fig. 4.6D), the interaction between growth and varieties was significant ($F(1) = 16.22$, $p<0.001$). The difference in growth was significant only after the first generation for Kenchen ($p=0$) and Siverka ($p=0.002$). As in untilled (S1) and tilled (S2), the NAG activity was less after the second generation.

In the control, (C) (Fig. 4.6E), the interaction effect was significant ($F(1) = 12.34$, $p=0.002$). The NAG activity was lower after first generation as in untilled (S1), tilled (S2) and mixed (S4).

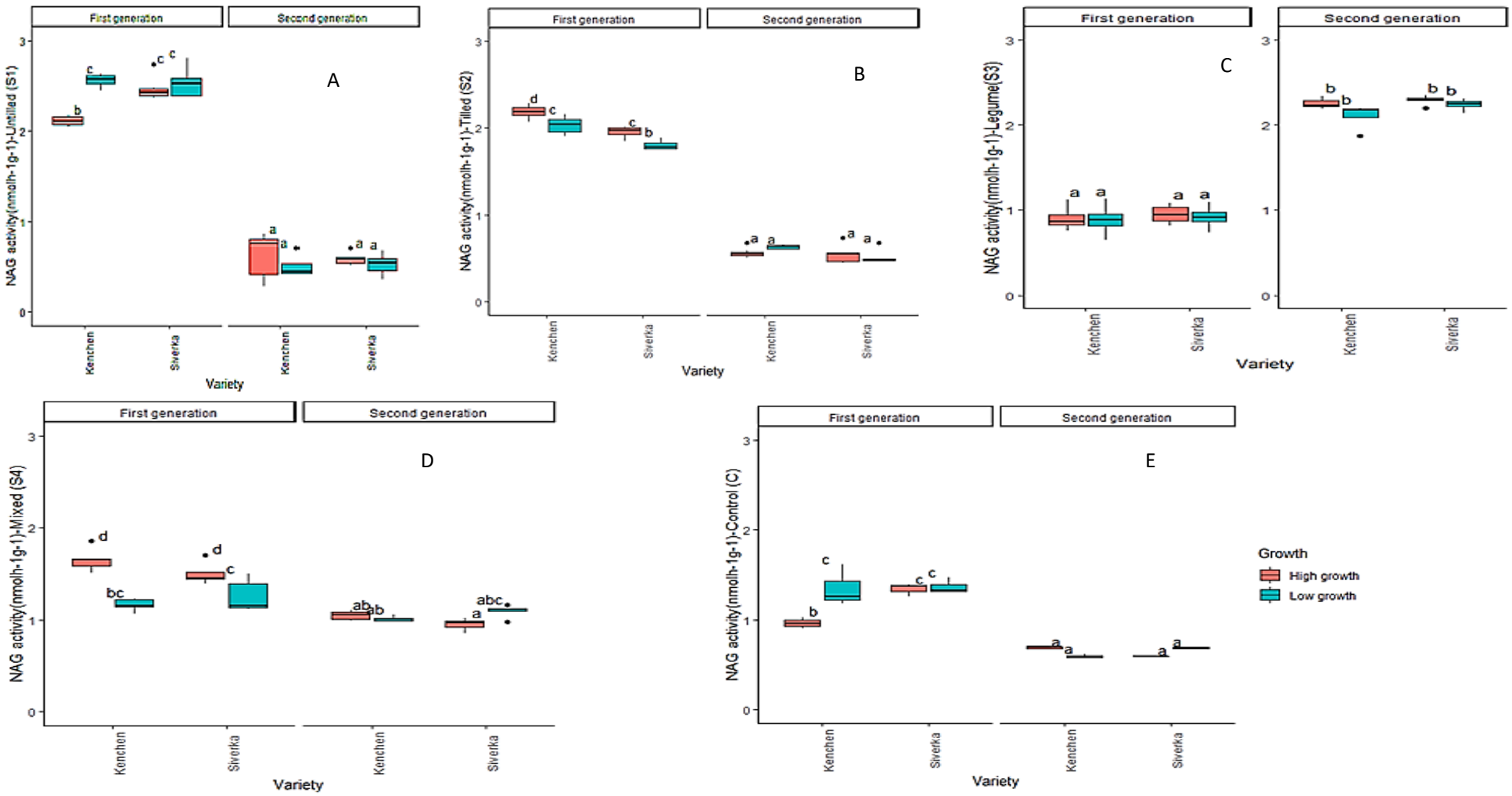


Fig. 4.6 **Effects of growth, variety and generation on N-acetyl beta glucosaminidase (NAG) enzyme activity in rhizosphere soils** A. Untilled(S1) B. Tilled(S2) C. Legume(S3) D. Mixed (S4) and E. Control(C). Control (C): autoclaved coir: sand without field soil suspension. Boxplots with the same letter were not significantly different from each other, `c` shows highest mean, obtained by Tukey method, significance level used: alpha = 0.05. n=6

4.3.2.2 Phosphatase (PHOS) activity

The interaction effect between soil type, growth, generation, and variety was significant (F (4) = 5.1585, p<0.001) on PHOS activity in rhizosphere soils. After the first generation of plant growth, highest PHOS activity was measured in control (C), and it was not significantly different from mixed (S4) (p=0.54). The PHOS activity in untilled (S1), tilled (S2) and legume (S3) was not significantly different after the first generation. The highest PHOS activity after the second generation of plant growth was in the rhizosphere of plants in tilled (S2) and it was not significantly different from legume (S3). The lowest was in untilled (S1) and it was not significantly different from control (C) and mixed (S4) (Fig.. 4.7).

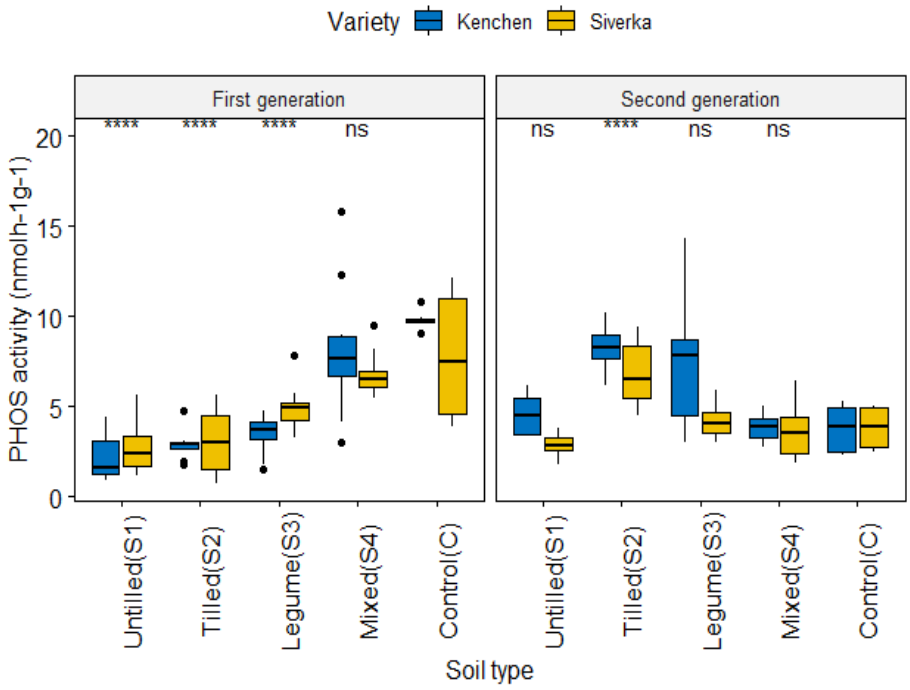


Fig. 4.7 . **Difference in Phosphatase (PHOS) activity between Control (C) and other treatments** in the rhizosphere soils of plants across two generations of plant growth in soybean varieties Kenchen and Siverka. Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05, ns- not significant

In untilled (S1), the difference in PHOS activity was significant between generations ($F(1) = 20.47$, $p<0.001$). Growth had less significant effect on PHOS activity ($F(1) = 6.73$, $p=0.013$). The interaction effect between growth, variety, and generation on PHOS activity was not significant ($F(1) = 0.0530$, $p=0.819$). Tukey results (Fig. 4.8A) showed that the difference in PHOS activity in the rhizosphere of high and low growing plants of variety Siverka was significant only after the first generation ($p=0.004$). There was no significant difference in PHOS activity between high and low growing plants for Kenchen in both generations.

There was a significant effect of growth ($F(1) = 11.58$, $p= 0.001$) and generations ($F(1) = 177.92$, $p<0.001$) on PHOS activity in tilled (S2) soil. Tukey results (Fig. 4.8B) indicated that the PHOS activity after the second generation of plant growth was significantly higher in the rhizosphere soil of tilled (S2) for both varieties, Kenchen ($p=0$) and Siverka ($p=0$). The difference in growth had significant ($p=0.0004$) effect on PHOS activity in the rhizosphere of Siverka plants after the first generation of plant growth but it was not significant ($p=0.27$) after the second generation. For variety Kenchen, the difference in growth did not have any significant effect on PHOS activity after both generations.

In legume (S3), the interaction between growth, variety and generation was significant ($F(1) = 5.96$, $p=0.019$) on PHOS activity. Tukey results (Fig. 4.8C) showed that the difference in PHOS activity was significant with growth only in variety Kenchen ($p=0.008$) after the second generation of plant growth. The growth had no significant effect on PHOS activity in variety Siverka after both generations of plant growth. The difference in PHOS activity between generations was significant only for variety Kenchen ($p<0.001$).

The interaction between growth, variety and generation was significant ($F(1) = 6.8069$, $p=0.013$) on PHOS activity in mixed (S4) with second generation recording less average PHOS activity. Tukey results (Fig. 4.8D) showed that the difference in growth had a significant affect only in variety Kenchen ($p<0.001$) after the first generation of plant growth.

In control (C) soil, the effect of growth ($F(1) = 85.96$, $p<0.001$), variety ($F(1) = 25.83$, $p<0.001$) and generation ($F(1) = 648.21$, $p<0.001$) was highly significant on PHOS activity. The interaction between these three factors was not significant ($F(1) = 2.94$, $p=0.105$). There was a decrease in PHOS activity after the second generation of plant growth (Fig. 4.8E).

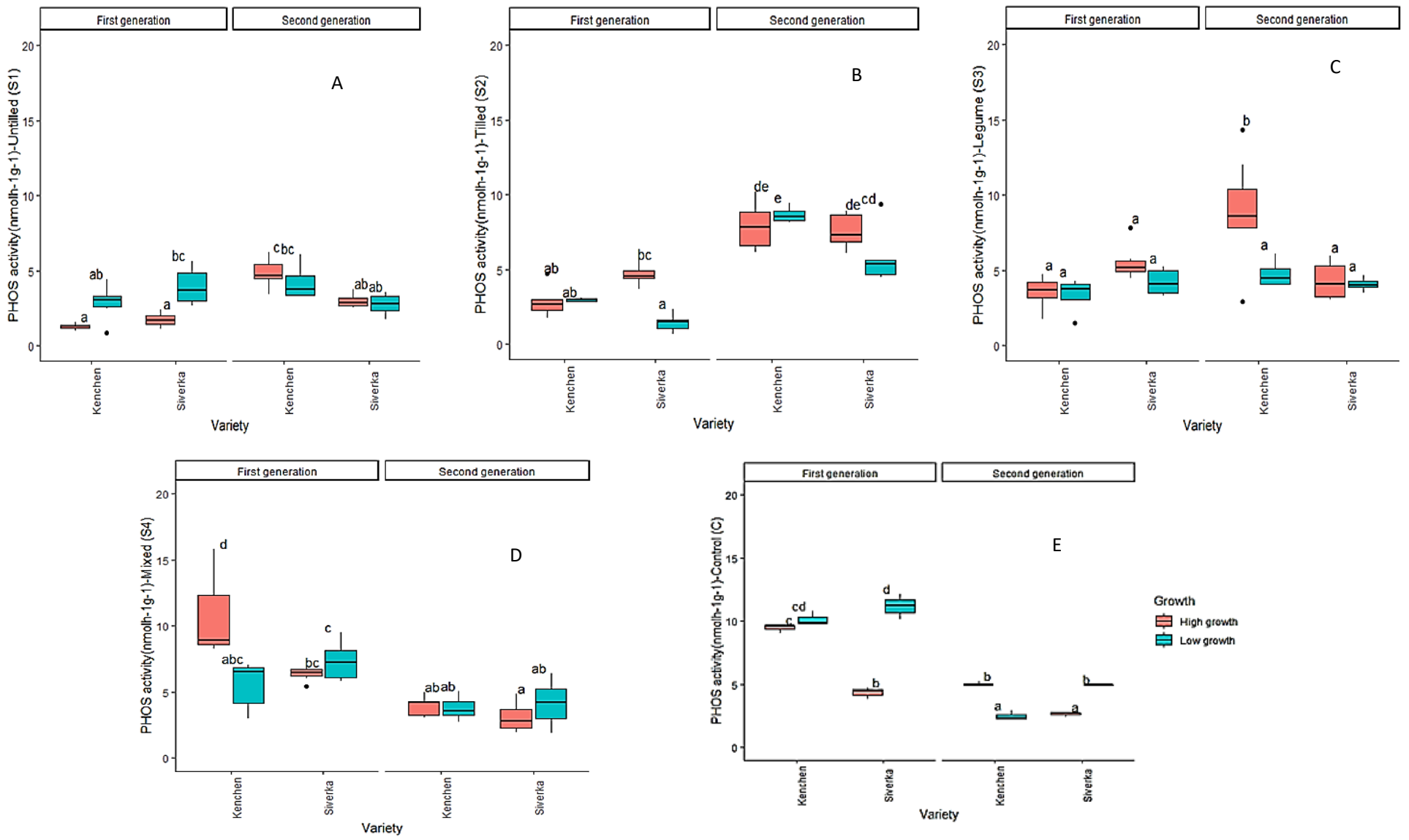


Fig 4.8 **Effects of growth, variety and generation on Phosphatase (PHOS) enzyme activity in rhizosphere soils** A. Untilled(S1) B. Tilled(S2) C. Legume(S3) D. Mixed (S4) and E. Control(C). Boxplots with the same letter were not significantly different from each other, `c` shows highest mean, obtained by Tukey method, significance level used: alpha = 0. 05, n=6

4.3.3 Rhizosphere pH and soil nutrients

4.3.3.1 Rhizosphere soil pH

Results indicated the interaction between soil type, growth, variety, and generation was significant (F (4) = 3.3153, p=0.013043) on rhizosphere soil pH. There was no significant interaction between soil type and generation (F (4) = 2.62, p=0.05) in the pH of fallow soils. After the second generation of plant growth all the soils (untilled (S1), tilled (S2), legume (S3), mixed (S4)) were significantly different from control (Fig. 4.9). Tukey results (Fig. 4.9) showed that there is no significant difference in pH in control (C)(p=0.688) and legume (S3) (p= 0.999) soils between generations. The average soil pH in the rhizosphere of plants in different soil types is given in Table 4.3.

Table 4.2 Average soil pH in the rhizosphere of plants and in fallow in different soil types after first and second generation

Soil_type	Mean pH	
	First Generation	Second Generation
Control(C)	8.51±0.04	8.63±0.04
Legume(S3)	8.81±0.04	8.83±0.04
Mixed(S4)	8.48±0.04	8.81±0.04
Tilled(S2)	8.77±0.04	8.46±0.04
Untilled(S1)	9.33±0.04	8.48±0.04
Control- fallow	8.76±0.04	8.65±0.03
Legume- fallow	8.92±0.04	8.69±0.03
Mixed- fallow	8.77±0.04	8.78±0.03
Tilled- fallow	8.80±0.04	8.58±0.03
Untilled- fallow	8.85±0.04	8.72±0.03

There was no significant effect of growth (F (1) = 0.5459, p=0.4672) or variety (F (1) = 0.1744, p=0.6799) on rhizosphere soil pH in untilled (S1). In tilled (S2), the interaction effect (F (1) = 5.0192, p=0.0345917) growth, variety, and generation was found to be significant on rhizosphere soil pH. Tukey test revealed that the

difference in pH with growth is significant only in variety Siverka in the first generation (p=0.0001). The difference in pH between varieties is significant only in the first generation (p<0.001).

In legume (S3), the interaction effect (F (1) = 10.5442, p=0.003) between growth, variety and generation was significant on rhizosphere soil pH. Tukey results showed that the difference in rhizosphere soil pH between varieties was significant only in the first generation as in tilled (S2). In mixed (S4), the difference in pH was significant with growth (F (1) = 24.48, p<0.001) and generation (F (1) = 131.97, p<0.001Tukey results indicated that the difference in pH with growth was significant only in the first generation for both Kenchen (p= 0.003) and Siverka (p= 0.041). The difference in pH with variety was significant (F (1) = 12.12, p= 0.002) in control (C)soil, but there was no significant interaction effect of growth, variety, and generation on soil pH. Tukey results for control (C) soil, indicated that there was no significant effect of growth, variety, or generation on pH in both varieties Kenchen and Siverka.

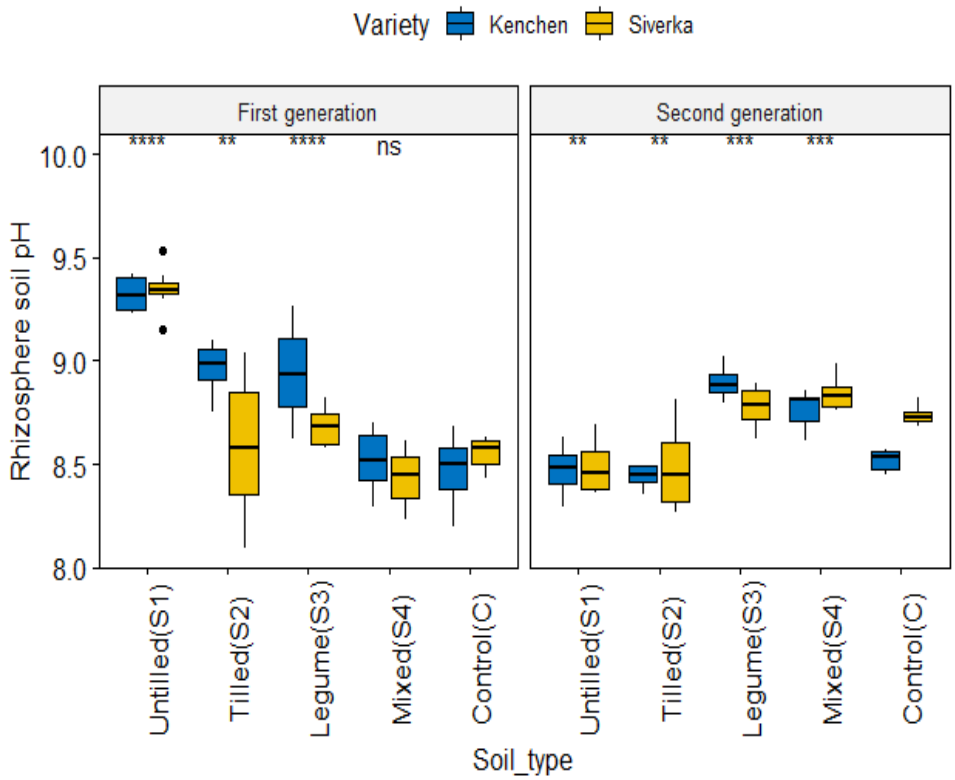


Fig. 4.9 Difference in rhizosphere soil pH between Control (C) and other treatments across both generations of plant growth in soybean varieties Kenchen and Siverka**.** Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05, ns- not significant

4.3.3.2 Available nitrate (NO_3) and Ammonium (NH_4)

The rhizosphere soil from both the low and high growing plants were analysed for available forms of nitrogen (NO_3 and NH_4). The levels in the soil were below the detection limits using a flow injection analyser (Skalar San SA1050 autosampler), <0.07 for NH_4 and <0.6 for NO_3, for both the generations.

4.3.3.3 Available Phosphorus (P)

After the first generation of plant growth, the levels of available P measured in rhizosphere soil solutions from various treatments were below the detection limits (<0.03 mgL^{-1}). Readings conducted on various occasions were inconsistent, and the data was considered unreliable for statistical comparisons.

After the second generation of plant growth, the available P measured in control soil solutions were below the detection limits (<0.03 mgL^{-1}). ANOVA on linear model with 4 soils (untilled (S1), tilled (S2), legume (S3), mixed (S4)) indicated, significant effect of soil type on available P ($F(3) = 37.43$, $p<0.001$). The interaction between soil type, variety, and growth was not significant ($F(3) = 0.0473$, $p=0.986$). Tukey tests showed that mixed (S4) (0.023 mgL^{-1}) was significantly lower in available P after the second generation of plant growth than legume (S3) (0.05 mgL^{-1}), tilled (S2) (0.049 mgL^{-1}) and untilled (S1) (0.065 mgL^{-1}). In general, the detected available P in rhizosphere soils were very low.

4.3.3.4 Available potassium (K)

The interaction effect of soil type, variety, growth, and generation was significant ($F(4) = 3.287$, $p=0.013$) on available K content. When compared to control soil, all soils (untilled (S1), tilled (S2), legume (S3), mixed (S4)) had significantly higher K content in rhizosphere soil after first generation of plant growth (Fig. 4.10). After the second generation, untilled (S1) and tilled (S2) differed significantly from control (C) in available K content, while available K in legume (S3) and mixed (S4) was not significantly different from control. Tukey tests showed that there was no significant difference ($p= 0.999$) in available K content between generations in C soil.

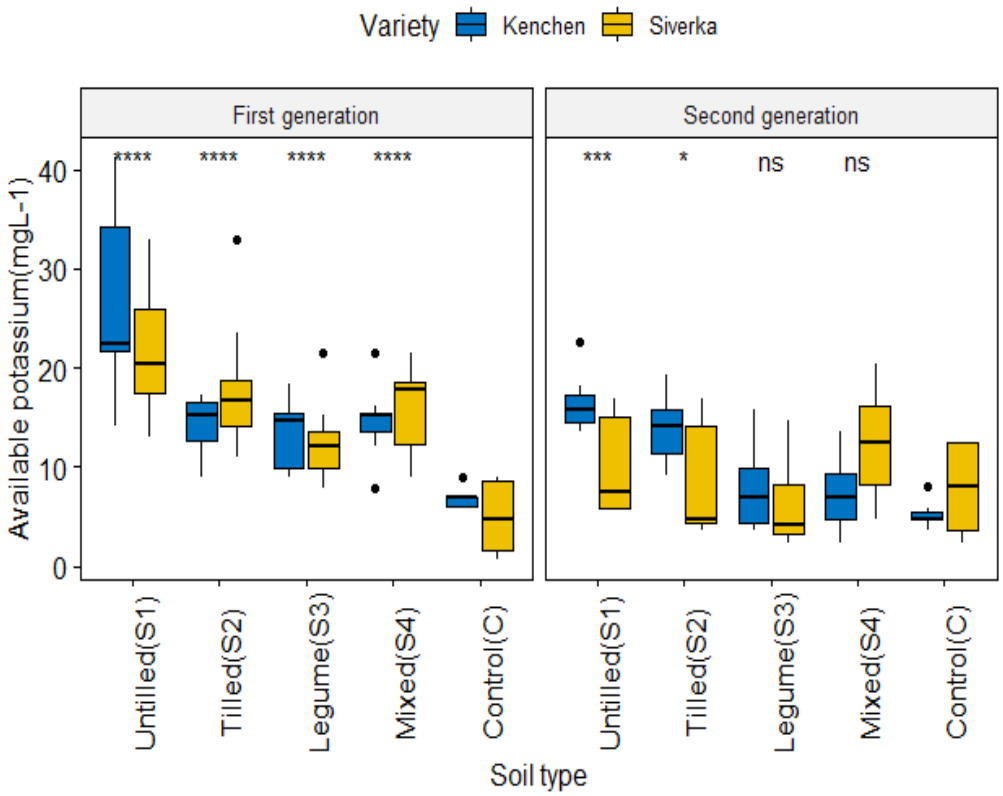


Fig. 4.10 **Difference in available potassium (mgL-1) in rhizosphere soils between Control (C) and other treatments across both generations of plant growth in soybean varieties Kenchen and Siverka.** Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05, ns- not significant

The difference in available K was not significant with growth in S1(F (1) = 0.153, p= 0.698), S2 (F (1) = 0.988, p=0.330), S3 (F (1) = 3.235, p=0.084) and S4 (F (1) = 0.010, p=0.920. The interaction between growth, generation and variety was significant (F (1) = 8.644, p= 0.009) on available K in control (C).

4.3.3.5 Total carbon (TC) in the rhizosphere soil

The interaction effect between soil type, growth, variety, and generation was significant (F (4) = 2.889, p= 0.024) on rhizosphere TC content. The average TC content in the rhizosphere increased after the second generation in control (C) (14.87 mgL^{-1}), legume (S3) (14.01 mgL^{-1}), and mixed (S4) (16.71 mgL^{-1}), whereas TC decreased in untilled (S1) (8.64 mgL^{-1}) and tilled (S2) (9.35 mgL^{-1}) after the second generation of plant growth. All the soils were significantly different from control after first generation. After the second

generation of plant growth, legume (S3) (p=0.974) and mixed (S4) (p=0.678) soils did not differ significantly from control (C) in TC content in the rhizosphere.

The effect of growth on TC content was different in different soils (Fig. 4.11). In control (C) (F (1) 0.802, p = 0.383), untilled (S1) (F (1) = 0.0002, p= 0.989) and tilled (S2) (F (1) = 0.0577, p= 0.811), there was no significant effect of growth on TC. In legume (S3), there was a significant effect of growth (F (1) = 18.25, p<0.001) on TC. The effect of growth was significant on TC in mixed (S4) (F (1) = 18.70, p<0.001) as in S3.

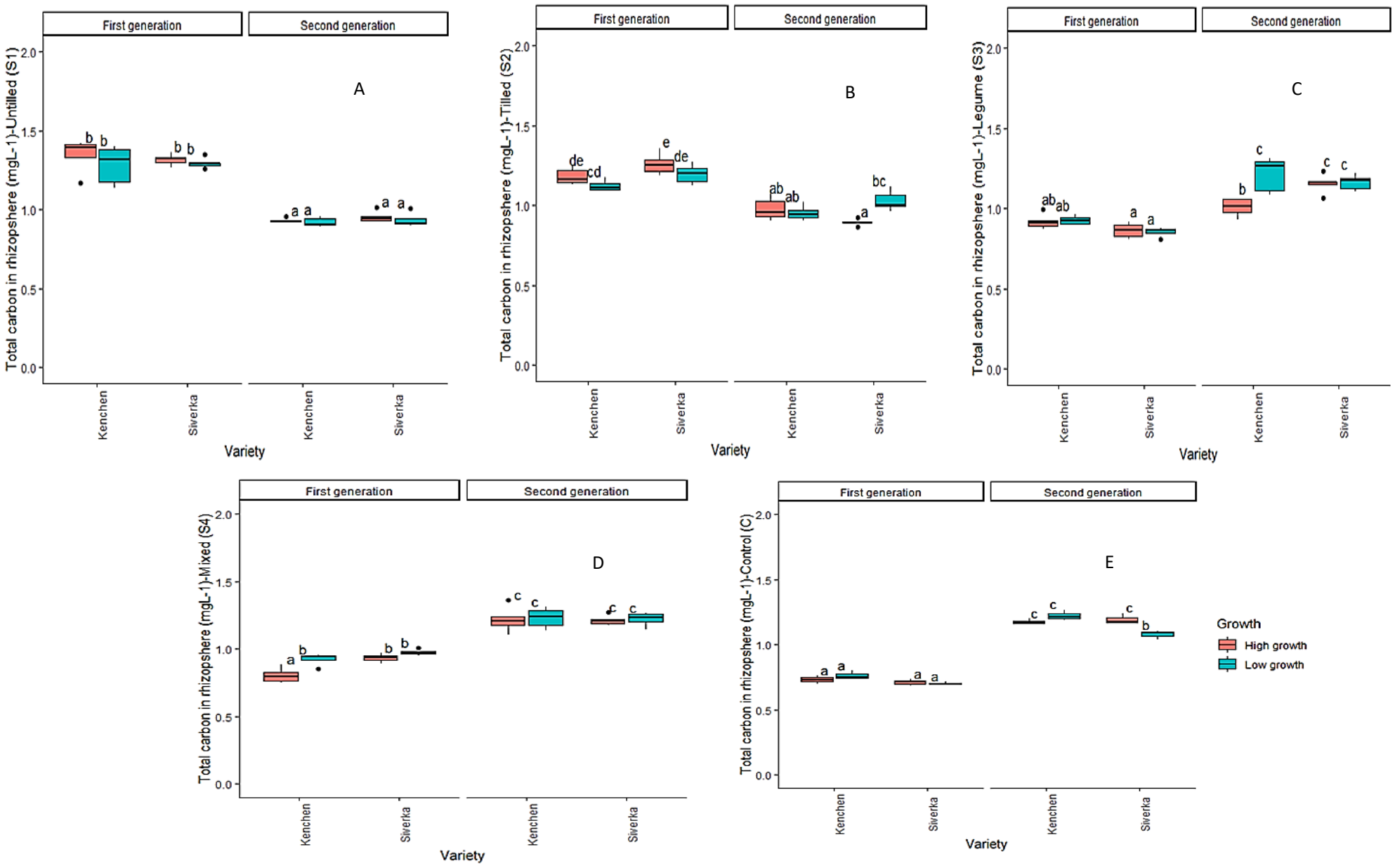


Fig 4.11 **Effects of growth, variety and generation on total carbon in rhizosphere soils** in **A. Untilled(S1) B. Tilled(S2) C. Legume(S3) D. Mixed (S4) and E. Control(C).** Boxplots with the same letter were not significantly different from each other, `c` shows highest mean, obtained by Tukey method, significance level used: alpha = 0. 05. n=6

4.3.4 Difference between fallow and cultivated in soil enzymes

4.3.4.1 Soil enzymes

In the first generation, both NAG ($F(4) = 4\ 10.42$, $p<0.001$) and PHOS ($F(4) = 4\ 18.005$, $p<0.001$) activity was found to be significantly influenced by the presence of plants and this again varied between soil types. In all the soils, the average NAG activity was higher in rhizosphere soils as compared to fallow soils (Fig. 4.12A). The PHOS activity (Fig. 4.12B) was significantly different between rhizosphere soil and fallow soil in control (C) (high in rhizosphere), untilled (S1) (high in fallow), and mixed (S4) (high in rhizosphere) after first generation. Both NAG and PHOS activity was high in the rhizosphere of control (C) soils, as compared to fallow.

In the second generation, significant difference in NAG activity (Fig. 4.12C) for fallow and rhizosphere soils was found only with soil type ($F(3) = 167.0356$, $p < 2e\text{-}16$). The results were different for PHOS activity (Fig. 4.12D) as there was significant interaction effect of soil type, variety, and cultivation ($F(3) = 94.755$, $p < 2.2e\text{-}16$) on PHOS activity. The average PHOS activity was found to be high in fallow soils in all soil types after second generation. The control fallow soils also had significantly higher PHOS activity than rhizosphere soils ($p= 0.005$). In general, PHOS activity in fallow soils, increased after the second generation.

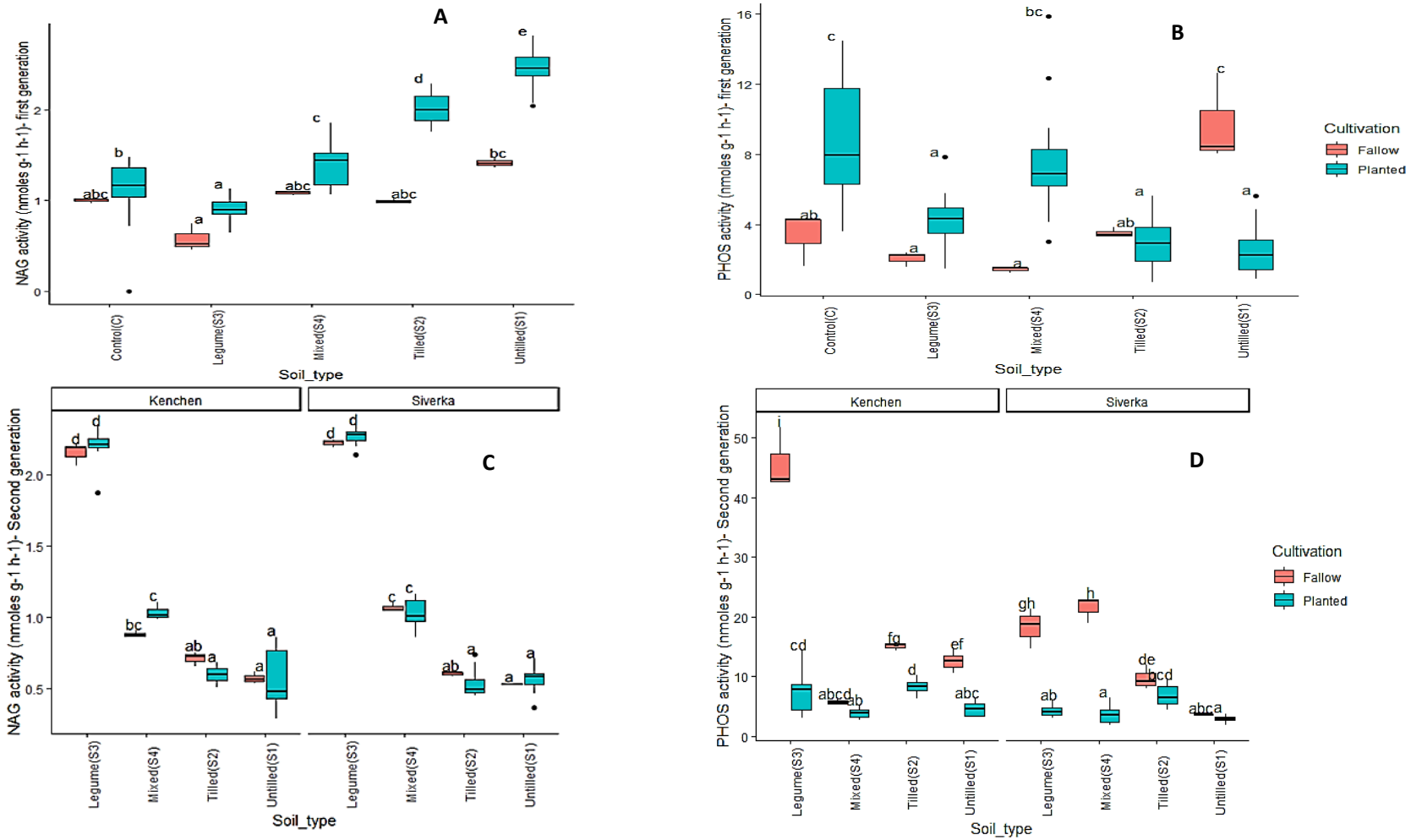


Fig. 4.12 **Difference in enzyme activity between fallow and cultivated rhizosphere soil in different soil types**, Untilled(S1), Tilled(S2), Legume(S3), Mixed(S4) and Control(C). Boxplots with the same letter were not significantly different from each other, `c` shows highest mean, obtained by Tukey method, significance level used: alpha = 0. 05. n= 3. A. First generation- N- acetyl beta glucosaminidase (NAG) activity; B. First generation- Phosphatase (PHOS) activity; C. Second generation-NAG activity; D. Second generation-PHOS activity; Fallow soils- with field microbiome and without plants, Second generation separate fallow soils for each variety, Kenchen and Siverka, control- autoclaved coir: sand without inoculation with soil suspension.

4.3.5 Correlation between soil enzymes and plant growth parameters

Based on Spearman's correlation analysis, plant height growth (n=76, r= 0.374, p<0.001) and above ground biomass (n=76, r=0.363, p=0.001) was significantly correlated to NAG activity in the rhizosphere after the second generation of plant growth. There was no significant correlation between enzyme activities and plant growth after the first generation. In both the generations, plant height and plant dry weight were positively correlated (Fig. 4.13)

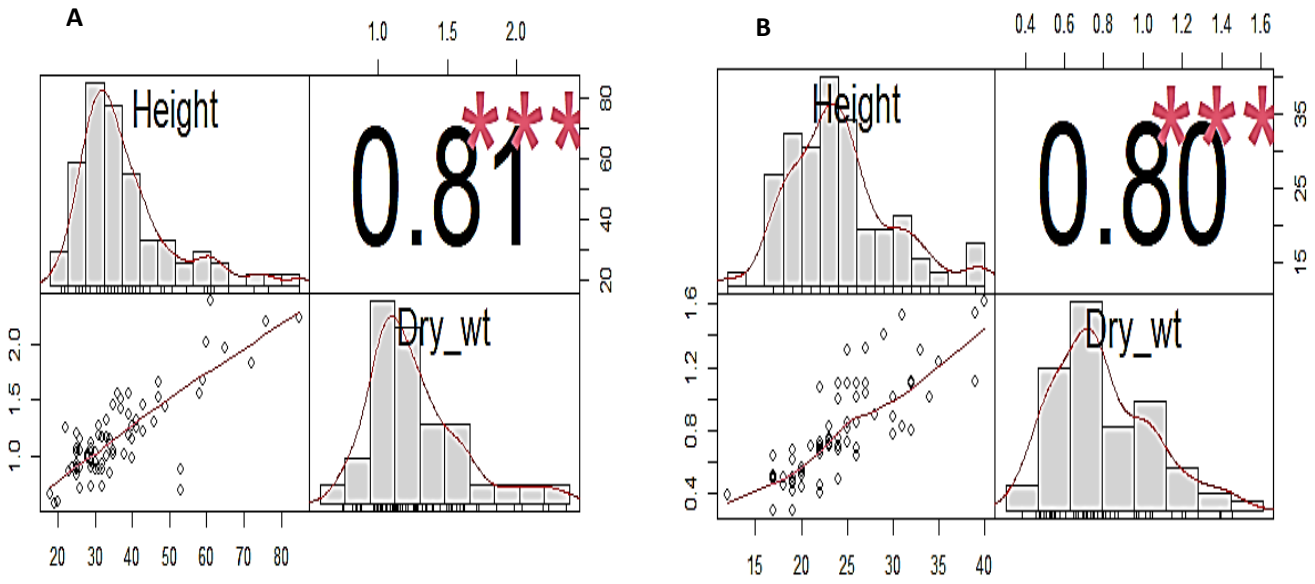


Fig. 4.13 **Correlation matrix showing dependence between plant growth variables (Height and above ground biomass (dry_wt))** *A. First generation of plant growth. B. Second generation of plant growth. n=76.* Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05, ns- not significant

4.3.6 Correlation between soil properties and enzymes

Results indicated that, NAG activity in both the generations was positively correlated to the TC (n=76, r=0.725, p<0.001; n=76, r=0.575, p<0.001 respectively for first and second generation), TOC (n=76, r=0.638, p<0.001; n=76, r=0.586, p<0.001 respectively for first and second generation), and pH (n=76, r=0.416, p<0.001; n=76, r= 0.741, p<0.001 respectively for first and second generation) in the rhizosphere soil. The enzyme PHOS was found to be negatively correlated to TC (n=76, r=-0.633, p<0.001; n=76, r= -0.381, p<0.001 respectively for first and second generation), and TOC (n=76, r=-0.672, p<0.001; n=76, r= -0.289, p<0.001 respectively for first and second generation) in both the generations of plant growth. There was a significant correlation between PHOS activity and pH (n=76, r=-0.726, p<0.001) after the first generation of plant growth but this relationship was not significant in the second generation (n=76, r= -0.128, p= 0.266). A significant positive correlation was seen between available K content in the rhizosphere and NAG activity after the first generation (n=76, r= 0.484, p<0.001), whereas there was no significant correlation noticed between NAG and K after the second generation of plant growth. Again, the PHOS activity was significantly negatively correlated (n=76, r= -0.407, p<0.001) to available K after first generation, whereas there was no significant correlation noticed between them after the second generation.

4.3.7 Correlation between soil properties in different soils

Principal component analysis (PCA) was used to identify directions along which there is maximum variation in the soil properties and based on this soils were grouped into clusters. The first two principal components (Axis Dim1 and Dim2) explained 78.9% of the variation seen in the data set from first generation (Fig. 4.14A). The variables, total carbon (TC), total organic carbon (TOC), inorganic carbon (IC), and NAG (N-acetyl beta glucosaminidase) activity contributed 81% to Dim1 and pH and PHOS (phosphatase) activity contributed 73% to Dim2. The bar plot on contribution of variables to both Dim1 and Dim2 (Fig. 4.14B) shows that TC, IC, pH and PHOS are the most important variables contributing to the variation in soils after first generation of plant growth. Available K had the least contribution to the variation. Positively correlated variables are grouped together (TOC, TC, IC, NAG, pH) and negatively correlated variable (PHOS) is placed on the opposite side of the quadrant. Tilled (S2) and Untilled (S1) soils are on the quadrant, which is high

in TC and NAG activity, whereas mixed (S4), legume (S3) and control (C) is differentiated mainly by the PHOS activity in their rhizosphere soil after the first generation of plant growth.

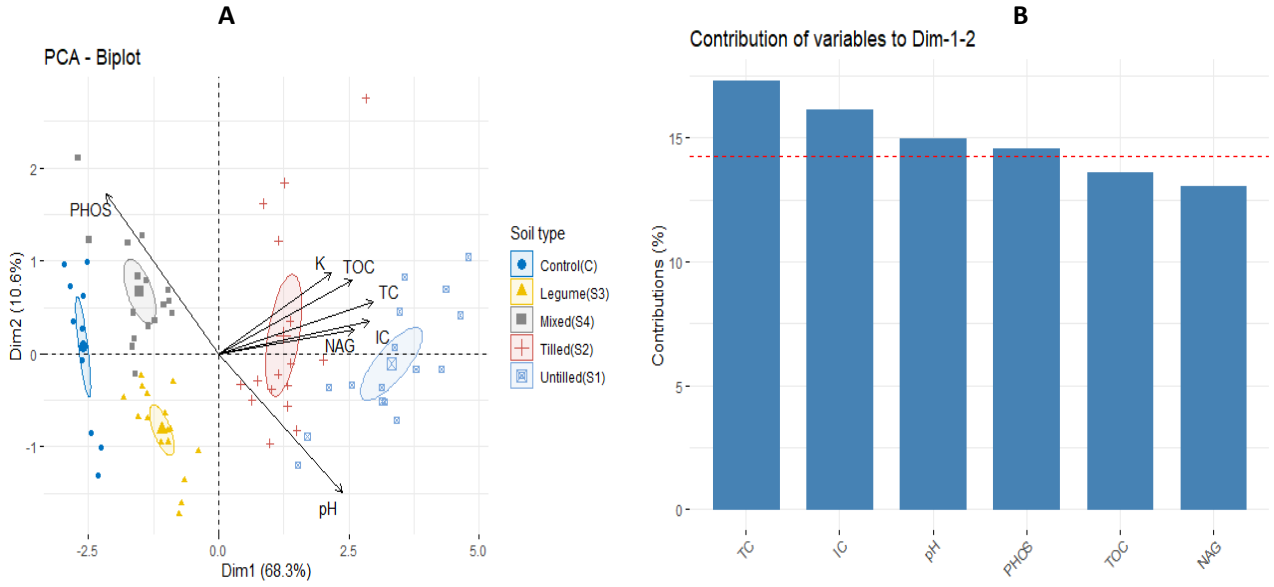


Fig. 4.14 **A. Principal component analysis biplot showing grouping of soil types and control due to variation in soil properties after first generation of plant growth**, Dim1- Dimension 1 (Principal component 1) showing 68.3% of total variation, Dim2- Dimension 2 (Principal component 2) with 10.6% of variation. 95% confidence ellipse shown around the mean of each soil type; there is clustering of soils based on difference in soil properties; Variables shown are K (available potassium), TOC (Total orgnic carbon), TC (Total carbon), IC (Inorganic carbon), NAG (N-acetyl beta glucosaminidase) enzyme activity, pH and PHOS (phosphatase) enzyme activity. **B**, Bar plot showing contribution of variables to principal components (Dim1 and Dim2), maximum contribution by TC, IC, pH and PHOS. The red dashed line on the graph above indicates the expected average contribution. For a given component, a variable with a contribution larger than this cutoff could be considered as important in contributing to the component.

The variables contributing to the difference were the same as those in the first generation with a change in their percentage of contribution (Fig. 4.15A). The first two principal components (Dim1 and Dim2) explain, 68.2% of the total variation. The variables, TC, TOC, pH, and IC contributed 84.5% to Dim1 and NAG and PHOS activity contributed to 85.28% to Dim2 (Fig. 4.15B). Most of the variation between soils after the second generation of plant growth can be explained due to the variation in carbon content, pH, and enzyme activities. Available K has little contribution to the total variation as in the first generation. The difference in legume soil (S3) is mainly due to the change in NAG activity after the second generation of plant growth. The change in carbon content was prominent for mixed (S4) and control (C), whereas tilled (S2) and untilled (S1) were again on opposite sides of the quadrant compared to the rest of the soils. Once again, pH and carbon content were positively correlated to NAG activity and negatively correlated to PHOS activity in rhizosphere soils.

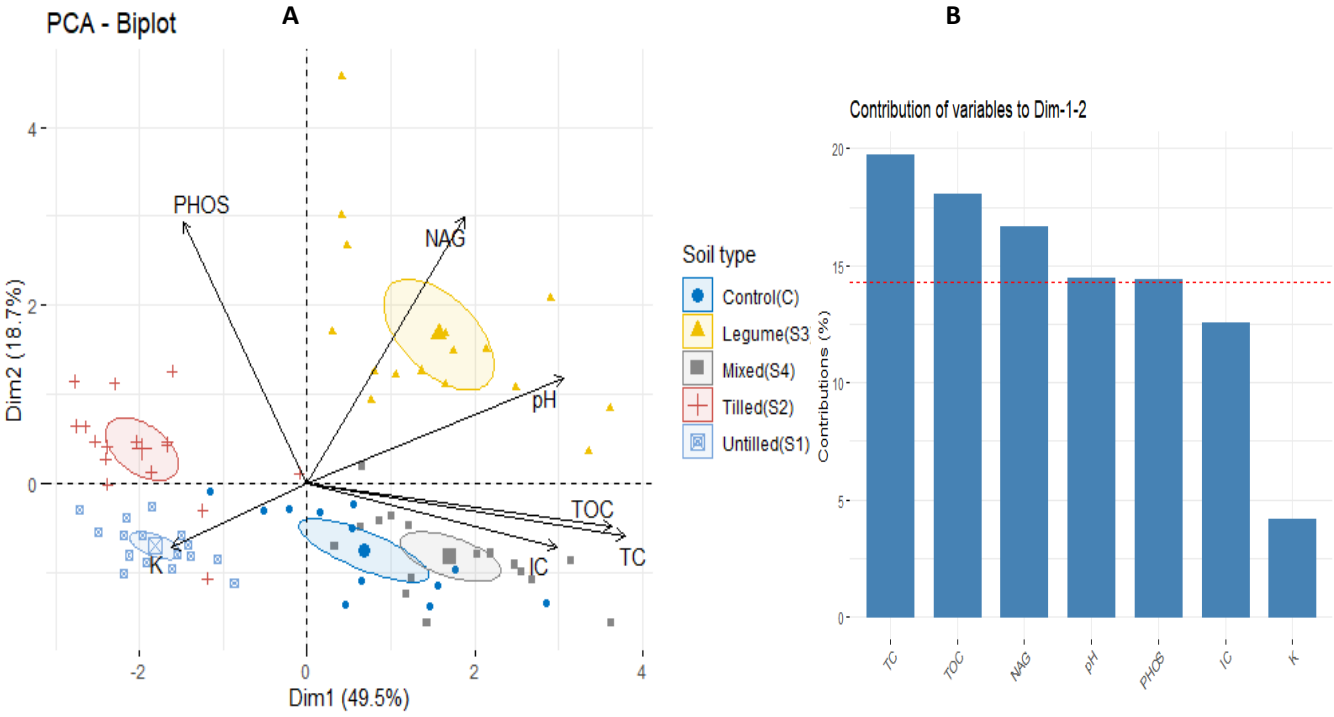


Fig. 4.15 **Principal component analysis biplot showing grouping of soil types and control due to variation in soil properties after second generation of plant growth**, A. Dim1- Dimension 1 (Principal component 1) showing 49.5% of total variation, Dim2- Dimension 2 (Principal component 2) with 18.7% of variation. 95% confidence ellipse shown around the mean of each soil type; there is clustering of soils based on difference in soil properties; Variables shown are K (available potassium), TOC (Total orgnic carbon), TC (Total carbon), IC (Inorganic carbon), NAG (N-acetyl beta glucosaminidase) enzyme activity, pH and PHOS (phosphatase) enzyme activity. **B**, Bar plot showing contribution of variables to principal components (Dim1 and Dim2), maximum contribution by TC, TOC, pH, NAG and PHOS. The red dashed line on the graph above indicates the expected average contribution. For a given component, a variable with a contribution larger than this cutoff could be considered as important in contributing to the component.

4.4 Discussion

Host mediated microbiome selection over multiple generations is an effective tool to study soil-based plant-microbial interactions. In the current study, the most important variable that influenced the activity of both studied soil enzymes (NAG and PHOS) was soil type. The interaction between plant growth and enzyme activity was significantly influenced by the soil type. We hypothesized that the difference in plant growth can be attributed to the difference in soil enzyme activities in the rhizosphere which was seen in earlier studies. The difference in enzyme activities between high growing and low growing plants, and how this differed when plants were grown in soil inoculated with microbiomes from different soil types, and the soil properties that are related to the enzymatic activities are discussed in the following sections.

4.4.1 Difference in phenotype during two generations of microbiome selection

The present study compared the activities of NAG and PHOS enzymes in the rhizosphere of soybean varieties grown in soils inoculated with microbiomes isolated from different soils and subjected to microbiome selection over two plant generations. The height and above ground dry mass of plants grown in soil inoculated with field microbiomes (untilled (S1), tilled (S2), legume (S3), and mixed (S4)) differed significantly between the two generations. The difference in plant height and above ground biomass of plants in control (C) did not vary significantly between two generations. This difference in plant height across generations in soils inoculated with microbiome indicates the effect of selection which was based on plant height. The plants were graded based on plant height and the microbiomes from six tallest plants were mixed and transferred to the plants in the second generation, with control plants receiving no microbiome. In a similar study in which the microbiome selection between generations was based on flowering, Wagner *et al*., (2014) found that the microbiome delayed flowering by 2.2 days and when sterilized soils were used as inoculum, there was no difference in the flowering time. Similarly, Panke-Buisse *et al*., (2015) found uniform flowering after multiple generations of selection. Unfortunately, in this study microbiome selection was only carried out over two generations. Uniformity in plant height across treatments is a posited outcome if experimentation were to be extended over further subsequent rounds

of plant generation. Mean differences in plant height between varieties was further reduced in the second generation. In a study on host mediated selection on tomato cultivars (Morella *et al*., 2020), through experimental passaging of the phyllosphere microbiome, it was found that host genotype shapes bacterial community composition early in passaging, explaining over 24% of variation among samples, but this diminishes over time. The difference between the varieties seems to be more prominent in the first generation than after the second generation in this study. It might be because the genotype- driven differences might become more subtle following selection (Morella *et al*., 2020).

4.4.2 Difference in soil enzyme activity after two generations of microbiome selection

The results suggested that there were significant differences in the activity of both NAG and PHOS in two generations. NAG activity in different soils after the first generation resembles the activity in the initial soil analysis conducted before any plants were grown in this medium (Chapter 3). The highest activity was recorded in untilled soil and the lowest in legume soil. This can be attributed to the enzymes associated with soil colloids and cell debris (Egamberdieva *et al*., 2010). Extracellular enzymes are easily adsorbed at organic and clay surfaces, and in this study, the untilled soil's high organic matter content helped to create soil colloids that included extracellular enzymes. The current methods available for measuring soil enzyme activity do not allow us to discriminate between the various contributions of enzyme locations (soil colloids, plant roots, microbes) to the total enzyme activity (Nannipieri *et al*., 2003). NAG activity decreased in the rhizosphere of plants grown in untilled (S1), tilled (S2), mixed (S4), and control after the second generation of plant growth, whereas it was found to be high in the rhizosphere of legume (S3) plants after the second generation. An increase in total nitrogen in the rhizosphere of plants was found to increase NAG activity in the rhizosphere (Dong *et al*., 2021). Increase in nitrogen due to the presence of Rhizobial strains in legume (S3) plants might have resulted in increased NAG activity in these soils. Egamberdieva *et al*. (2010), reported production of NAG enzyme by soybean roots. This also might have contributed to the enzyme activity in the rhizosphere which is evident in this study where there is decreased NAG activity in fallow soils (soils without plants). The activity of both the enzymes were low in fallow soils in control providing further evidence for the influence of plants in modulating nutrient cycling in soil.

PHOS enzyme activity was high in the control, a factor which can be attributed to the high organic acids present in our coir: sand mixture (Vidhana and Somasiri, 1997). This changed after the second generation of plant growth and highest activity was recorded in tilled (S2) and legume (S3). Dick *et al*., (1983) noted that the alkaline phosphatase in soils is not produced by plants, but instead is completely derived from microorganisms. Juma and Tabatabai (1988) showed that sterile corn (*Zea mays*) and soybean (*Glycine max*) roots contain acid phosphatase. The present study showed that phosphatase (alkaline) activities were enhanced in fallow soils after the second generation. A higher phosphatase activity could be associated with the basic transformation of P, mainly originating from the hydrolysis of esters and anhydrides of phosphoric acid (H_3PO_4) (Solangi *et al*., 2019) which is caused during the storage of coir. Van Holm, 1993, reported accumulation of fulvic and humic acids (mixture of organic acids) with aging of coir. These organic acids can act as substrate for the action of microbial phosphatase. The correlation between PHOS activity and available phosphorus is complex (Solangi *et al*., 2019). A study conducted by Sarapatka (2003) failed to find any correlation between colonies of bacteria actively expressing phosphatase and acid and alkaline phosphatase activity in the investigated soils.

4.4.3 Interaction between soil enzymes and plant growth

Soil type was found to be the most important factor contributing to differential enzyme activity. The interaction effect between soil, plant growth, variety, and generation were also found to be significant indicating that the difference in plant growth is attributed to the difference in enzyme activities in the rhizosphere (Panke-Buisse *et al*., 2015; Swenson. *et al*., 2000). Mueller *et al*., (2016), while selecting for plants with increased salt tolerance found that the selected microbiomes can confer salt tolerance after 1-3 selection cycles. In this study, our imposed selection pressure provided us with a microbial community better able to grow under nutrient deficit compared to the ancestral community. The plants in the second generation were found to flower earlier compared to the first generation. Across both generations, control plants were late to flower. Plants may be evolving to complete their life cycles more quickly due to enhanced nutrient cycling in the rhizosphere. In this study, NAG activity was significantly correlated to plant height and above ground biomass indicating the influence of enzyme activity on the growth of plants. The production of extracellular enzymes like NAG and PHOS is a major mechanism by which microorganisms

gain access to soil nutrients. Under nutrient limiting conditions, these organisms can access and utilise nitrogen or phosphorus that would otherwise be inaccessible for biological uptake (Sinsabaugh *et al*., 2008).

4.4.4 Influence of soil properties on enzyme activities

The results from the correlation test indicated that both enzymes NAG and PHOS were correlated to pH and total carbon during microbiome selection. One of the most important factors affecting nitrification in soil is pH (Tabatabai, 1985). There is a lag period between addition of crop residues and mineralization and this lag period was reduced by increasing the soil pH from 4 to 8 (Fu *et al*., 1987). Thus, an increase in soil pH might have induced favourable changes to the soil environment of the microorganisms and the associated enzyme systems resulting in nitrogen transformations. This is reflected in this study, where the field soils were generally acidic (pH 5.4-5- 6) and these were added to coir: sand mix (pH 8.5). The resulting changes in NAG enzyme activity proved to be beneficial for plants with NAG activity showing a positive relation with pH and plant growth. Soil organic matter is an important source of carbon in soil and is a substrate for enzymatic activity. Increased plant growth means increased root activity below ground, and this promotes turnover of litter leading to increased microbial respiration and increased soil enzymatic activity (Zhang *et al*., 2020). The result of this study shows similar influences of plant growth on NAG activity. It was observed in this study that soil carbon had a positive correlation with NAG activity and a significant negative correlation with phosphatase activity. This was observed in other studies (Peng *et al*., 2016; Feng *et al*., 2019). There have been many studies on influence of organic matter on soil PHOS activity and the results are still controversial depending on soil amendments, plant species, management practices and soil properties (Christopoulou *et al*., 2021). Chatzistathis *et al*., (2020) found a positive correlation between phosphatase activity and organic P and suggested that organic P fractions may be more useful tools to evaluate phosphatase activity.

PCA analysis showed that the grouping of soils is distinct after the second generation of plant growth and the difference is attributed to differences in soil carbon, pH, and enzyme activities. In the first generation, tilled and untilled soils were clustered closer and variables contributing to their variation were soil carbon and NAG activity. This can be explained based on the initial soil properties measured in these soils. The

source of microbiome inoculum for tilled and untilled soils was from tilled and untilled field soils and they were high in organic matter content as compared to legume soil which explains their difference in soil carbon. The rhizosphere soils from legume and mixed differed in PHOS activity. It is postulated that in legume crops (nitrogen fixing crops), nitrogen fixation entails high phosphatase activity to enhance phosphorus acquisition from organic sources (Png *et al*. 2017). The authors measured the root phosphomonoesterase activity of leguminous and non-leguminous plants and found a clear shift from nitrogen to phosphorus limitation of plant growth. They also suggested that the relatively greater root phosphatase activity of legumes compared to non-legumes is likely a phylogenetically conserved trait and not directly linked to nitrogen fixation. It was not possible to measure the nitrogen fixing ability of plants in legume and mixed in this study. However, presence of nodules in the roots in the plants in these soils suggests nitrogen fixation in these soils which might be the reason for their difference in PHOS activity.

In the second generation 68.2% of the total variation was explained by the difference in soil enzyme activities and total carbon content in soils. The properties of rhizosphere soil in legumes differed mainly by NAG activity. The increased growth of plants due to rhizobial activity might have increased the nitrogen demand in the rhizosphere which resulted in increased NAG activity. Zang *et al*. (2019) reported an increased NAG activity on addition of nitrogen to soil. Mixed soils were high in total carbon. The increase in carbon content in these soils can be attributed to the increased growth of plants in these soils which led to increased root exudation in these soils. Our soil mix (S4) led to changes in soil properties, and which reflected in the better growth of plants in these soils providing further evidence that mixing of microbiomes generates novel combinations of microbes with novel beneficial effects on a host (Mueller and Sachs 2015).

4.4.5 Conclusions

Host mediated microbiome engineering is used to indirectly select microbiomes based on plant phenotype (Mueller and Sachs, 2015). Host phenotype is used to indirectly select for beneficial microbiome-host interactions over multiple generations. In the current study, plant height was used as a trait to select for a microbiome that can produce better plant growth under nutrient limiting conditions. In this study, mixing and inoculating plants with soil from high-growth plants' rhizospheres caused a noticeable difference in the height of the plants in the second generation. Host mediated microbiome selection allows microbiomes

to change through both ecological (e.g., diversity, relative abundance) and evolutionary (e.g., extinction events, alterations in allele frequency, mutation, horizontal gene transfer) processes (Mueller and Sachs, 2015). Previous studies demonstrated that it is possible to select microbiomes for pH tolerance (Swenson *et al*., 2000) and early flowering (Panke-Buisse *et al*., 2015) using host mediated selection and the biomass of plants in these studies were suggested to be dependent on soil enzyme activities. The results from this study have enough evidence to back this suggestion that the rhizosphere enzyme activity is a strong indicator of biological activity in the rhizosphere and is responsible for nutrient content in the rhizosphere. The enzyme NAG showed significant positive correlation with plant height and above ground dry mass in this study.

The most important factor that contributed to the variation in plant growth and soil enzyme activities was the soil type or the source of microbiome inoculum. The interaction between soybean varieties and growth was significant in the first generation of plant growth and this interaction was not found to be significant after the second generation indicating that genotype- driven differences might become subtler after fewer selection cycles (Morella *et al*., 2020). The most important variables contributing to the difference between soils was soil carbon content and pH. NAG was found to be positively correlated to both pH and total carbon in the soil whereas PHOS was negatively correlated to these variables. The rhizosphere soils were found to be high in NAG activity as compared to fallow soils indicating the influence of plants on this enzyme activity. PHOS activity was found high in fallow soils after second generation suggesting microbial activity on coir producing organic acids which can act as substrates for PHOS activity.

Chapter 5: Influence of soil type and host-mediated microbiome selection on root exudation in the rhizosphere of soybean plants

5.1 Introduction

Plants modify the soil environment through their root system, changing its physical properties and exuding compounds that promote or inhibit the growth of certain microorganisms. The area near the roots which is influenced by root exudates is referred to as the `rhizosphere` (Turner *et al*., 2013). Plants release up to 20% of their photosynthetically fixed carbon in root exudates and this helps in the establishment of plant–microbe interactions that benefit plant growth, for example by increasing the availability of mineral nutrients, production of phytohormones, degradation of phytotoxic compounds and suppression of soil-borne pathogens (Bais *et al*., 2006). Plants can react to changes in their environment by releasing root exudates such as sugars, amino acids, flavonoids, aliphatic acids, proteins, and fatty acids (Badri *et al*., 2009). These compounds can attract microbes and initiate both symbiotic and pathogenic interactions within the rhizosphere (Bais *et al*., 2006). The observation that microbes are more abundant in the rhizosphere than in distant soil was initially made by Hiltner (1904), and later, Knudson (1920) and Lyon and Wilson (1921) reported a link between root exudation and microbial abundance.

Rhizodeposition consists of border cells, root debris, and root exudates and is the major source of organic carbon to enter the soil (Uren, 2007). It comprises a high carbon cost to the plant (Lynch and Whipps, 1990), but this cost is compensated by the service offered by microbes that live on the carbon deposits. In an experiment using grazing tolerant grass (*Poa pratensis*), Hamilton and Frank (2001) demonstrated that under herbivore attack, this grass species could attract microbes that facilitated the uptake of nitrogen in its rhizosphere. Some plants like White lupin (*Lupinus albus*) decreased soil pH by releasing organic acids into the rhizosphere to discourage microbial growth and thus decreased the competition for Phosphorus acquisition (Weisskopf *et al*., 2006).

5.1.1 Factors affecting root exudation

The composition and concentration of root exudate changes with the age of the plant, soil type, and the interaction of the plant with biotic and abiotic stressors (Badri *et al*., 2009; Chaparro *et al*., 2012). Soil nutrient availability has also been shown to affect plant root exudate composition. In *in vitro* studies, it has been found that different growth media can change the composition of root exudates of a particular plant species indicating the importance of soil nutrient content in root exudation. It has also been reported that nutrient deficiency enhances exudation of certain metabolites, particularly those that increase the availability of nutrients for uptake by plant roots (Jones 1998). For example, in case of iron deficiency, grape roots reduced the exudation of sugars and amino acids, to reduce microbial competition for iron (Marastoni *et al*., 2020). Neumann, *et al*. (2014) demonstrated that soil type had a significant influence on root exudation in lettuce (*Lactuca sativa* L. cv. Tizian). Zhu *et al*., (2016) reported that in maize total root exudation of sugars, sugar alcohols, and phenolic compounds was positively and significantly correlated with increasing nitrogen levels. Pine (*Pinus radiata*) roots were found to increase the exudation of amino acids under phosphorus deficit conditions (Bowen, 1969). Carvalhais *et al*., (2011) hypothesized that an increase in root exudation of carbohydrates in maize plants grown under P-deficient conditions may be a plant strategy to stimulate growth and activity of Arbuscular mycorrhizal fungi (AMF). Root architecture is genetically determined, but soil physical and chemical characteristics ultimately shape root growth in response to soil nutrients (Canarini *et al*., 2019).

By studying the exudation of ^{l4}C applied as $^{14}CO_2$ to the atmosphere surrounding the shoots of 8-day-old wheat plants, McDougall (1970) reported the effect of change in pH on root exudation. He found that at pH 5.9, ^{l4}C-containing compounds exuded by roots accounted for 20,306 disintegrations per minute (dpm), at pH 6.4 the count was reduced to 7057, and at pH 7.0 to 8595, ie there was change in the concentration of root exudation with change in pH. McDougall postulated that the change in pH in the surrounding solution near roots might have altered the ionic state of compounds released by roots and thus affected their reabsorption by roots. Meier *et al*., (2020) found that in beech forests, root exudation was negatively related to soil pH and nitrogen availability. Root exudate is a main source of organic carbon in the

rhizosphere (Chaparro *et al*., 2014) and microbial production of soil enzymes like hydrolases that depolymerize chitin and cellulose to bioavailable forms depends on total carbon content and pH (Sinsabaugh *et al*., 2010).

Root exudates and the response of micro-organisms to the exudates as well as to root morphology were shown to shape rhizosphere microbial communities (Berg and Smalla, 2009). In *Arabidopsis thaliana* accessions, genetic variants of the same plant species were found to select for markedly different rhizobacterial assemblages. (Micallef *et al*., 2009). The *Arbidopsis* accessions used in their study were from different geographical locations and hence, Micallef *et al* postulated that the discrepancies in exudation among *Arabidopsis* accessions may be indicative of co-evolutionary mechanisms that have occurred over time between plants and their local microbiota. The root exudate composition varies from plant to plant and affects the relative abundance of microorganisms in the vicinity of the root (Somers *et al*., 2004). Some plant species also contain unique antimicrobial metabolites in their exudates. Many of them are used as medical plants, for example camomile, thyme, and eucalyptus (Berg and Smalla, 2009).

Other than the factors discussed above, root exudation is also affected by changes in environmental conditions like biotic or abiotic stresses. The various factors influencing root exudation is given below in Fig. 5.1.

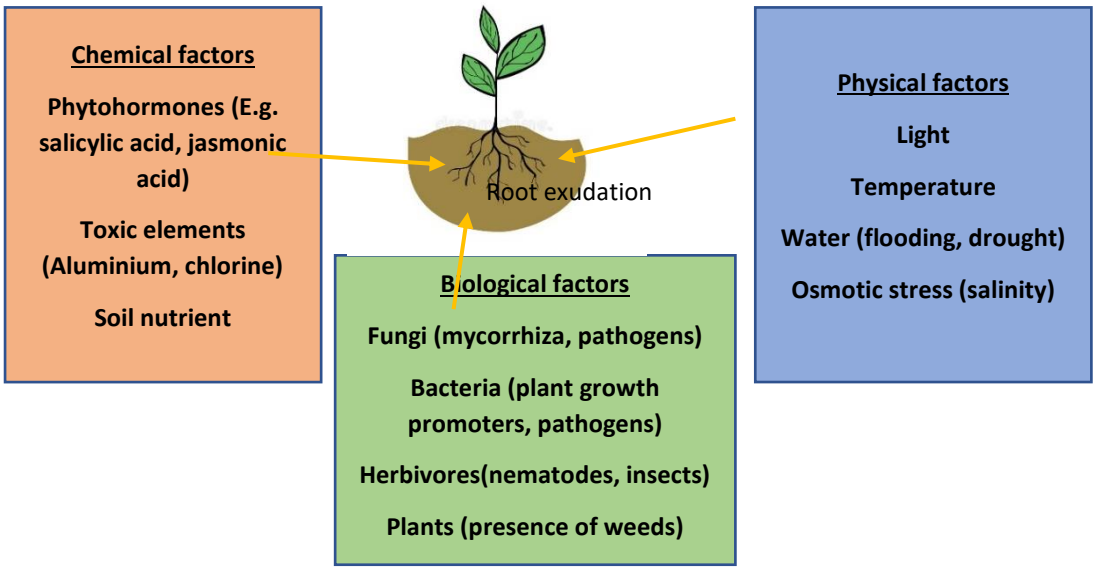


Fig. 5.1 **Factors affecting root exudation processes**. Root exudation is affected quantitatively and qualitatively by different physical, chemical, and biological factors. Vives-Peris *et al*., (2020)

5.1.2 Primary and secondary metabolites in root exudate

The long-distance transport of carbon produced at source organs (leaf) occurs through the phloem by a difference in turgor between sink (root) and source organs generated by concentration gradients, which are determined by source-sink activities (De Schepper *et al*., 2013). Sugars, amino acids, and organic acids are the major metabolites, and they operate as signalling molecules in the rhizosphere.

Amino acids are one of the main components of root exudates and are present in the soil environment at low concentrations. They are considered as important triggers of plant responses to changing N availability in soil. It has been demonstrated that amino acids are the main indicator of the N status of plants, which is important for the regulation of plant N uptake (Nacry *et al*., 2013). The export of amino acid from the leaf to the phloem depends on the N-level belowground (Caputo and Barneix, 1997). In Poplar trees (poplar hybrid, *Populus tremula*×*P. alba*), exogenous application of amino acids reduced the uptake of nitrate by plants (Dluzniewska *et al*., 2006). Aslam *et al*., (2001) suggested that it is not a single amino acid, but their overall concentration that regulates root system architecture.

Uptake of different N forms by plant roots can also affect root exudation patterns. Mahmood *et al*., (2002) found that switching plants from ammonium to nitrate nutrition in hydroponics rapidly downregulated sugar efflux by 30-fold. An important mechanism noticed during P deficiency in soils was the exudation of organic acid malate (Mora-Macias *et al*., 2017). They found that malate exudation is involved in the signalling of P deficiency. Malate is involved in the accumulation of iron in the root apical meristem, causing root apical meristem exhaustion (arrests cell proliferation) and inhibits primary root growth ultimately resulting in lateral root proliferation for P acquisition. Carbohydrates were also found to initiate a similar response to P deficiency but the exact pathway through which sugars can activate the P starvation response is yet unknown (Karthikeyan *et al*., 2007).

Phenols are an important class of secondary metabolites exuded by plants that can alter microbial abundance, activity, and community composition (Zwetsloot *et al*., 2020). Phenolics can also inhibit enzyme activity by inducing the precipitation or oxidation of proteins (Salminen and Karonen, 2011), slowing down organic matter decomposition and decreasing nutrient availability. Flavonoids are the largest group of naturally occurring phenolic compounds and are found to have many biological activities including an antimicrobial, antiulcer, antiarthritic, antiangiogenic, anticancer, and involved in protein kinase inhibition and in mitochondrial adhesion inhibition (Sulaiman and Balachandran, 2012). Flavonoids shape rhizosphere microbial community structure by providing a potential source of carbon and some of these compounds are toxic to microbes (Shaw *et al*., 2006). Legume plants exude flavonoids continuously and the concentrations in the rhizosphere increase significantly in the presence of compatible rhizobial strains (Schmidt *et al*., 1994). Phytoalexins and phytoanticipins are a group of isoflavanoids that are produced by plants, especially legumes in response to diseases (Dixon *et al*., 2002).

5.1.3 Importance of studying soil-plant-microbe interactions

Root tips function as control centres for sensing external nutrient concentrations and help in altering the root architecture and act as exudation site of sugars, amino acids, and organic acids (Canarini *et al*., 2019). The concentrations of exudates at root tips depend on the nutritional status of the plant and the soil environment. Thus, the flow of root exudates determines the growth and the development of the root by

affecting temporary concentrations of organic solutes at the root tip (Canarini *et al*., 2019). Soil microorganisms also play a major role in this by creating a difference in source – sink concentration by their consumption of organic solutes from exudates. Root-microbe interactions can affect whole plant growth, particularly via effects of rhizosphere microbes on root system architecture and consequent effects on plant aboveground performance (Verbon and Liberman, 2016). These interactions also have strong effects on the whole plant and on nutrient cycling. Guyonnet *et al*., (2018) observed that root exudation is linked as a functional trait to nutritional strategies in six grass species belonging to the Pooideae subfamily. Thus, an ecosystem is influenced by key interactions between plant nutritional strategy (and therefore its root exudates) and microbes (Canarini *et al*., 2019).

Microorganisms in the rhizosphere play important roles in the ecological fitness of their plant host (Nihorimbere *et al*., 2011). Plants have been observed to attract and cultivate a diverse rhizosphere population, which may explain why it is difficult to introduce beneficial root-colonizing bacteria and fungi in a field experiment (Hirsch and Mauchline, 2012).The plant-microbe interactions are complex, and many of the experiments studied these interactions by simplifying the interactions that occur to an individual plant-microbe relationship, but these interactions involve a vast array of microbes, and often produce synergistic effects (Mendes *et al*., 2011). It is therefore necessary to move away from the potentially simplistic view of individual plant-microbe interactions and consider all the factors that influence this complex ecosystem. As described in previous chapters, host mediated microbiome engineering provides an excellent platform to study the influence of the complex plant-soil-microbe interactions on various parameters. Understanding the role of root exudates in plant nutrient status and microbial competition has great significance in agricultural productivity. With calls for sustainable productivity, it is important to understand below ground interactions that facilitate nutrient cycling. More research is needed to completely understand the significance of root exudates in nutrient foraging by microbes and plant recognition, as well as to successfully construct combinations of plant species and soil microbes that promote sustainability in food production (Chen *et al*., 2018). Host mediated microbiome selections provides us with a platform to study soil-plant-microbe interactions. The metabolic changes happening during this interaction might give us an insight into how plants recruit specific microbial communities for

their requirement (Mueller *et al*., 2016). In many of the root exudation studies, plants were grown in either pure nutrient solution or in semi-hydroponic conditions with a solid support (e.g., quartz sand, perlite). These techniques may provide results that are not representative for soil conditions, since the quantity and quality of root exudates may largely differ from a soil environment (Mimmo *et al*., 2011). In this study, my focus was on the difference in exudation of carbohydrates, phenols, and amino acids by soybean varieties during microbiome selection in two generations of plants grown in different soil types. These soils were from different areas of a farm that differed in their cultural practices and cropping history. Plants were grown in autoclaved coir: sand mix which was inoculated with the soil suspensions from these soil types.

The aims of the study were:

1. To study the effect of host mediated microbiome selection on root exudation of carbohydrates, amino acids, and phenols. Previous studies on microbiome selection have found a decrease in alpha diversity (species richness) of microbes during the selection process (Jochum *et al*., 2019). The exudation in plant is believed to be controlled by the concentration gradient between source (leaf) and sink (rhizosphere) and this gradient is created by the utilisation of compounds from exudates by microbes in the rhizosphere (Canarini *et al*., 2019) I postulate that, due to the difference in species richness during selection process, there will be a change in the utilisation of exudate compounds in the rhizosphere which might cause a difference in root exudation from plants in the second generation.
2. To test if there are differences in root exudation between plants grown in different soil types. Based on results from previous studies showing effects of soil nutrients on exudation patterns (reviewed above), I hypothesise that there will be difference in exudation pattern in the soils studied.
3. To investigate differences in phenols, carbohydrates and amino acid content between fallow soils and rhizosphere soils. Since fallow soil is not under the influence of root exudation, any differences in the composition and concentrations of compounds between the two soil types is likely to be caused by root exudation.

5.2. Methodology

5.2.1 Plant growth and collection of root exudates

Soybean crop (varieties Kenchen and Siverka) were grown in a glasshouse in pots containing microbiome inoculum from different field soils (pot preparation, microbiome inoculation and planting explained in Section 2.4). The methods used for exudate collection and analysis are explained in Chapter 2 (Section 2.5, section 2.9).

5.2.2 Estimation of amino acids

The amino acid content in root exudates was measured using Gas Chromatography- Mass Spectrometry (GCMS) and it was possible to do the estimation for only the second generation in this study. The methodology is explained in Chapter 2 (Section 2.9.3). The ionic mass (m/z) and retention time (RT) of amino acids quantified is given in table below (Table 5.1).

Table 5.1 List of amino acids identified in root exudates of plants using gas chromatography-mass spectrometry (GCMS) with mass to charge ratio (m/z) of quatified ion and their retention time (RT) in minutes.

Amino acids	RT (min)	Quant ion (m/z)
Alanine	1.48	130
Glycine	1.59	116
Valine	1.80	158
Leucine	2.02	172
Isoleucine	2.08	172
Threonine	2.30	101
Serine	2.34	146
Proline	2.42	156
Asparagine	2.52	69
Aspartic acid	3.09	216
Methionine	3.13	61
Glutamic acid	3.47	230
Phenylalanine	3.50	148
Glutamine	4.14	84
Ornithine	4.54	156
Lysine	4.81	170
Tyrosine	5.30	107
Tryptophan	5.60	130

5.2.3 Statistical analysis

All the statistical analyses were carried out using R software (Version 4.1.2, R Core Team, 2021). Linear models were used in the study. Analysis of variance (ANOVA) was carried out to test for the influence of soil type, plant varieties, growth, and generations on concentrations of carbohydrates, phenols, and amino acids. Graphs were plotted using ggpubr package (version 0.4.0) and package ggplot2 (version 3.3.5, Wickham (2016)).

5.3 Results

The two varieties of Soybean were grown in glasshouse under controlled conditions in autoclaved coir: sand mixture inoculated with soil suspension (microbiome inoculum) from field soils. Autoclaved coir: sand mixture without any microbiome inoculum was used as control. Rhizosphere soil from the tallest plants in the first generation was used as the microbiome inoculum for the second generation. Root exudates were collected from plants immediately after rhizosphere soil sampling.

5.3.1 Effect of soil type, growth, and variety on root exudation

5.3.1.1 Phenols

The interaction between soil, growth, variety, and generation had a significant effect ($F = 5.45$, $df=4$, $p<0.001$) on total phenol exudation. The total phenol exudation was highest in plants grown in legume soil (S3)., whereas exudation of phenols from plants in other soils did not differ significantly from each other (Fig. 5.2). Tukey tests revealed that there was no significant difference in total phenol content between varieties in both the generations. In general, the difference in exudation of phenols was not significant between generations.

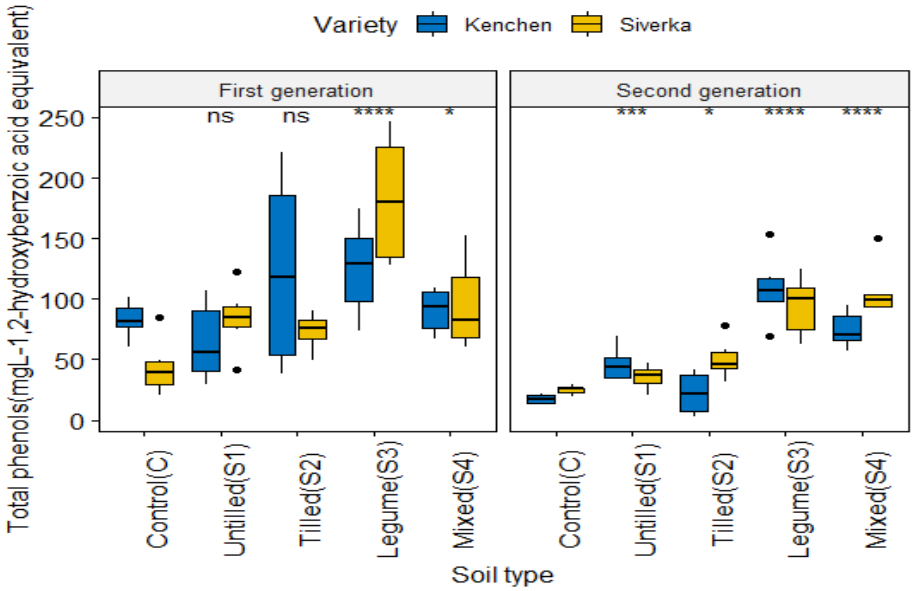


Fig. 5.2 **Difference in total phenols in root exudates of plants in Control (C) and other treatments in two generations of plant growth in soybean varieties Kenchen and Siverka.** Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05, ns- not significant

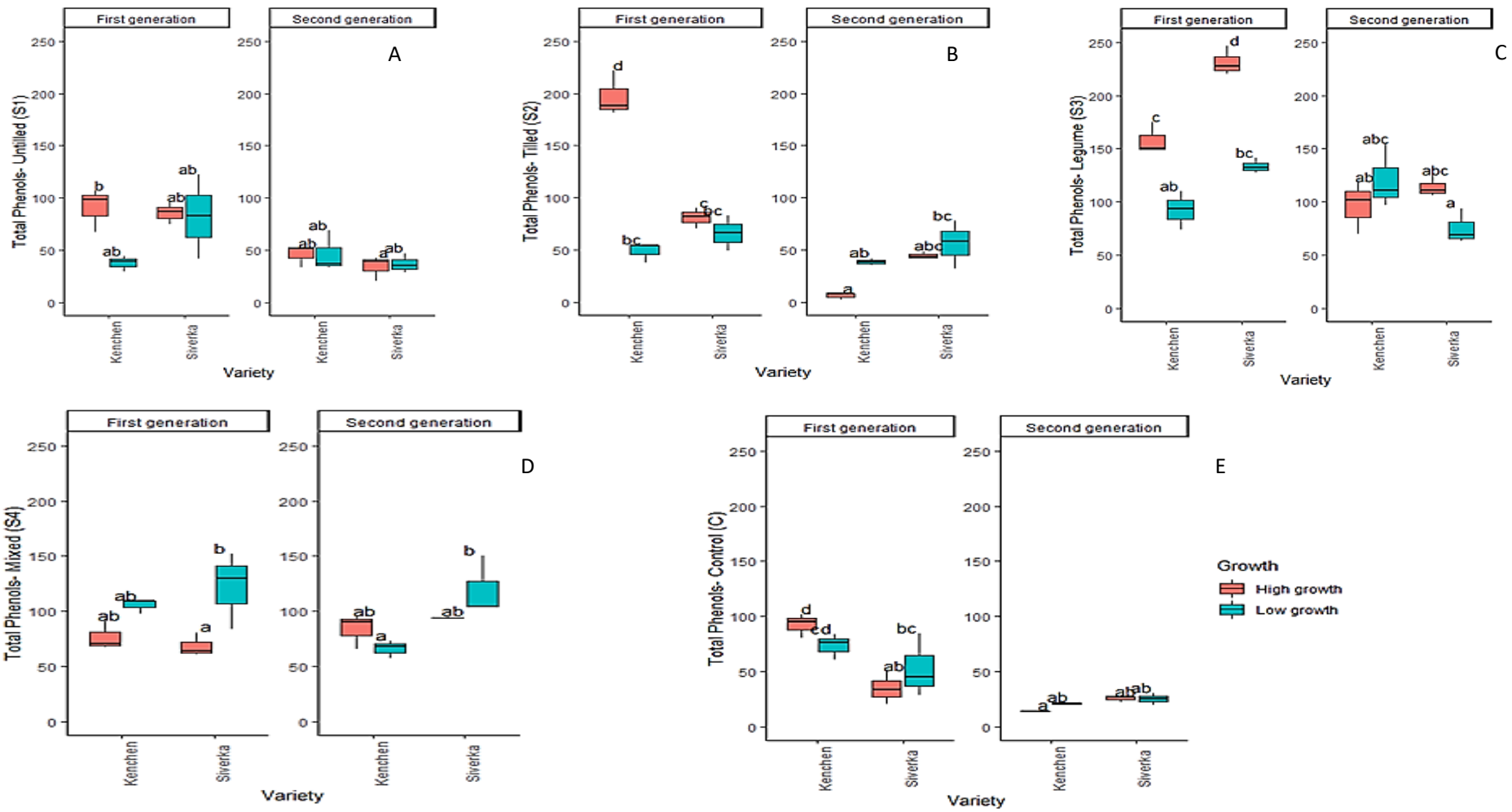


Fig 5.3 **Effects of growth, variety and generation on total phenols in root exudate of plants grown in different soil types A**. Untilled(S1) **B**. Tilled(S2) **C**. Legume(S3) **D**. Mixed (S4) and **E**. Control(C). Phenols measured as mg L-1, 2-hydroxybenzoic acid equivalent. Different letters show significant influences ($p < 0.05$) using Tukey's multiple range tests. n=6

The total phenol content from plants in each soil type was analysed separately to see the effect of growth. In Untilled (S1) (Fig. 5.3A), the effect of growth was not significant ($F = 2.9069$, $df = 1$, $p = 0.107$) on root exudation of total phenols. In tilled (S2), Tukey test results (Fig. 5.3B) showed that the effect of growth was significant on total phenol content only in variety Kenchen in first generation ($p=0$).

In legume (S3), the effect of growth had significant effect on total phenol content ($F = 35.84$, $df = 1$, $p<0.001$) in first generation of plant growth, with High growth plants (tall plants) having significantly higher phenol content than Low growth plants (short plants). Tukey results (Fig. 5.3C) indicated that the difference in total phenol content was not significant with growth in the second generation.

In both mixed (S4) and control (C), the effect of growth was not significant within each variety (Fig. 5.3D, E).

5.3.1.2 Carbohydrates

The interaction between soil type, growth, variety, and generation was significant ($F = 4.62$, $df = 4$, $p = 0.002$) on total carbohydrate content in root exudate. The highest exudation was noticed in plants grown in legume (S3) soil followed by mixed (S4). The lowest was in control (C). In first generation, the root exudation of carbohydrates was significantly higher than control (C) in all other soils (untilled (S1), tilled (S2), legume (S3), and mixed (S4)), whereas in second generation, plants in legume (S3) and mixed (S4) had significantly higher exudation of carbohydrates than control (C) (Fig. 5.4). There was no significant difference in root exudation of carbohydrates by plants between two generations within soil types.

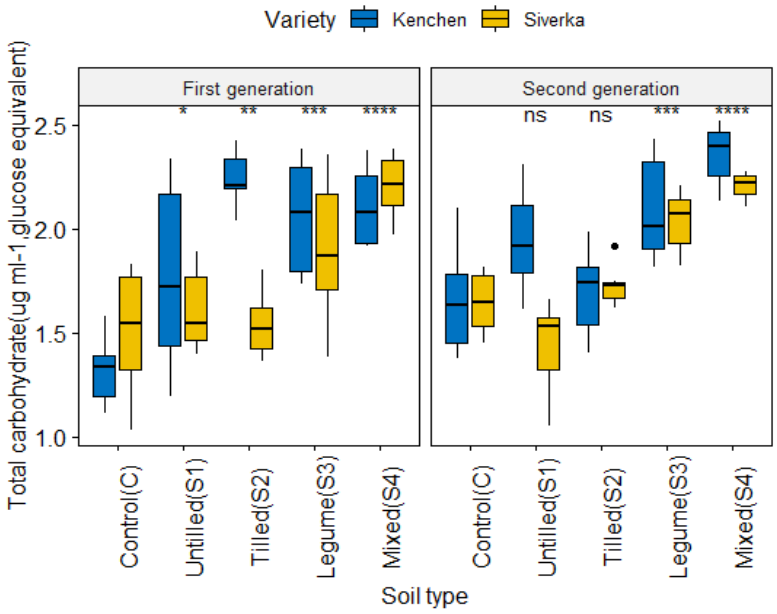


Fig. 5.4 **Difference in total carbohydrates in root exudate of plants in Control (C) and other treatments in two generations of plant growth in soybean varieties Kenchen and Siverka.** Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05, ns- not significant

The data from each soil was analysed separately to understand the effect of growth in each soil type. ANOVA results indicated that there was significant difference in root exudation with growth and variety in soil untilled (S1). Tukey results (Fig. 5.5A) showed that the difference was significant between High growth plants in Kenchen and Siverka and within varieties, only Kenchen showed significant difference in carbohydrate exudation with growth in first generation (p= 0.029).

In tilled (S2), the interaction between varieties and growth was significant only in first generation. Tukey results (Fig. 5.5B) indicated that the difference in root exudation of carbohydrates was not significant with growth in the second generation.

In legume (S3) (Fig. 5.5C), there was a significant difference in root exudation with growth, for both Kenchen ($p=0.002$) and Siverka ($p<0.001$) in first generation, whereas in second generation, the effect of growth was significant in Kenchen ($p=0.009$) and not significant in Siverka ($p=0.26$).

In mixed (S4), the difference in growth affected root exudation of carbohydrates only in variety Kenchen in first generation ($p=0.01$). The difference in carbohydrate exudation by roots was not affected by growth for both varieties in second generation (Fig. 5.5D). The difference in growth was significant ($F = 19.8127$, $df = 1$, $p<0.001$) on carbohydrate exudation in control (C) soil (Fig. 5.5E).

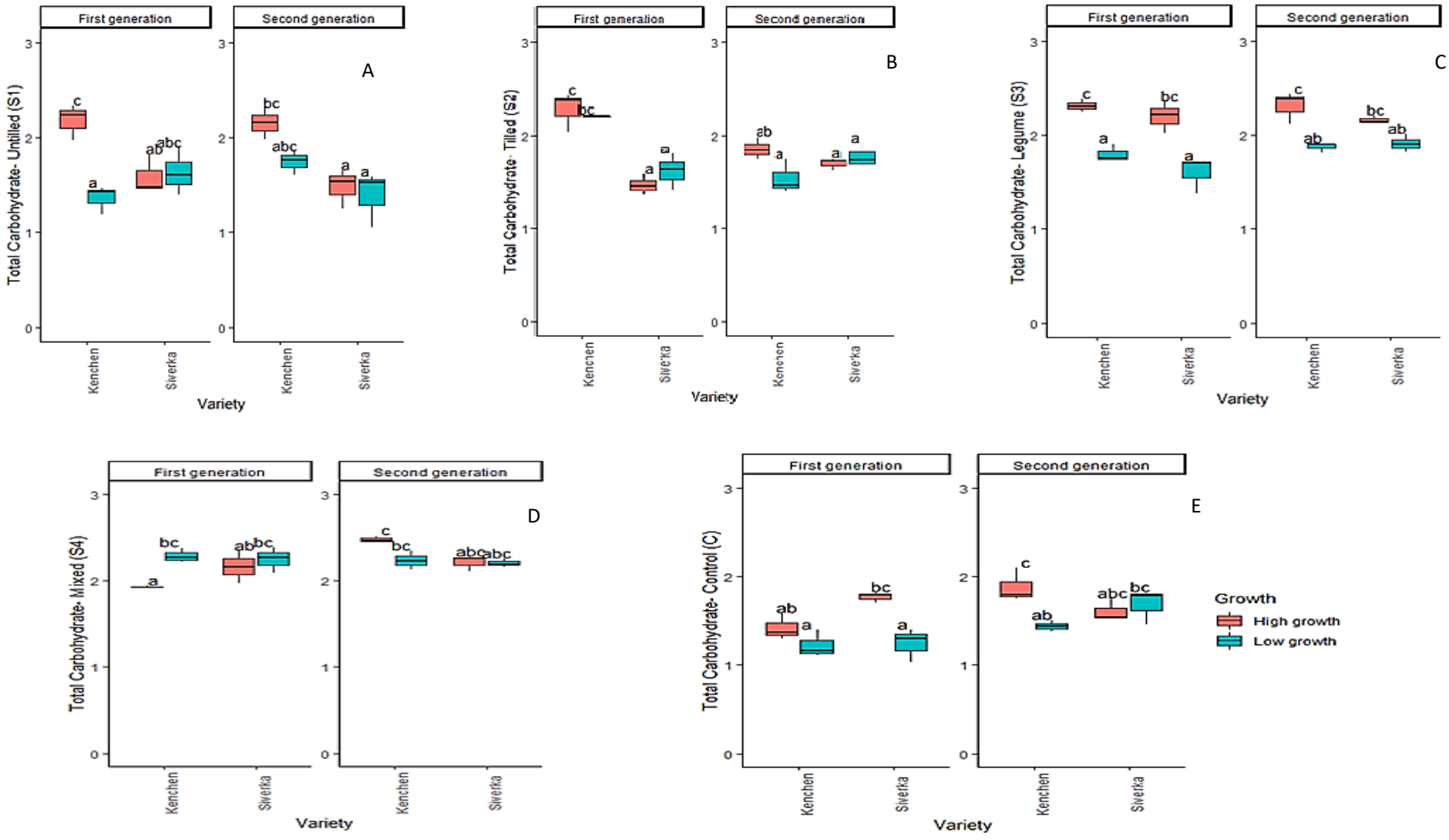


Fig 5.5 **Effects of growth, variety and generation on total carbohydrates in root exudate of plants grown in different soil types A**. Untilled(S1) **B**. Tilled(S2) **C**. Legume(S3) **D**. Mixed (S4) and **E**. Control(C). Carbohydrates measured as ug ml-1 glucose equivalent Different letters show significant influences ($p < 0.05$) using Tukey's multiple range tests. n=6.

5.3.1.3 Amino acids

A larger number of amino acids were detected in legume (S3) and mixed (S4) soil than in other soil types. Amino acids tryptophan, tyrosine, ornithine, methionine, lysine, and asparagine were detected in legume (S3) and mixed (S4) but was not detected in other soils. The relative concentration (%) of amino acids in each soil type is given in Fig. 5.6. Glycine and serine were found in high relative percentage concentration in untilled (S1) (13.75% and 13.62% respectively), tilled (S2) (8.83% and 6.8% respectively) and C (9.08% and 6.63% respectively). In legume (S3), percentage relative concentration was high for tryptophan (6.8%) and in mixed (S4), highest percentage was for glycine (3.75%). Total amino acid concentration (Fig. 5.6) in root exudates was significantly affected by soil type ($F = 13.29$, $df = 4$, $p<0.001$), variety ($F = 12.63$, $df = 1$, $p<0.001$), and growth ($F = 8.35$, $df = 1$, $p<0.001$). The interaction between growth, soil type, and variety had no significant influence on total amino acid content ($F = 2.29$, $df = 4$, $p= 0.075$).

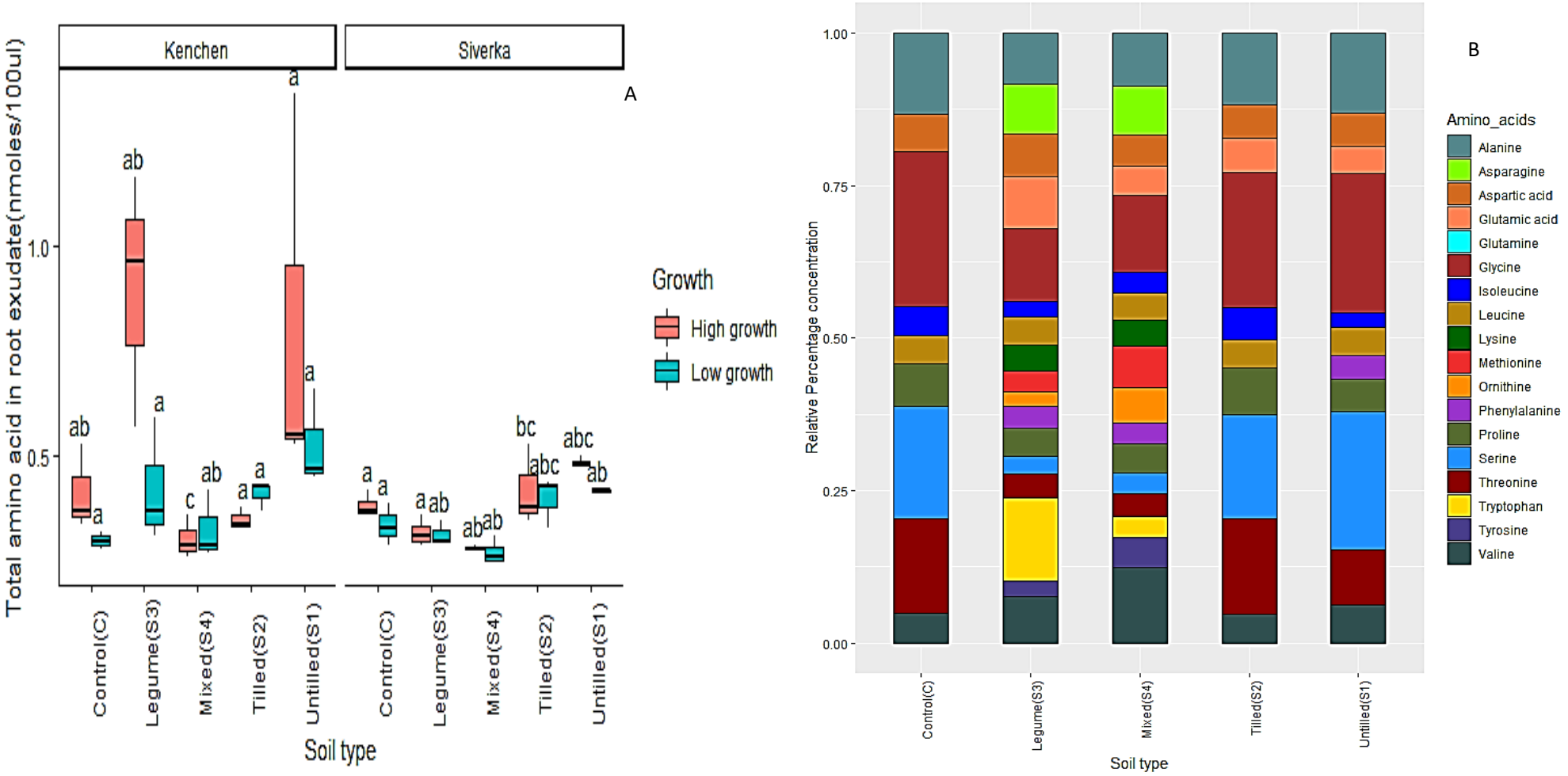


Fig. 5.6 ***A. Effects of growth and variety on total amino acids in root exudate in Untilled(S1), Tilled(S2), Legume(S3), Mixed (S4) and Control(C) soils after second generation of plant growth*** **in soybean genotypes, Kenchen and Siverka**. Total amino acidss *measured as* nmoles per 100 µl of root exudate *Different letters show significant influences ($p < 0.05$) using Tukey's multiple range tests*. *n= 6*. B. ***Relative*** **concentration (%) of amino acids identified in the root exudates of soybean plants grown in different soils in the second generation.** Control (C), Untilled (S1), Tilled (S2), Legume (S3), and Mixed (S4). Data pooled from varieties (n=6).

The concentration of amino acids detected in the root exudates of plants were low, ranging from 0.01 n moles per 100µl of exudate to 0.13 n moles per 100µl of exudate. The effect of growth and variety was not significant in untilled (S1), tilled (S2), mixed (S4), and control (C). There was significant effect of growth and variety in exudation of amino acids in legume (S3) soil, but results from Tukey test showed that the difference was significant only in variety Kenchen ($p=0.03$).

5.3.2 Phenols, amino acid, and carbohydrate in fallow soils

The phenol and carbohydrate contents in fallow soil was lower than in root exudates. In both the generations, total phenol content was not significantly different between fallow soils in all the soil types (Fig. 5.7. A,C). The difference in phenol content in fallow soils between two generations was significant ($F = 70.57$, $df = 4$, $p<0.001$). The difference in phenol content between fallow soils and root exudate was significant only for legume (S3) and mixed (S4) soils.

The carbohydrate content in fallow soils of all soil types was not significantly different in the first generation (Fig. 5.7B). The carbohydrate content in root exudates were high compared to fallow soils in all soils. In the second generation, the carbohydrate content in legume (S3) fallow soil and untilled (S1) fallow soil was significantly higher than other soils (Fig. 5.7D).

Amino acids detected in fallow soils is given in Fig. 5.8. No peaks were identified in control fallow soils. The percentage concentration of Glycine, serine and threonine detected were high in tilled (S2) and untilled (S1). In legume (S3), the amino acids tryptophan and methionine, which were detected in root exudate were not detected in fallow soils. Amino acid glycine was found to be high in all fallow soils.

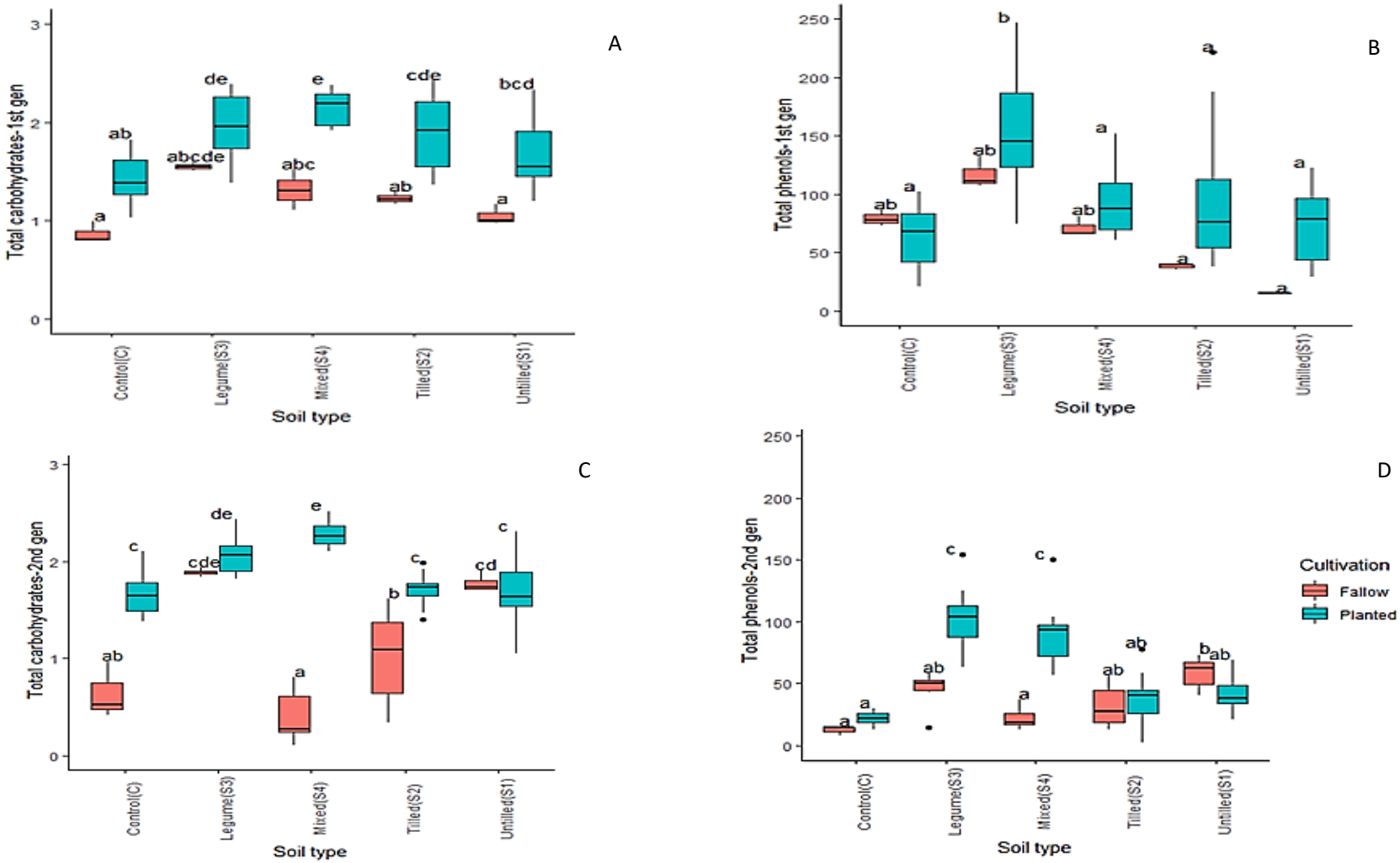


Fig. 5.7 **Difference in total phenols and carbohydrate content between fallow and root exudates in different soil types**, Untilled(S1), Tilled(S2), Legume(S3), Mixed(S4) and Control(C). A. First generation- Total carbohydrates in fallow soil and root exudates; B. First generation- Total phenols in fallow soil and root exudates; C. Second generation- Total carbohydrates in fallow soil and root exudates; D. Second generation- Total phenols in fallow soil and root exudates. Carbohydrates measured as ug ml-1 glucose equivalent; Phenols measured as mg L-1, 2-hydroxybenzoic acid equivalent. Different letters show significant influences ($p < 0.05$) using Tukey's multiple range tests. n= 3.

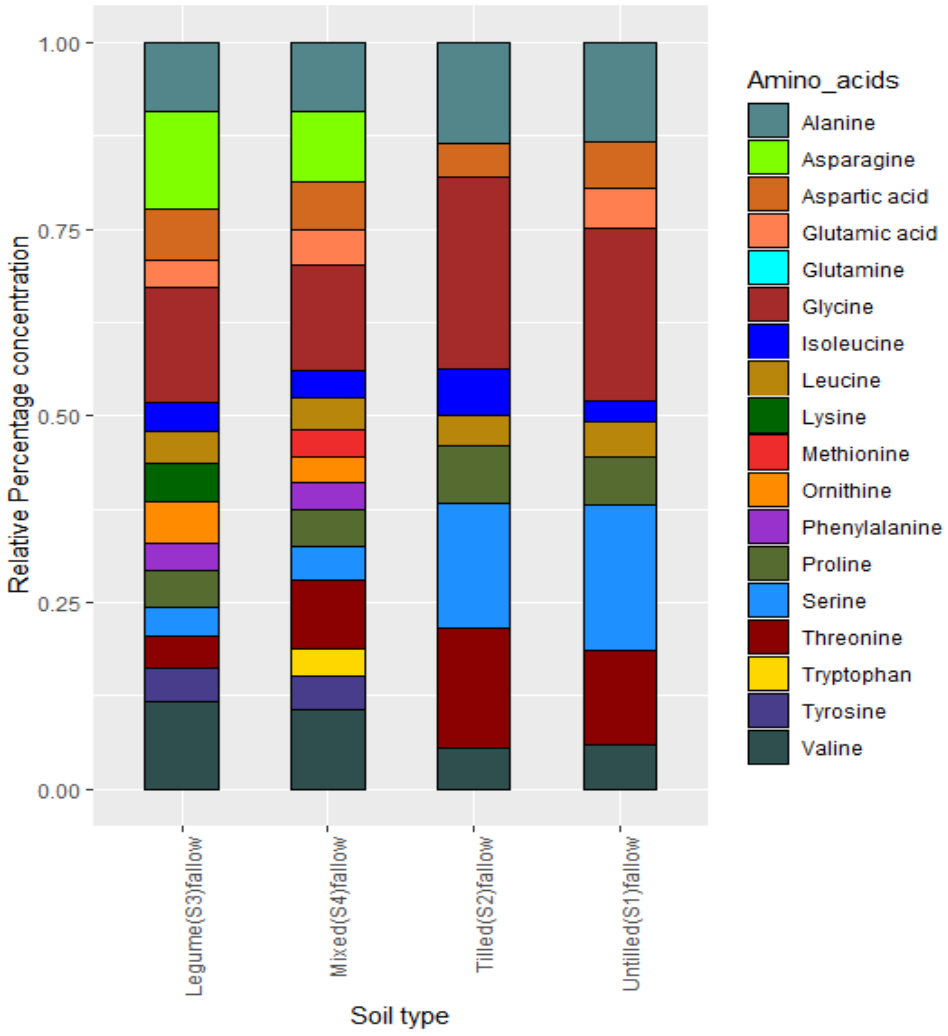


Fig. 5.8 **Second generation- amino acids detected in fallow soils of legume(S3), mixed (S4), tilled (S2) and untilled (S1).** Peaks not quantified in control fallow

5.4 Discussion

Root exudation is an important process that determines rhizosphere functions and plays an important role in plant-soil-microbe relationships. It is one of the main sources of organic carbon in the soil. This carbon released is in the form of low and high molecular weight compounds like sugars, organic acids, amino acids, phenols, fatty acids, enzymes, and growth regulators (Bais *et al*., 2006). These compounds in the root exudate creates a rich but also a competitive environment for microbial growth. In this study, the purpose was to understand the changes in root exudation by soybean varieties, Kenchen and Siverka, during microbiome selection based on host phenotype. Root exudation depends on the concentration gradient between source (leaf) and sink (rhizosphere) (De Schepper *et al*., 2013). This means that there will be an increase in root exudation based on the demand in the rhizosphere and previous studies on microbiome selection (Jochum *et al*., 2019) has recorded a change in alpha diversity (species count) during microbiome selection. Based on this it was hypothesised that there will be difference in root exudation between generations of plant growth and this will depend on soil type, plant growth and plant variety.

5.4.1 Effect of host mediated selection of microbiome on root exudation

In this study, the difference in root exudation of carbohydrates and phenols between two generation of microbiome selection was studied. In general, the concentration of both phenols and carbohydrates in root exudates did not differ significantly between generations in all soils. But plants in both legume (S3) and mixed (S4) had higher concentration of phenols and carbohydrates than control in both the generations.

Each generation of host mediated microbiome selection is associated with changes in taxonomic diversity and composition (Jochum, *et al*. 2019). During microbiome selection, second generation of plants received selected microbiome from the first generation and as a result there was dilution of microbiome inoculum in between generations. This might result in a reduction of alpha diversity between generations (Jochum, *et al*. 2019). This will be reflected in the exudation pattern by plants. In the current study, the concentration of carbohydrates and phenols exuded were not significantly different between generations. But the

analysis method used in this study does not allow identification of different types of sugars and phenols exuded. Identification of different sugar and phenolic compounds will help to understand the difference due to microbiome selection better. In this study, it can only be said that there was no significant difference in the concentration of phenols and carbohydrates in the root exudates of plants between generations of microbiome selection.

5.4.2 Effect of soil type, variety, and growth on root exudation

The exudation of compounds from roots of nutrient starved plants is an important way by which plants can respond to their environment. In the previous chapter, we saw that the difference in growth of plants between first and second generation was not significant in control (C) soil as compared to other soils. The competition for nutrients in soil might have been less in control soil due to low plant growth and less microbial competition. This explains the low concentration of phenols and carbohydrates found in the exudates of plants grown in control. In the current study, plants were grown under nutrient deficit conditions. This might have created a concentration gradient near the rhizosphere of plants in soils with microbiome, induced by the demand for nutrients in the rhizosphere by plants and microbes, which resulted in increased exudation in soils with microbiome. In a study using bean plants, Juszczuk, *et al*. (2004) found that the plants under phosphorus and nitrogen deficient conditions increased exudation of phenolic compounds by roots. Some of the phenolic compounds (e.g. flavonoids) have antibiotic function and may prevent the microbial degradation of other organic compounds exuded by the roots, e.g. organic acids and phosphatases, involved in phosphorus mobilization (Neumann and Römheld, 2001). The activity of enzyme phosphatase measured in this study was high in legume (S3) which might also be the result of increased phenolic exudation by plants in this soil. As discussed above, demand of nutrients in the rhizosphere also plays a major role in root exudation and hence low exudation recorded in control plants.

The exudation of phenols and carbohydrates were high in legume (S3) and mixed (S4) soil. There was nodulation in plants grown in these soils. Legume (S3) soil was from an area in the field where red clovers (a legume crop, *Trifolium pratense*) were grown during sampling time and this explains the presence of rhizobia in these soils. Results from next generation sequencing showed presence of *Bradyrhizobium*

species in soils. Mixed (S4) soil was prepared by mixing the field soils (untilled (S1), tilled (S2), and legume (S3)) in equal proportions. Mixing of microbiomes might generate novel combinations of microbes with novel beneficial effects on a host (Mueller and Sachs, 2015), or merge previously separate networks of microbes into a superior compound network (called community-network coalescence; Rillig *et al*., 2016) or generate novel competitive interactions between microbes that increase microbiome stability (Coyte *et al*., 2015). In this study, the presence of nodules in plants in mixed (S4) and better growth of plants in these soils suggests that mixing of field microbiomes resulted in a novel network of microbes in S4 and plants with the help of exudates were able to select their rhizosphere microbiome beneficial to their growth and development. When nitrogen is scarce in the soil, legumes exude a series of phenolic compounds (flavonoids and isoflavanoids) into the rhizosphere that act as signalling molecules for rhizobia and activate the transcriptional regulator Nodulation protein D (nod D) in them, triggering the transcription of genes required for Nod factor synthesis. (D'Haeze *et al*., 2002). Nod factors are lipochitooligosaccharides secreted by rhizobia and perceived by receptors present in the plasma membrane of root cells and perception of Nod factors by legume root cells initiates the formation of nodules in their roots. The increased concentration of phenols in soybean plants grown in legume (S3) and mixed (S4) soils in this study might be due to the presence of rhizobia in these soils. The analysis of amino acids in these soils in the second generation showed presence of tryptophan, ornithine, tyrosine, methionine, lysine, and asparagine which were not detected in other soils (untilled (S1) and tilled (S2)). Phenylalanine and tryptophan were reported as precursors to many of the important flavonoid compounds involved in rhizobia-legume symbiosis (Mandal *et al*., 2010). There have been many studies that reported asparagine, glutamic acid, and tryptophan to be chemotactic to plant-associated bacteria, including *Rhizobium leguminosarum*, *Bacillus subtilis*, *B. megaterium*, and *Azospirillum brasilense* (Carvalhais *et al*., 2015). Detection of asparagine and tryptophan in exudates of plants in legume (S3) and mixed (S4) suggests their role as signalling molecules, and it also explains the increased concentration of phenols in their exudates. The lack of nodulation in other two soils, untilled (S1) and tilled (S2), suggest the difference in their microbiome population. Based on these results, it can be concluded that the effect of soil type was significant on root exudation by plants in this study.

The concentration of amino acids found in the root exudates was very low in the second generation. A decrease in release of amino acids from nitrogen depleted plants has also been reported in maize by Carvalhais *et al*., (2011). Von Wiren *et al*., (2000) suggested that the decrease in amino acid content in root exudate under nitrogen deficit conditions might be as a consequence of the lower amount of amino acids being produced in nitrogen deficient roots.

The effect of growth was significant on root exudation of phenols and carbohydrates in untilled (S1), tilled (S2), legume (S3) and mixed (S4) in the first generation. The effect of growth was not significant on amino acid exudation in the second generation. This is comparable to the observations made regarding plant growth in the preceding chapter. The earlier flowering observed in plants in all soils except control suggests that the selection cycle is providing a microbiome that can grow better under nutrient deficit conditions. In a study for selecting plants with increased salt tolerance, Mueller *et al*., (2016) were able to select plants with salt tolerance after 1-3 selection cycles. During host mediated selection of microbiome, each selection cycle is associated with changes in taxonomic diversity and composition (Jochum *et al*., 2019). Jochum *et al*., hypothesised that during host mediated selection, plants might be selecting microbes with functionalities than taxonomies. The concentration of root exudates studied were not significantly affected by growth in the second generation might suggest that the microbiome activity is tending towards uniformity through selection as we saw in the case of plant height.

5.4.3 Difference between fallow soils and root exudates in phenols, carbohydrates, and amino acid content

The phenol and carbohydrate contents in fallow soil was lower than in root exudates. There was no significant difference in phenol and carbohydrate content of control fallow soil and fallow soils of untilled (S1), tilled (S2), legume (S3), and mixed (S4). Amino acids were not detected in control fallow soils. The results suggest that root exudates are driving variations in the concentration of these compounds in soil. The variation in the spectrum of plant exudates is responsible for the diversity of the rhizosphere microbial community (Zverev *et al*., 2021) and the demand generated by their activity is responsible for the difference in exudation patterns seen in plants grown in different soils. The result from this study also

shows that the compounds are found in fallow soils even in low concentration. One important factor that needs to be taken into consideration here is that the fallow soils received microbiome inoculum like soils with plants. This again ascertain the fact that the microbial activity is influenced by the root exudates. As was previously observed, the type of soil inoculum in this study significantly influenced the root exudation of phenols, amino acids, and carbohydrates.The absence of `rhizosphere effect` is evident in fallow soils in this study with them recording low concentration of primary and secondary metabolites. Bulk soil (here fallow soil which is not under the influence of root exudates) is generally poor in carbon compared to rhizosphere and majority of bacteria in this soil is oligotrophs that can survive in low levels of nutrients and characterised by slow growth (Zelenev *et al*., 2005). Shi *et al*., (2011) found that in *Fritillaria thunbergia* (a Chinese medicinal plant) microbial communities differed between the rhizosphere and bulk soil. Microbial diversity and root exudates have a close link (Eisenhauer *et al*., 2017), but it is still unknown at what level they influence each other (Canarini *et al*., 2019).

5.4.4 Limitations in this study

While discussing the results from this study, it is also important to investigate some of the limitations presented in this study which might influence the results. The most important limitation was in the identification of different components of root exudates especially phenols and carbohydrates. The method used did not distinguish between various components and it is possible that the concentration of some of these components might vary with soil and growth. Sugiyama (2019) found that daidzein and genistein are the two important isoflavanoids secreted by soybean (*Glycine max*) to attract rhizobia. Quantifying their amount in the root exudates might have given a better picture of difference between soil types studied and how it varied with growth. Highly variable forms of sugars, including glucose, pinitol, arabinose, galactose, sucrose, and oligosaccharides, were detected in soybean root exudates (Timotiwu and Sakurai, 2002). The difference in the concentration of these components in the root exudates of plants in different soils and how they change with growth should be considered in the future. An efflux of amino acids glycine and serine were noticed in this study. Numerous investigations have found substantial levels of glycine and serine in root exudates from many species, including rape, ryegrass, and alfalfa (Bobille *et al*., 2016). In

plant cells, calcium (Ca2+) and magnesium (Mg2+) are said to have antagonistic interactions and maintaining a balance between these two elements is critical for optimal plant growth and development. (Tang and Luan, 2017). Glycine and glutamate together act as plant ligands and control movement of Ca2+ between membranes and helps maintaining Ca2+ homeostasis in plants and plays important role in root growth and development (Lesuffleur *et al*., 2007). Ca2+ signalling was found important during interactions with Arbuscular myccorhizal fungi and plants (Navazio and Mariani, 2008). Lesuffleur *et al*., (2007) suggested that high plant glycine efflux could act not only in plant-microbe interactions, but also for plant growth via soil calcium uptake. Further studies to understand the mechanism and the role played by glycine and serine efflux in plant nutrition and interactions with soil microbes is necessary.

5.4.5 Conclusions

In this study, my aim was to understand the difference in exudation of primary (carbohydrates and amino acids) and secondary metabolites (phenols) by plants grown in different soil types and how it changes during microbiome selection. The results clearly shows that there is significant impact of different soil inoculums on root exudation from plants. The plants grown in legume (S3) soils and mixed (S4) soil led to the highest recorded concentration of exudates. The nodulation observed for plants in this study indicates the presence of rhizobia in these soils and this might have influenced their root exudation. The low exudation recorded in plants in control and fallow soils (soil without plants but with inoculum from soil suspensions) again shows the importance of the plant-microbiome interaction in controlling the root exudation in plants. Unfortunately, it was not possible to completely understand the difference in exudation patterns between two generations of microbiome selection. The concentration of root exudates studied were not significantly affected by growth in the second generation, which might suggest that the microbiome activity is tending towards uniformity through selection as I saw in the case of plant height. Further study is required to understand the impact of selection on different components of phenols and sugars.

Chapter 6. Influence of Host mediated microbiome selection on rhizosphere soil bacterial communities in soybean genotypes.

6.1 Introduction

Plants are hosts to diverse communities of microorganisms that influence their health and development. These host-associated-microbial-communities, referred to as the microbiome (Mueller and Sachs, 2015) can alter host development, physiology, and systemic defences. All tissues of a plant host a microbial community. Phyllosphere refers to the aerial surfaces of the plant and is subjected to extremes of temperature, moisture, and radiation. Rhizosphere is the region of soil largely influenced by plant roots through deposition of exudates, mucilage, and sloughed cells. Microbial diversity found in rhizosphere and phyllosphere are considered as epiphytes and the microbes residing inside plant tissues are called endophytes (Turner *et al*., 2013). In this study we are concentrating on rhizosphere soil communities.

6.1.1 Soil microbial communities

Soil microbial communities are diverse both in terms of their taxonomic structure and biological functions (nutrient recycling, organic matter decomposition, suppression of pests and pathogens etc.). The most abundant microbes in these communities are bacteria, but fungi, oomycetes, algae, protozoa, nematodes, and viruses are also important contributors (Mueller *et al*., 2016). It is estimated that a single gram of soil might contain tens of thousands of bacterial and archaeal species (Berendsen *et al*., 2012) and play important roles in soil nutrient cycling, soil formation and influence plant health and performance. It has been shown that plants grown in sterile soil may only produce half the biomass of plants grown in the presence of soil microbes (Carvalhais *et al*., 2011). The major bacterial phyla found in soil are Proteobacteria, Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Planctomycetes, Gemmatimonadetes and Firmicutes (Janssen, 2006). Actinobacteria are reported to play an important role in soil nutrient mobilisation and are among the most efficient groups in producing secondary metabolites that relate to plant disease suppression (Palaniyandi *et al*., 2013). Many plant growth promoting bacteria

(PGPB) like *Pseudomonas fluorescens, Bacillus subtilis*, and nitrogen fixing bacteria like *Rhizobium* and *Bradyrhizobium* belong to the phyla Actinobacteria, Proteobacteria and Firmicutes.

Soil microbial diversity and biomass controls organic matter decomposition, nutrient cycling, and gaseous fluxes in soil (Bastida *et al*., 2021). The supply of organic compounds by plants into the soil ecosystem provide necessary nutrients for the corresponding microbiological processes that are vital for soil ecosystem functioning (Jones *et al*., 2009). The important activities involving rhizosphere bacteria include parasitism, antagonism, competition for nutrients and space, and activation of plant defence. (Whipps, 2001). Some of the microbes that act against root pathogens and root feeding insects have also been shown to promote plant growth. The plant growth promoting effect of *Trichoderma harzianum* (Harman *et al*., 2004) has been suggested to be based on the production of antagonistic compounds against root pathogens, which also serve as plant hormones (peptides and low molecular weight compounds), increasing root growth. Some of the free-living bacteria like *Azospirillum*, *Burkholderia* and *Bacillus* promote plant growth by nitrogen fixation and by inducing root branching and phosphorus solubilization in soil (Larsen *et al*., 2015).

6.1.2 Effect of soil type and soil management on soil microbial population

Microbial-driven ecosystem functioning activities including soil nutrient cycling, disease suppression and plant growth promotion make microbes critical for the maintenance of soil health in both natural and managed agricultural soils. The microbial community structure of soil is influenced by the physicochemical properties of soil and its structure, the presence and age of specific plant species, and crop management and field rotations (Garbeva *et al*., 2004).

Soil structure, pH, cation exchange capacity, and organic matter content can all influence soil microbial populations, either directly by providing a specific habitat that selects specific microbes, or indirectly by impacting plant root functioning and exudation in a soil-specific manner. Gelsomino *et al*., (1999) suggested that the structure of bacterial communities in soil is determined by soil type and that similar soil types tend to have similar communities. In a study comparing the influence of soil type, cultivar, and growth stage of maize on the population size and structure of rhizosphere bacterial communities, soil type was found to

have the greatest effect on bacterial density and community structure, with no significant differences between the effect of maize cultivars being observed (Chiarini *et al*., 1998). Soil type was the most important factor that affected the diversity of *Paenibacillus* populations in maize (da Silva *et al*., 2003) and diversity of the populations of culturable fluorescent *Pseudomonas* in tomato (Latour *et al*., 1999). Soil nutrients, pH and organic matter content were the major factors contributing to the dominant effect of soil type on microbial activity.

Soil management practices, such as crop rotation, tillage, fertilizer, compost, manure, or pesticide applications and irrigation greatly affect soil microbial communities (Gajda *et al*., 2018). It has been postulated that management practices such as crop rotation, increase aboveground biodiversity of plants and this in turn increases the diversity belowground (Hooper *et al*., 2000). It has been shown that organic amendments (compost and cover crop residues) to soil can shift bacterial and fungal community composition and influence soil microbial interactions and resilience of microbial communities (Schmidt *et al*., 2019). Tillage practices change soil moisture, soil aggregation and distribution of pore sizes, which thereby influence access of soil microbes to oxygen, water, and nutrients (Ghimire *et al*., 2014). Higher fungal and bacterial activities were observed in no tillage systems (Dang *et al*., 2015). However, long term no tillage can also result in the accumulations of nutrients at the soil surface, nutrient runoff, compromised soil quality and decreased agronomic productivity (Dang *et al*., 2015). Minimum-till or no-till cultivation was also reported to increase disease severity by pathogens that survive better when infested crop debris remain on or near the soil surface (Garbeva *et al*., 2004). Consequently, it is important to understand the management-induced shifts in soil microbiome and how plant selection changes across management systems. It is important to consider the effect of interaction between plant selection and agricultural management on rhizosphere microbial communities to promote both plant productivity and agroecosystem sustainability (Schmidt *et al*., 2019).

6.1.3 Host-mediated microbiome selection as a tool to study plant-microbe interactions

When a plant is studied in conjunction with its microbiome across multiple generations, new forms of interactions can be observed between plants and microorganisms shaping plant development (Panke-Buisse *et al*., 2015). In host-mediated microbiome selection, the microbiome is selected based on specific plant traits (for example plant growth, disease resistance, flowering) and the microbiome associated with this trait is passed onto new plant hosts through multigeneration selection (Panke-Buisse *et al*., 2015; Swenson *et al*., 2000; Mendes *et al*., 2011). Thus, progressively enriching plants or their growth environment with microbiota associated with a specific plant trait facilitates the use of more complex communities instead of a single microbial strain and provides a potential platform for exploring plant–microbiome interactions. The microbial communities and plant–microbe interactions are highly dependent on the entire ecosystem (Bulgarelli *et al*., 2013) and it has been shown that complex microbial inoculums can improve plant disease resistance and promote growth compared to single species inoculums (Sergaki *et al*., 2018). The generation of microbial communities with beneficial activities will serve as a powerful tool to enhance sustainable agriculture. Only a few studies have used this tool of selection to shape microbiomes (Panke-Buisse *et al*., 2015; Swenson *et al*., 2000; Mendes *et al*., 2011; Lau and Lennon, 2012; Jochum *et al*., 2019). Many of the mechanisms underlying plant-microbe interactions in the rhizosphere are still poorly understood. To understand the benefits of the plant microbiome in agriculture, it is necessary to know the functional and mechanistic aspects of the interaction between microbes, plant, environment, and agricultural practices.

In this study, focus is given on understanding the difference in bacterial populations in field soils collected from areas differing in their management practices and cropping history. Soybean plants were grown in an autoclaved coir: sand mix which was inoculated with the soil suspensions from these soil types. The microbiomes from the first plant generation were selected based on a plant trait associated with high fitness (plant height) and used as inoculum for the growth of the second generation of plants. This study used next generation sequencing to characterise bacterial communities in the soil samples collected from the field and to study the changes in community composition during microbiome selection.

The study had three broad aims

1. To study the difference in bacterial populations of soils differing in management practice and cropping history. Based on results from studies in other systems/species (Schops *et al*., 2018, Zhang *et al*., 2013), I predict that bacterial diversity and composition will differ in field collected soil samples which varied historically in management and cropping practices.
2. To study the difference in diversity and abundance of bacterial taxa between initial soil sample and rhizosphere soil samples after microbiome selection. Previous studies (Jochum *et al*., 2019; Panke-Buisse *et al*., 2015) found that there was difference in bacterial diversity and composition during microbiome selection rounds. So, in this study, I propose that during microbiome selection, microbes contributing to the plant trait (better plant height) is selected by plants with the help of root exudates and this selection might exert changes in bacterial population. There will be differences in bacterial diversity and composition between initial field soil and rhizosphere samples.
3. To study the effect of soil pH, total carbon, and soil enzyme activities on bacterial population. The quality and productivity of soil are attributed to the physical and chemical properties of the soil as well as its biological activity (Garbeva *et al*., 2004; Schloter *et al*., 2017). In this study, we propose that the difference in bacterial composition between soils during microbiome selection will be affected by soil properties.

6.2 Methodology

6.2.1 Soil sampling and DNA extraction

The methodology for initial soil sampling is explained in Section 2.1. The field soil samples were used as microbiome inoculum for growing soybean plants. Soybean crop (Varieties Kenchen and Siverka, Soy UK) were grown in the Crop and Environment Laboratory (CEL) glasshouse, Whiteknights Campus, University of Reading, in plastic plant pots containing microbiome inoculum from different field soils (pot preparation, microbiome inoculation and planting explained in Section 2.4). The rhizosphere soils from the 2nd generation of plants were collected and stored at -80^{o}C for DNA analysis (Section 2.4). DNA extraction and preparation of samples for Next generation sequencing (NGS) is explained in Section 2.10.

6.2.2 PCR and sequencing

The polymerase chain reaction procedure for sequencing was developed and optimised by NOVOGENE, Cambridge, UK for Amplicon Metagenomics Sequencing. The workflow is shown in Fig. 6.1.

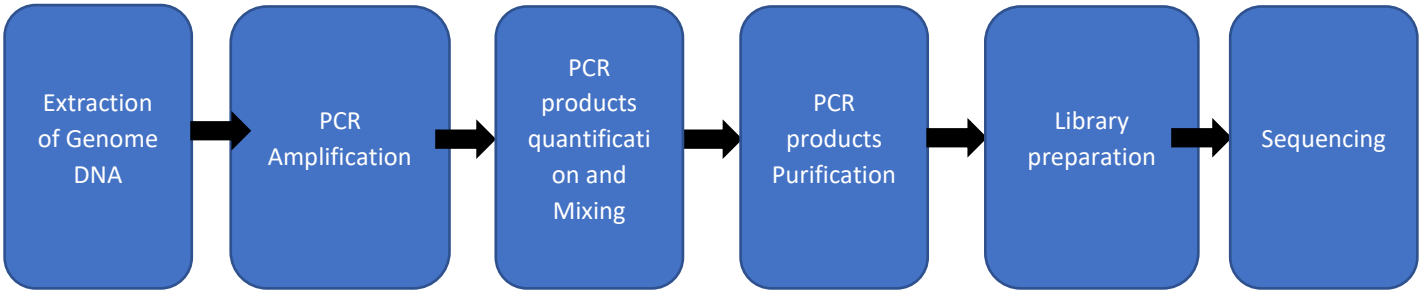


Fig. 6.1 **Workflow showing procedures from DNA extraction to sequencing**.

According to the concentration, DNA was diluted to 1ng/µL using sterile water. For bacterial species, the V3-V4 region of the 16S rRNA gene were targeted for amplification using specific primer pair (16sV3-V4: 341F (5'-CCTAYGGGRBGCASCAG-3'), 806R (5'-GGACTACNNGGGTATCTAAT-3')) with the barcode. All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). PCR products were subjected to electrophoresis on 2% agarose gels and bands between 400-450bp were

purified with Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries were generated using NEB Next Ultra DNA Library Pre ® Kit for Illumina, following manufacturer 's recommendations and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. The library was sequenced on an Illumina platform and 250 bp paired-end reads were generated.

6.2.3 Bioinformatic analysis

The bioinformatic analysis was performed by NOVOGENE, Cambridge, UK using the following protocol: Paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. Paired-end reads were merged using FLASH (V1.2.7, Magoc and Salzberg, 2011) and the splicing sequences were called raw tags. Quality filtering on the raw tags were performed under specific filtering conditions to obtain the high-quality clean tags according to the QIIME (V1.7.0, Caporaso *et al*., 2010) quality-controlled process. The tags were compared with the reference database (Gold database) using the UCHIME algorithm (UCHIME Algorithm, Edgar *et al*., 2011) to detect chimera sequences, any of which were removed (Haas *et al*., 2011) to obtain effective tags. Sequences analyses were performed by Uparse software (Uparse v7.0.1001, Edgar, 2013). Sequences with ≥97% similarity were assigned to the same operational taxonomic units (OTUs). A representative sequence for each OTU was screened for further annotation. For each representative sequence, the GreenGene Database (DeSantis *et al*., 2006) was used based on RDP 3 classifier (Version 2.2, Wang *et al*., 2007) algorithm to annotate taxonomic information.

6.2.4 Statistical analysis

All the statistical analysis were carried out using R software (Version 4.1.2, R Core Team, 2021). Rarefaction curves were produced for each OTU table to see whether sufficient sequencing depth was reached to allow a reasonable estimate of the number of different OTUs. The sequencing depth of the samples differed considerably (Appendix F.1) and to adjust for these differences, OTU tables were rarefied to an even sequencing depth prior to analysis using the vegan package (version 2.5-7, Oksanen *et al*., (2020)) in R to

avoid biases resulting from differences in sample size. Data were normalized using a standard of sequence number corresponding to the sample with the least sequences. Subsequent analysis of alpha diversity and beta diversity were all performed based on this output normalized data. Alpha diversity indices were calculated as the number of observed species (species richness) in each sample and the Shannon-Wiener diversity index which estimates species diversity. Data were analysed using linear models. Continuous variables were tested for normality before model fitting and transformations were applied as necessary. Analysis of variance (ANOVA) was carried out to test for differences in number of observed species and Shannon diversity between soil types, plant growth and varieties. OTU tables for initial soil sample and rhizosphere soil samples after second generation of plant growth were analysed separately. Linear models were used to analyse OTU richness and diversity correlations with soil parameters. Linear discriminant analysis (LDA) effect size (LEfSe) was used to elucidate significantly different relative abundances of bacterial taxa, associated with soil types. LEfSe analysis was carried out in R using package microbiomeMarker (version 1.0.1, Cao, 2020). These analyses are presented in a bar plot and the parameters set with default p-value, $\alpha = 0.05$, and an LDA score of 4.0 with LEfSe (Segata *et al.*, 2011).

Dissimilarities in OTU composition between samples (beta diversity) was calculated based on the Bray-Curtis dissimilarity index. Dissimilarities between samples were visualised in a non-metric multidimensional scaling (NMDS) plot using 2 axes (k=2) and ensuring that the stress value was close to or below 0.2. Bray-Curtis dissimilarities were analysed using permutational multivariate analysis of variances (PERMANOVA) using the adonis function in the vegan package (Oksanen *et al.*, 2020), with 999 permutations. PERMANOVA model was used to test the effect of soil variables (NAG, PHOS, TOC, TC, and pH) on soil bacterial community composition. Distance-Based Redundancy Analysis in the vegan package (dbRDA; Legendre and Anderson, 1999) is an extension of Redundancy Analysis (RDA) method that aims to detect linear relationships between environmental variables and Bray-Curtis dissimilarities. dbRDA was used to visualise how environmental variables constrain variation in community composition between soil types. Soil variables were normalised by z-score transformation. Correlation between environmental variables was accounted for by calculating variance inflation factors (VIFs) and then removing selected terms from the model until all VIF scores were < 10.

6.3 Results

6.3.1 Difference in bacterial diversity and composition between initial soil samples

The sequencing reads from initial field soil samples clustered into 6404 OTUs and the OTU matrix was rarefied to the lowest sequencing depth of 29328, leaving 3979 OTUs for analysis. Of these OTUs, 31.78% were assigned to Actinobacteriota, 26.88% to Proteobacteria, 11.04% to Firmicutes, 10.17% to Acidobacteriota, 4.83% to Verrucomicrobiota, 3.29% to Myxococcota, 1.87% to Bacteroidota and the remaining 10.13% allocated to Nitrospirota, Chloroflexi, Desulfobacterota, Methylomirabilota, and other unassigned groups. Fig. 6.2 shows the relative abundance (percentage of each OTU represented in each soil) of top ten class divisions and Fig. 6.3 shows the abundance of top 10 orders identified in each soil.

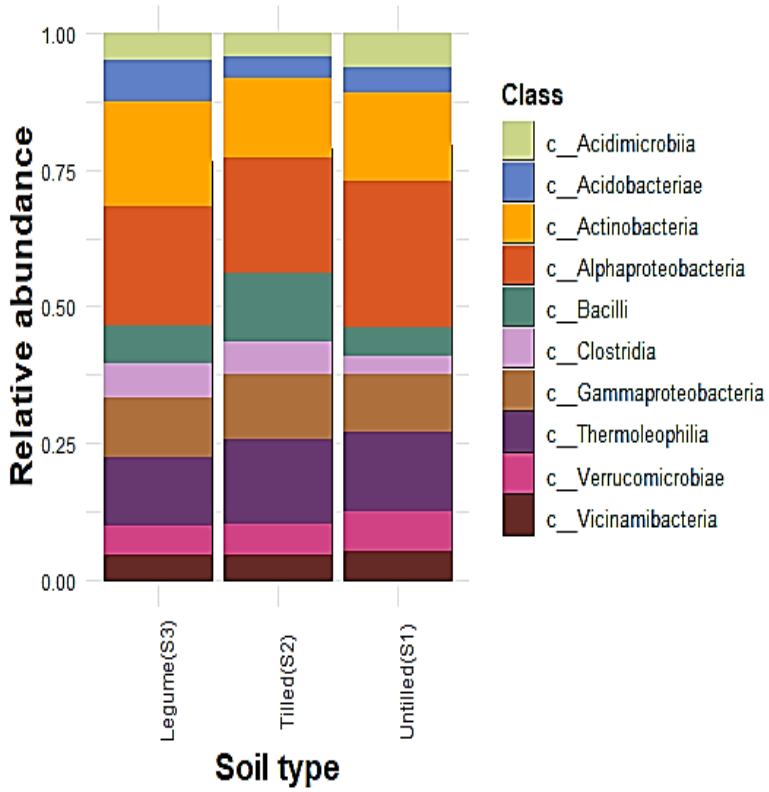


Fig. 6.2 **Relative abundance of top 10 class divisions for bacterial OTUs in different field soil types**, Untilled (S1), Tilled (S2), Legume (S3). n=3

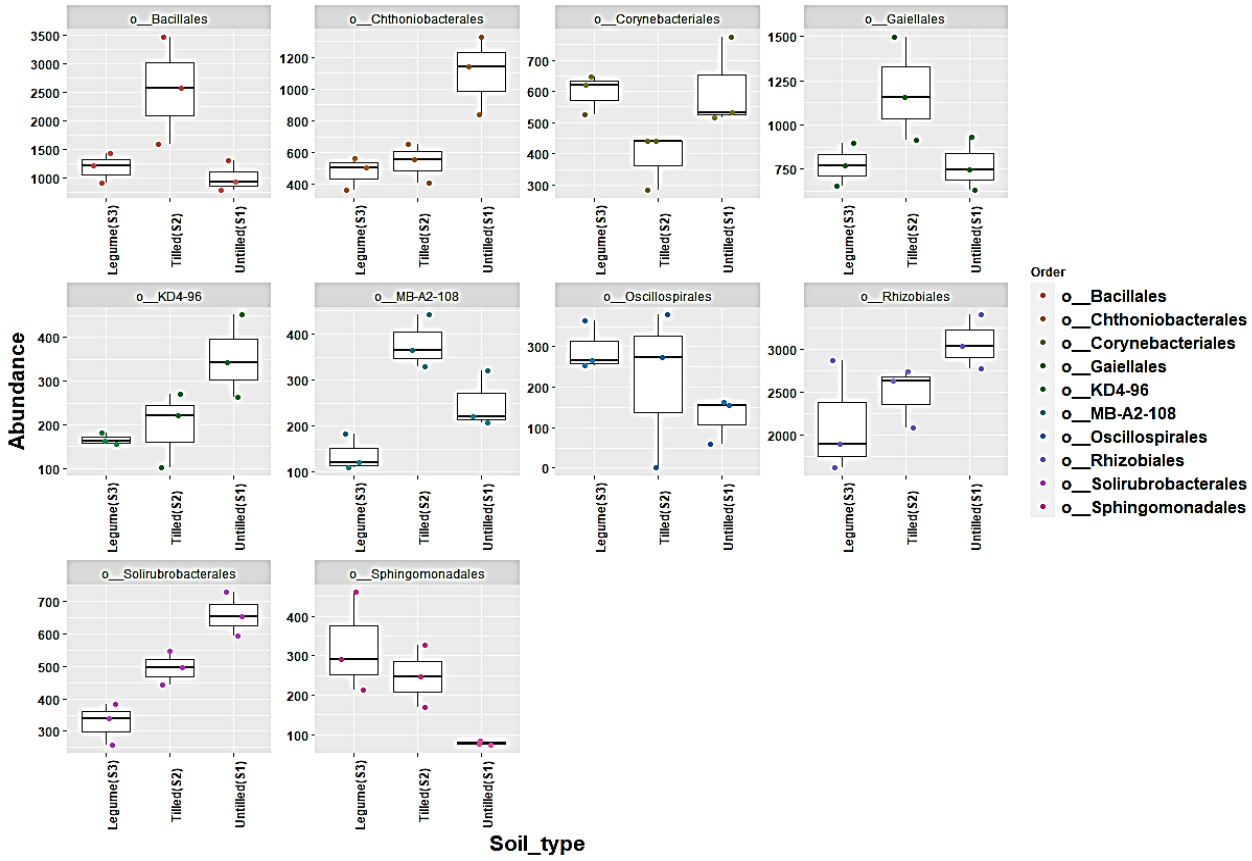


Fig. 6.3 **Difference in the relative abundance of top 10 orders associated with different field soil types.** Each facet grid is represented by one order (o) given in the legend. Y axis represents, abundance of each order in soil, n=3

6.3.1.1 Effect of soil type on species richness and diversity

Richness, or the number of distinct species (OTUs) in each sample, and evenness, or the distribution of those species, constitute diversity. There was no significant difference in species richness between our three soil types, untilled, tilled and legume soils (F (2) = 1.61, p= 0.27, Fig. 6.4A). The bacterial diversity was significantly influenced by soil type (F (2) = 11.28, p= 0.009). Tukey test results revealed that the difference in diversity was significant between legume- tilled and legume- untilled (Fig. 6.4B), and there was no significant difference in diversity between tilled and untilled soils.

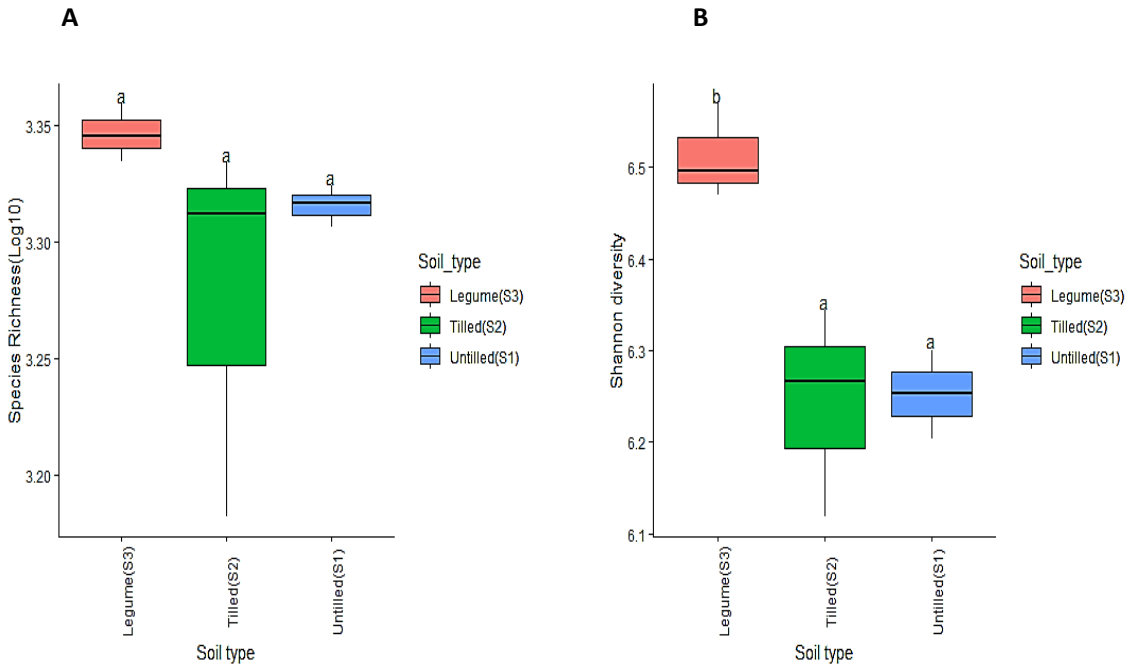


Fig. 6.4 **Effects of soil type on A. Species richness and B. Shannon diversity in different field soils**, Untilled(S1), Tilled(S2), and Legume(S3). Different letters show significant influences ($p < 0.05$) using Tukey's multiple range tests. n=3

6.3.1.2 Effect of soil type on bacterial composition

PERMANOVA revealed that the bacterial composition was significantly influenced by soil type (F (2) = 2.84, p=0.003). 2147 OTUs were shared between three soil types. 212 OTUs were shared between untilled and tilled soils, untilled and legume soils shared 327 OTUs and 311 OTUs were shared between tilled and legume soils. Each soil type had number of unique OTUs which was not shared by the other two (Fig. 6.5).

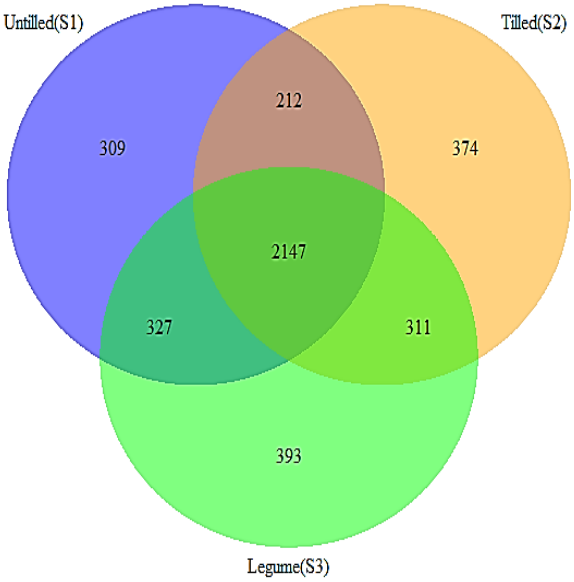


Fig. 6.5 **Venn diagram showing number of shared OTUs (mean relative abundance > 1%) by each soil type, Untilled(S1), Tilled(S2), and Legume(S3).**

6.3.2 Differences in bacterial diversity and composition between rhizosphere soil samples following second generation of plant growth

The sequencing reads from rhizosphere soil samples clustered into 6404 OTUs and the OTU matrix was rarefied to lowest sequencing depth of 45770, leaving 5333 OTUs for analysis. Proteobacteria accounted for the highest number of identified OTUs (47.87%). Firmicutes accounted for 32.1% of OTUs, followed by

Verrucomicrobiota (5.51%), Bacteroidota (4.95%), Actinobacteriota (3.39%), Myxococcota (2.67%), with the remainder 3.43% of OTUs assigned to Chloroflexi, Acidobacteriota, Abditibacteriota, Desulfobacterota, and other unassigned phyla. The relative abundance of top ten class divisions identified in the rhizosphere soils is given in Fig. 6.6. Fig. 6.7 shows the top ten order identified in rhizosphere soil of soybean plants. The relative abundance of order Bacillales was high in the rhizosphere of Control (C) soil. The relative abundance of orders Caulobacterales, Polyangales, Rhizobiales, Sphingomonadales and Xanthomonadales were high in the rhizosphere of Mixed (S4) soils. In Legume (S3) rhizosphere soils, the relative abundance of Verrucomicrobiales and Micrococcales were found high.

Linear discriminant analysis effect size (LEfSe) results showed that the rhizosphere soils of plants in each soil type enriched distinct bacterial taxa (Fig. 6.8). Legume soils were found significantly high in Actinobacteriota, Rhizobiales (Xanthobacteraceae including unknown genus and *Pseudolabrys*) and Verrucomicrobiota. The relative abundance of Proteobacteria (Rhizobiales, Burkholderiales) and Myxococcotta (Polyangiales) were significantly high in mixed soils. Rhizosphere of untilled soils were more dominated by Gammaproteobacteria (many unidentified order and genus) and Verrucomicrobiota (order Opitutales). Tilled soil rhizospheres were colonised by Bacteroidota, Bacilli (*Paenibacillus*, *Lysinibacillus*) and Rhizobiales (family Devosiaceae). The rhizosphere of plants in control showed high abundance of Bacilli (*Bacillus asahii*) and Rhizobiales (unidentified genus). High LDA scores reflects high abundance of that taxa in the soil. In untilled (S1) rhizosphere, Gammaproteobacteria was highest in abundance with a LDA score more than 4.5. High LDA scores were for Brevibacillales (> 4.5) in tilled (S2). Phylum Proteobacteria (> 4.5) was highest in mixed (S4) and legume (S4) had high abundance of Verrucomicrobiae (> 4.5). Control (C) rhizosphere soils had high LDA scores (> 5) for phylum Firmicutes and class Bacilli.

6.3.2.1 Effect of soil type, plant growth and varieties on species richness and diversity

Species richness was significantly influenced by both soil type ($F(4) = 5.27$, $p = 0.002$) and plant growth ($F(1) = 6.04$, $p = 0.018$). Tukey test results (6.9A) showed that rhizosphere soil from plants in Control (C) soil had low species richness than other soils (Untilled, tilled, legume and mixed). There was a significant effect

of soil type (F (2) = 14.31, p= <0.001) on Shannon diversity. Shannon diversity was not significantly different with growth and varieties. Control (C) had the lowest species diversity (Fig. 6.9B).

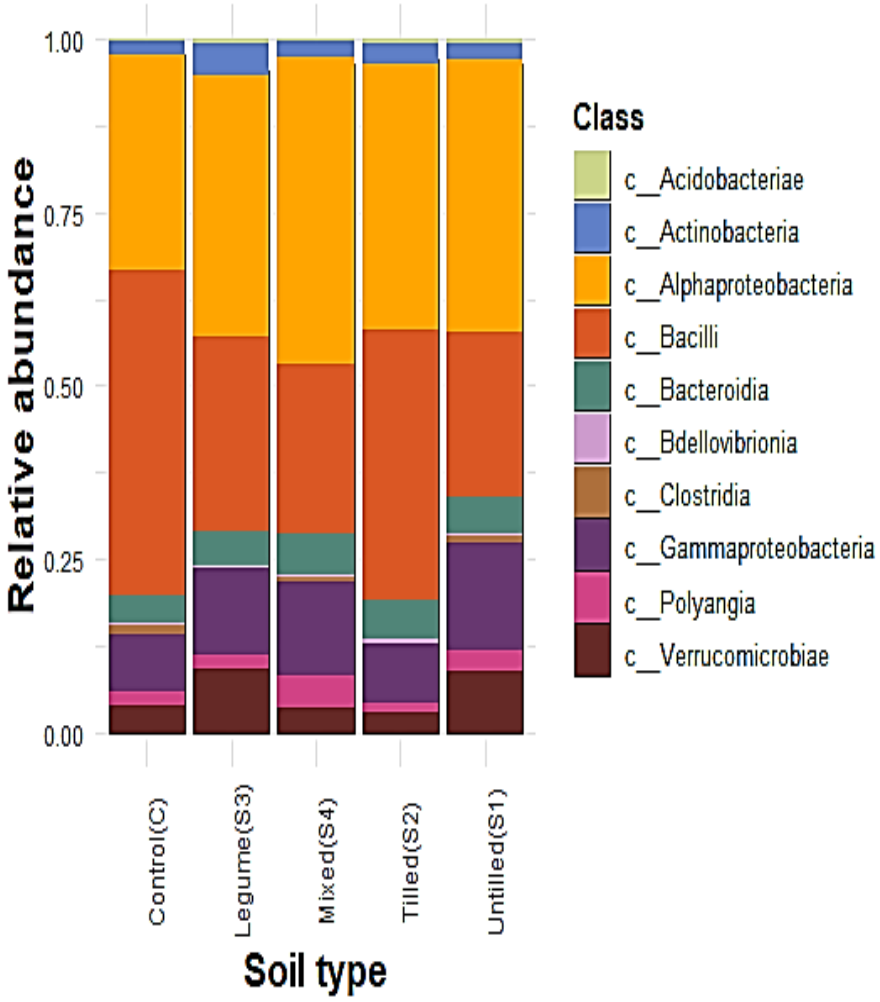


Fig. 6.6 **Relative abundance of top 10 class divisions for bacterial OTUs in the soybean rhizosphere of different soil types after second generation**, Control (C), Legume(S3), Mixed (S4), Tilled (S2), and Untilled(S1). n=3

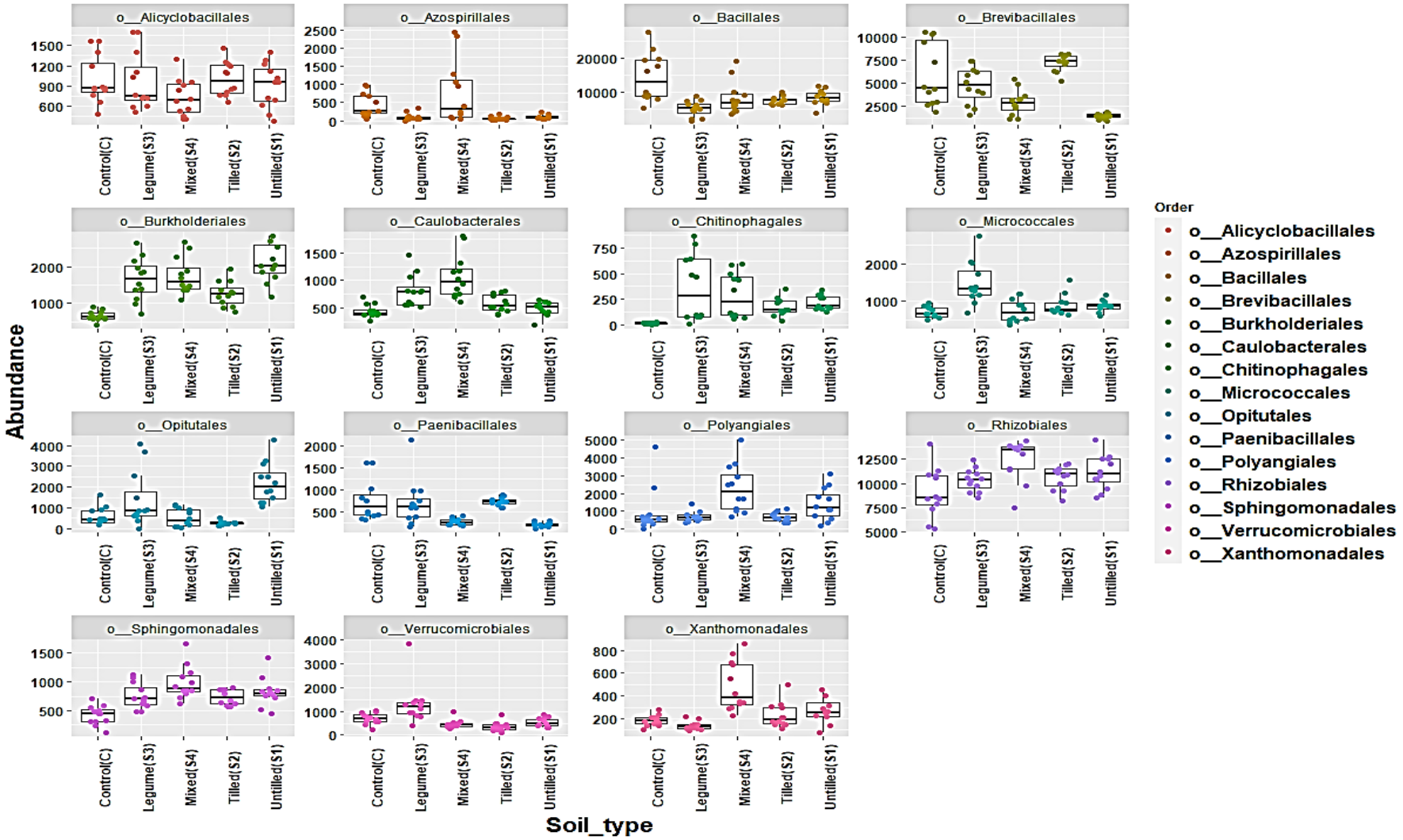


Fig. 6.7 **Abundance of top 15 Order in the soybean rhizosphere of different soil types after second generation**, Control (C), Legume(S3), Mixed (S4), Tilled (S2), and Untilled(S1). Each facet grid is represented by one order (o) given in the legend. Y axis represents, abundance of each order in soil. n=3

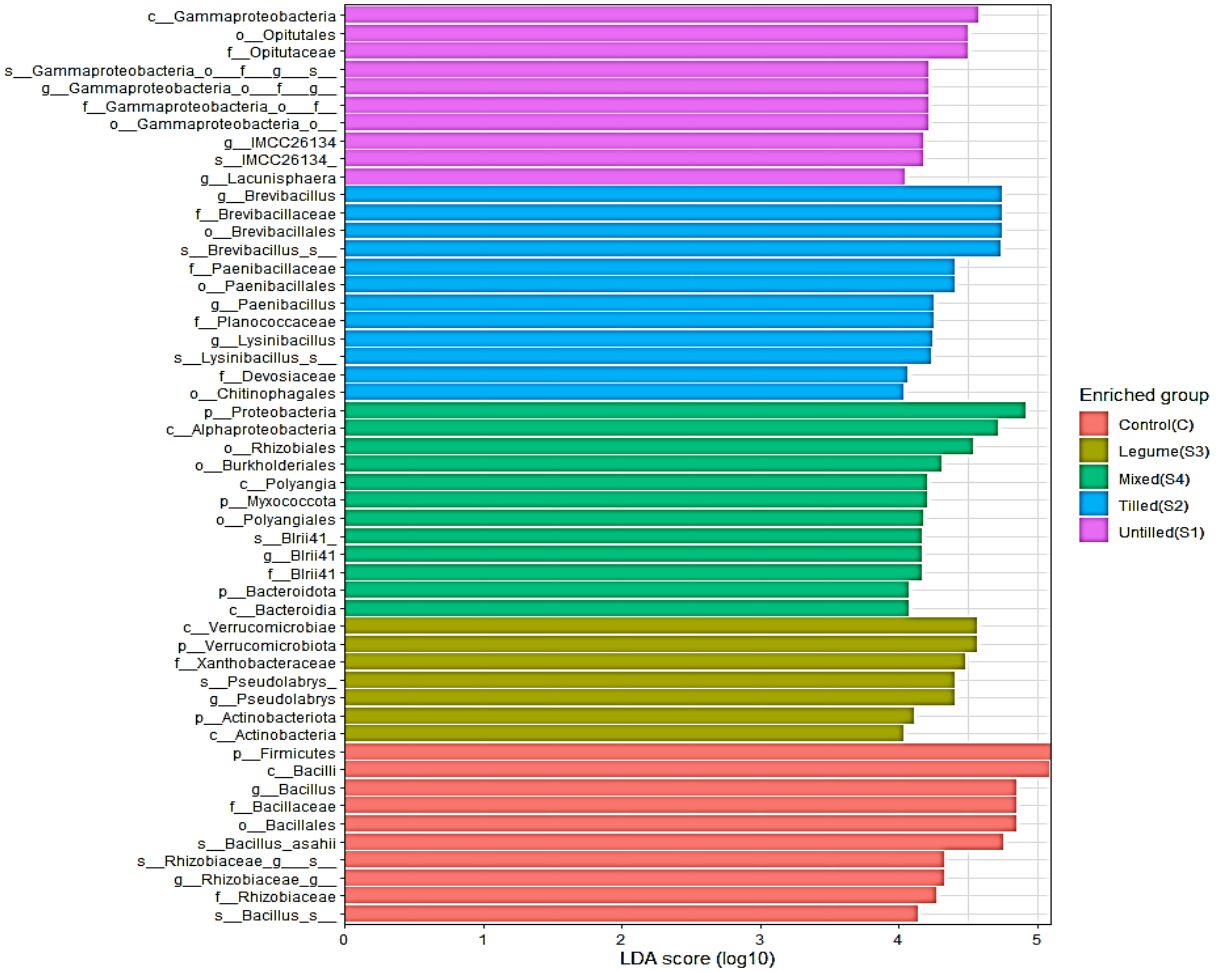


Fig. 6.8 **Graphics of linear discriminant analysis (LDA) effect sizes (LEfSe) for rhizosphere soils of different soil types after second generation**. Horizontal bars represent the effect size for each taxon. The length of the bar represents the log10 transformed LDA score. Each colour represents the soil, that taxa was found to be more abundant compared to other soils. The different soil types included were Untilled(S1), Tilled (S2), Legume (S3), Mixed (S4), and Control (C). The threshold on the logarithmic LDA score for discriminative features was set to 4.0. The taxon of bacteria with statistically significant change ($p < 0.05$) in the relative abundance is written alongside the horizontal lines. The name of the taxon level is abbreviated as p-phylum; c-class; o-order; f-family, g-genus, and s-species.

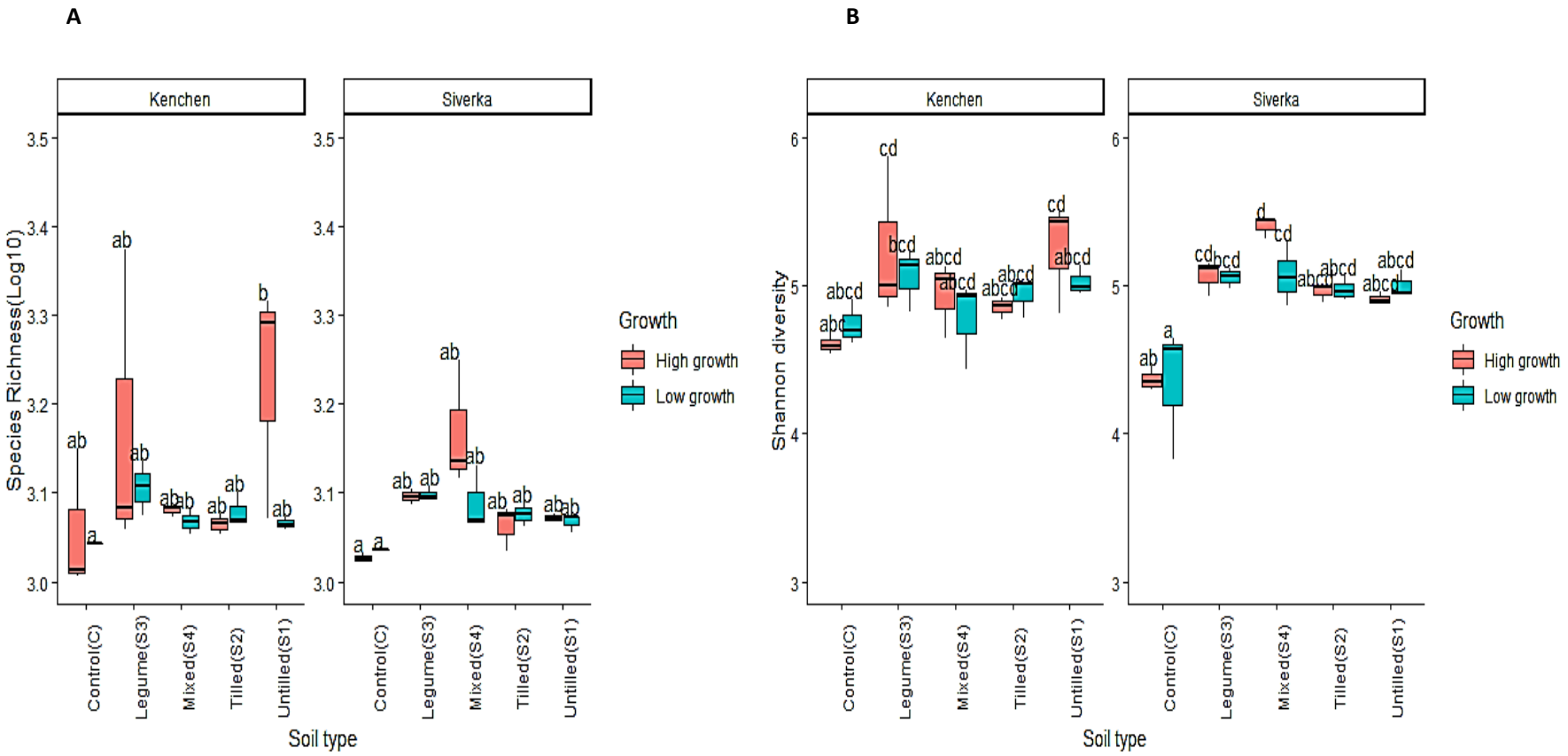


Fig. 6.9 **Effects of soil type, plant growth and varieties on A. Species richness** and **B. Shannon diversity in the rhizosphere of plants grown in different soil types after second generation**, Control (C), Legume(S3), Mixed (S4), Tilled(S2), and Untilled(S1). Different letters show significant influences ($p < 0.05$) using Tukey's multiple range tests. n=3

6.3.2.2 Effect of soil type, plant growth and varieties on bacterial composition

There was significant effect of interaction between soil type and growth (F (4) = 1.55, p=0.018), and soil type and variety (F (4) = 4.50, p=0.001) on bacterial composition (Fig. 6.10) in the rhizosphere of plants in different soil types after second generation of plant growth. 1817 OTUs were shared between the four soil types (untilled, tilled, legume and mixed soil). Each soil had a unique set of OTUs, untilled had 433, tilled had 106, legume had 692 and mixed soil had 408. Fig. 6.11 shows the OTUs shared between soils. All the four soil types shared few OTUs with control (Fig. 6.12).

6.3.3 Effect of soil properties on bacterial composition of rhizosphere soil samples

PERMANOVA results showed that diversity was significantly affected by rhizosphere total organic carbon (TOC) (F (1) = 9.74, p= 0.001), total carbon (TC, F (1) = 4.11, p= 0.001), N- acetyl beta glucosaminidase activity (NAG, F (1) = 7.80, p= 0.001), phosphatase activity (PHOS, F (1) = 6.11, p= 0.001), and pH (F (1) = 2.28, p= 0.022).

The plot based on distance-based redundancy analysis (dbRDA) showed the grouping of soils based on variation in community composition as affected by soil variables (Fig. 6.13). The first and second axes explained 39.13% and 28.25% of the variance respectively. Total carbon, NAG and PHOS activity were the major contributors to the variance in community between soils. Control soil is oriented towards the negative axis of RDAaxis1 and RDAaxis2. Legume soil is oriented towards the positive axis of both RDA1 and RDA2. Untilled soil is grouped in the positive axis of RDA1 and negative axis of RDA2. Mixed soil is oriented towards the negative axis of RDA1 and positive axis of RDA2. Tilled soil is grouped in the centre and oriented more towards positive axis of both RDA1 and RDA2. Legume soil is characterized by high NAG activity and tilled soil showed high PHOS activity. Mixed soil shared the same quadrant as pH and TC. In general, clustering of soils in different quadrants indicates difference in their community composition and the major soil properties contributed to this variance were TC and soil enzyme activities.

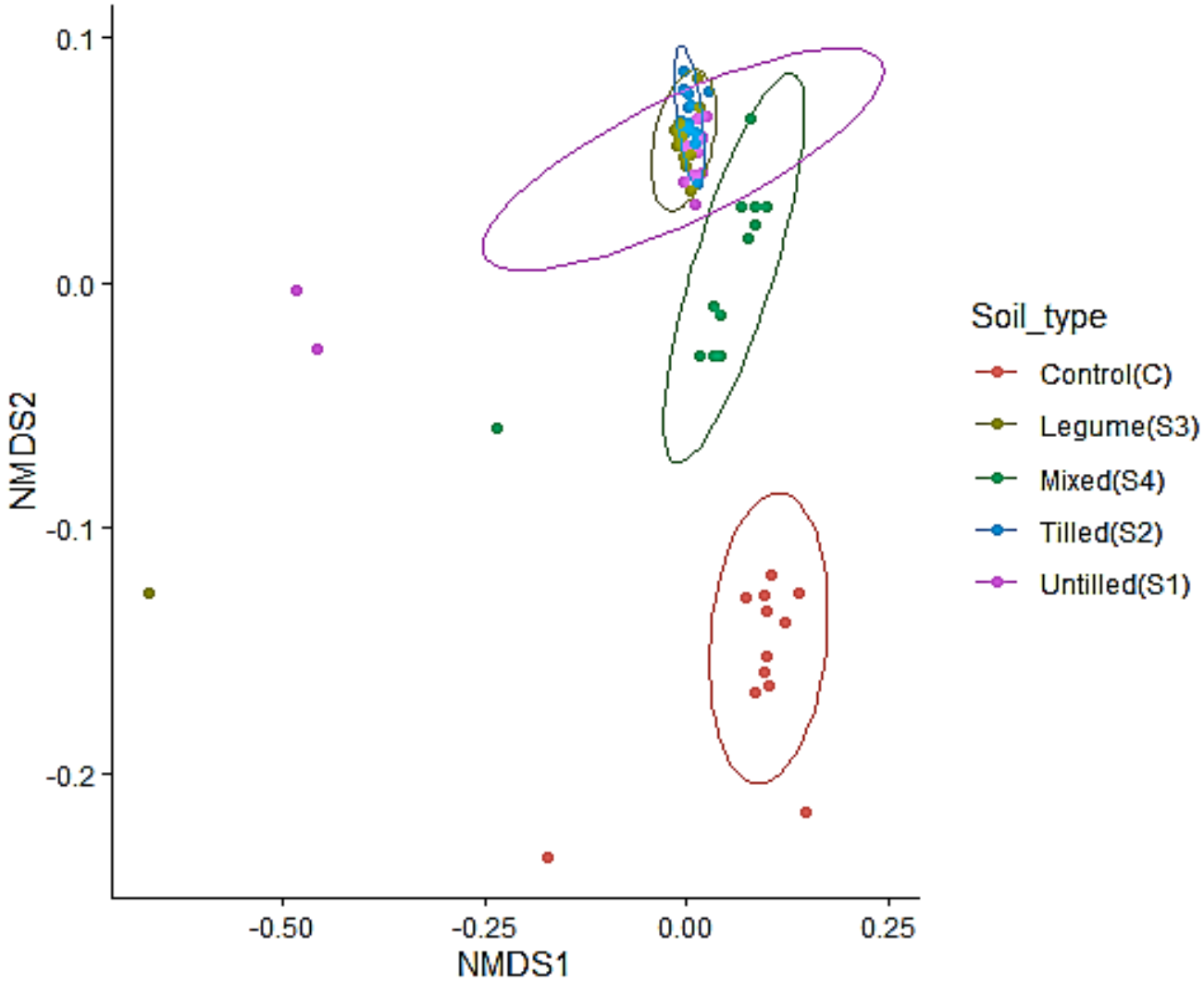


Fig. 6.10 **NMDS score computed using the Bray-Curtis index, representing dissimilarities in the rarefied bacterial species (OTU) community between soil types** (Untilled(S1), tilled(S2),legume (S3), mixed (S4), control (C)). The stress value associated with this representation was 0.082. The ellipsis represents a 95% confidence interval

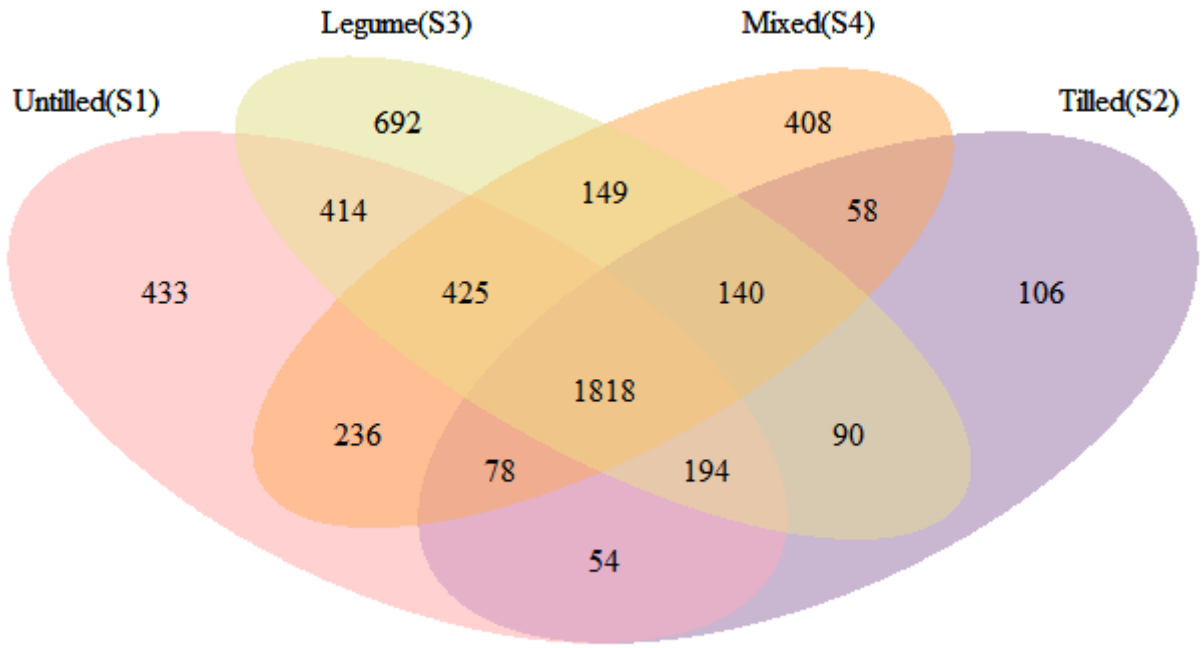


Fig. 6.11 **Number of shared OTUs (mean relative abundance > 1%) by each soil type, Untilled(S1), Tilled(S2), Legume(S3) and Mixed (S4).**

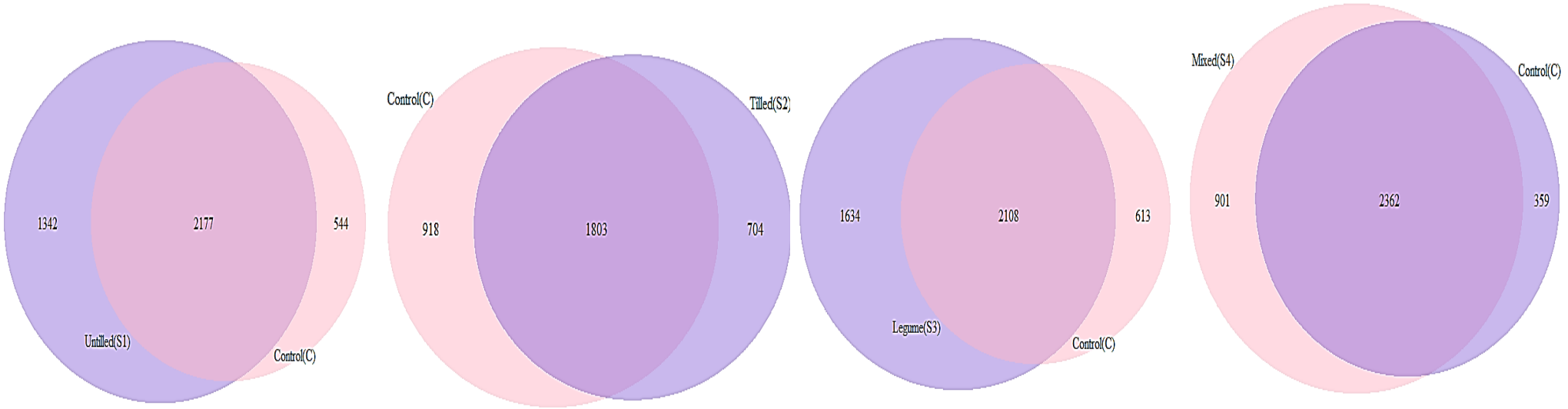


Fig. 6.12 **Number of shared OTUs (mean relative abundance > 1%) with Control (C) by each soil type, Untilled(S1), Tilled(S2), Legume(S3) and Mixed (S4).**

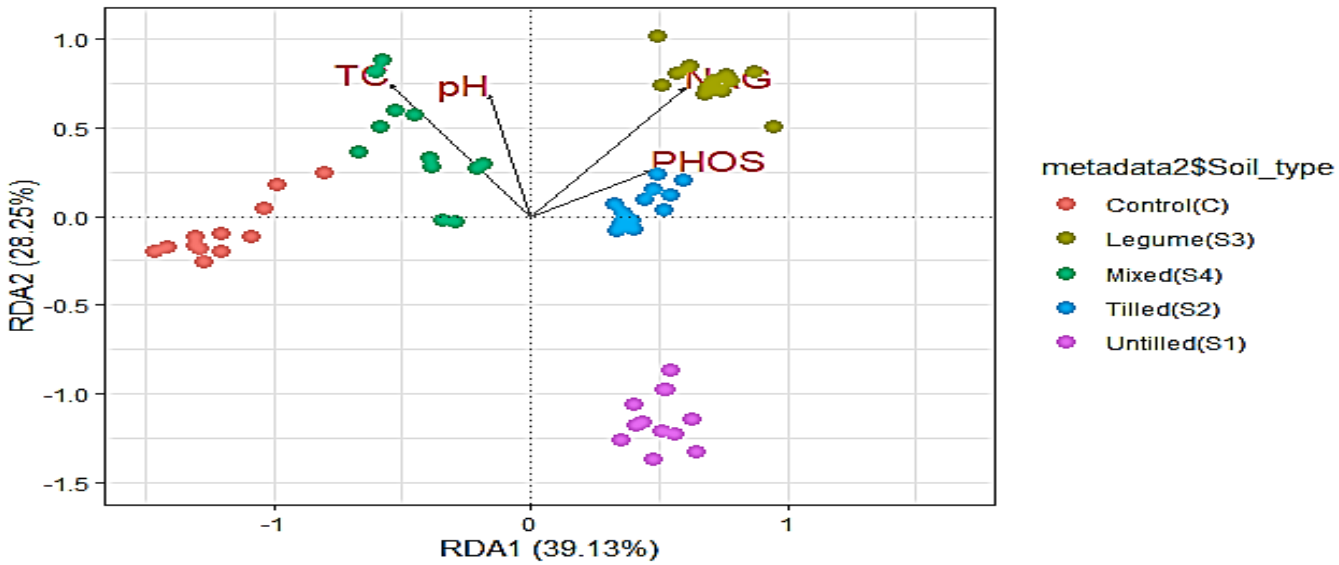


Fig. 6.13 **dbRDA plot based on dissimilarity or distance matrix, representing dissimilarities in the rarefied bacterial species (OTU) community between soil types** (Untilled(S1), tilled(S2),legume (S3), mixed (S4), control (C)) and their relationship with soil properties, Total organic carbon (TOC), pH, N-acetyl beta glucosaminidase (NAG) and phosphatase (PHOS) activity. Samples are represented as points and explanatory variables (soil properties) as vectors. Objects ordinated close together are expected to have similar variable values.

6.3.4 Difference in alpha and beta diversity between fallow soils

OTU matrix was rarefied to lowest sequencing depth of 45770, leaving 4288 OTUs for analysis. The top five phyla identified in fallow soils were Proteobacteria (53.24%), Firmicutes (23.73%), Bacteroidota (6.01%), Myxococcota (5.62%), Verrucomicrobiota (3.80%) and Actinobacteriota (3.4%).

The difference in relative abundance of top ten classes of bacterial taxa between fallow and cultivated soils is given in Fig. 6.14. The relative abundance percentage of Bacilli and Verrucomicrobiae were high in planted soils than fallow soils. Relative abundance of Actinobacteria and Gammaproteobacteria were high in planted soils of legume (S3) (4.16%, 11.69% respectively) as compared to fallow soil (1.34%, 8.18% respectively).

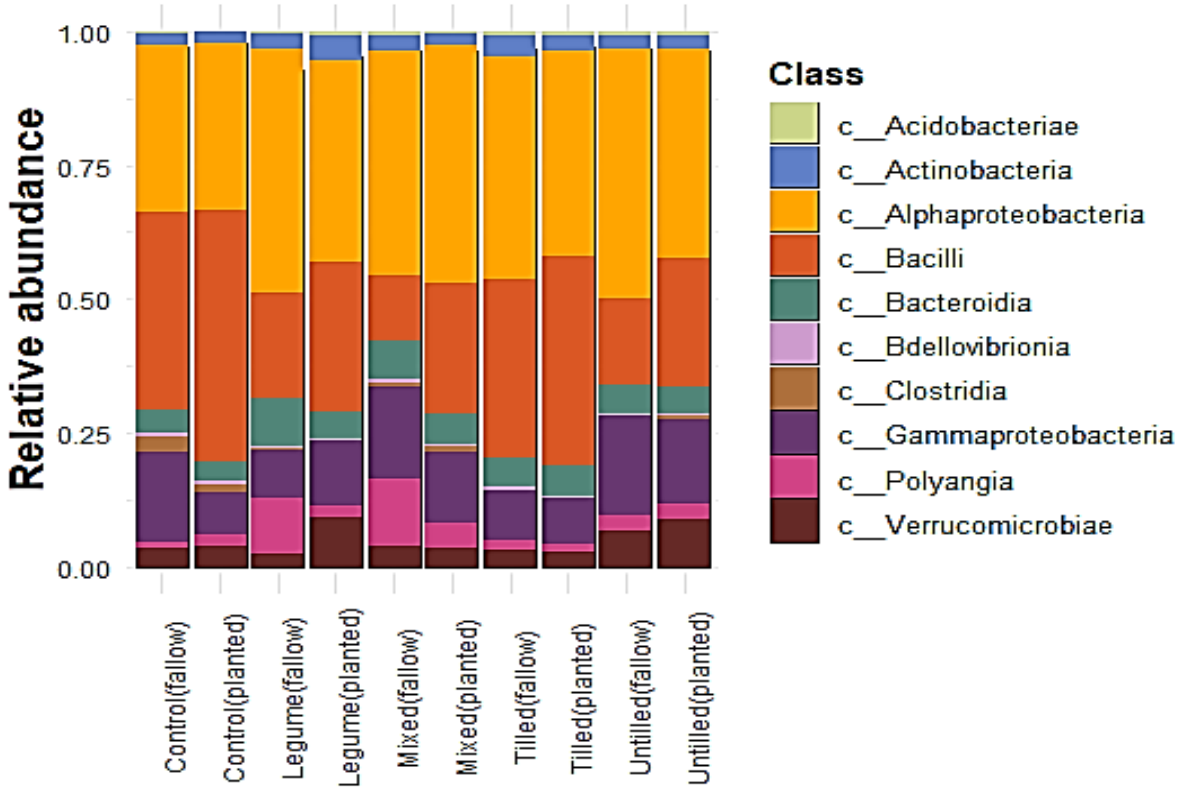


Fig. 6.14 **Relative abundance of top 10 class divisions for bacterial OTUs in fallow and planted soils of different soil types**, Untilled(S1), Tilled(S2), Legume(S3), Mixed (S4) and Control (C) after second generation.

6.3.4.1 *Difference in alpha diversity between fallow soils*

The species richness (F (4) = 0.50, p= 0.732) and Shannon diversity (F (4) = 1.25, p= 0.315) were not significantly affected by soil types in fallow soils (Fig. 6.15).

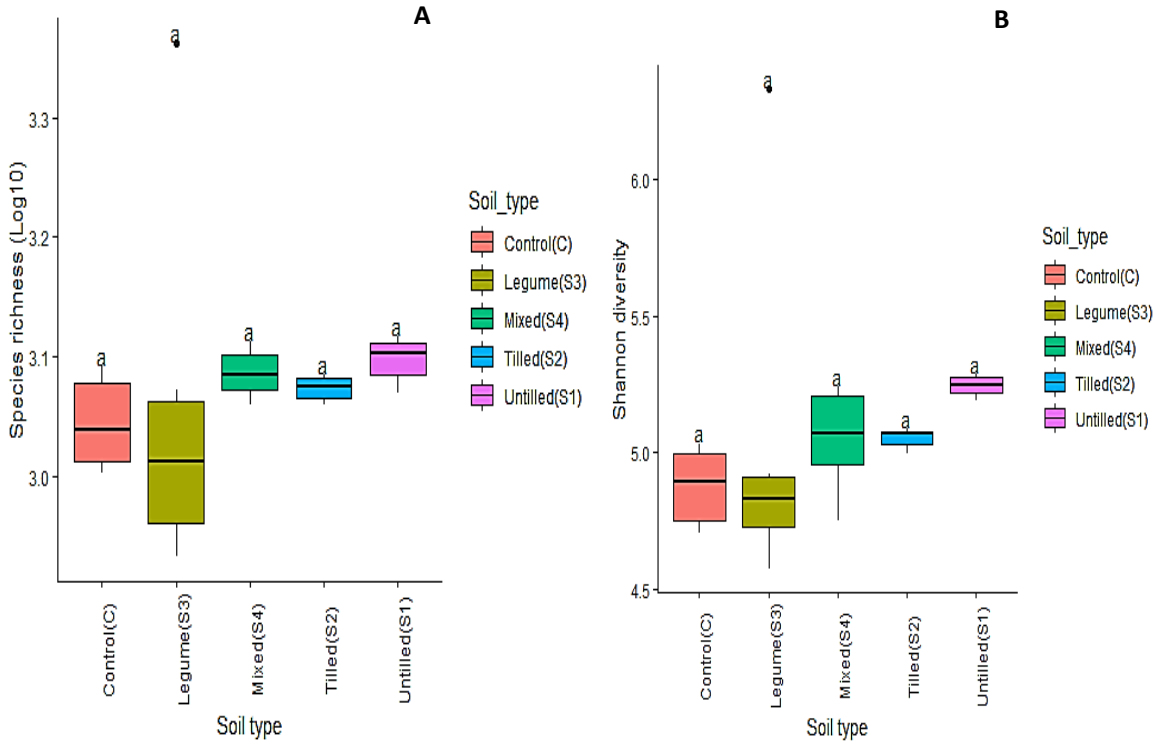


Fig. 6.15 **Effects of soil type on A. Species richness and B. Shannon diversity in the fallow soils of, Untilled(S1), Tilled(S2), Legume(S3), Mixed (S4), and Control (C).** Different letters show significant influences ($p < 0.05$) using Tukey's multiple range tests.n=3

6.3.4.2 *Difference in bacterial composition between fallow soils*

PERMANOVA analysis showed that the species composition differed significantly between soil types in fallow soils (F (4) = 13.13, p=0.001). Fig. 6.16 shows the PCoA plot of diversity plotted with Bray Curtis distance. It represents dissimilarities in the rarefied bacterial communities between soil types.

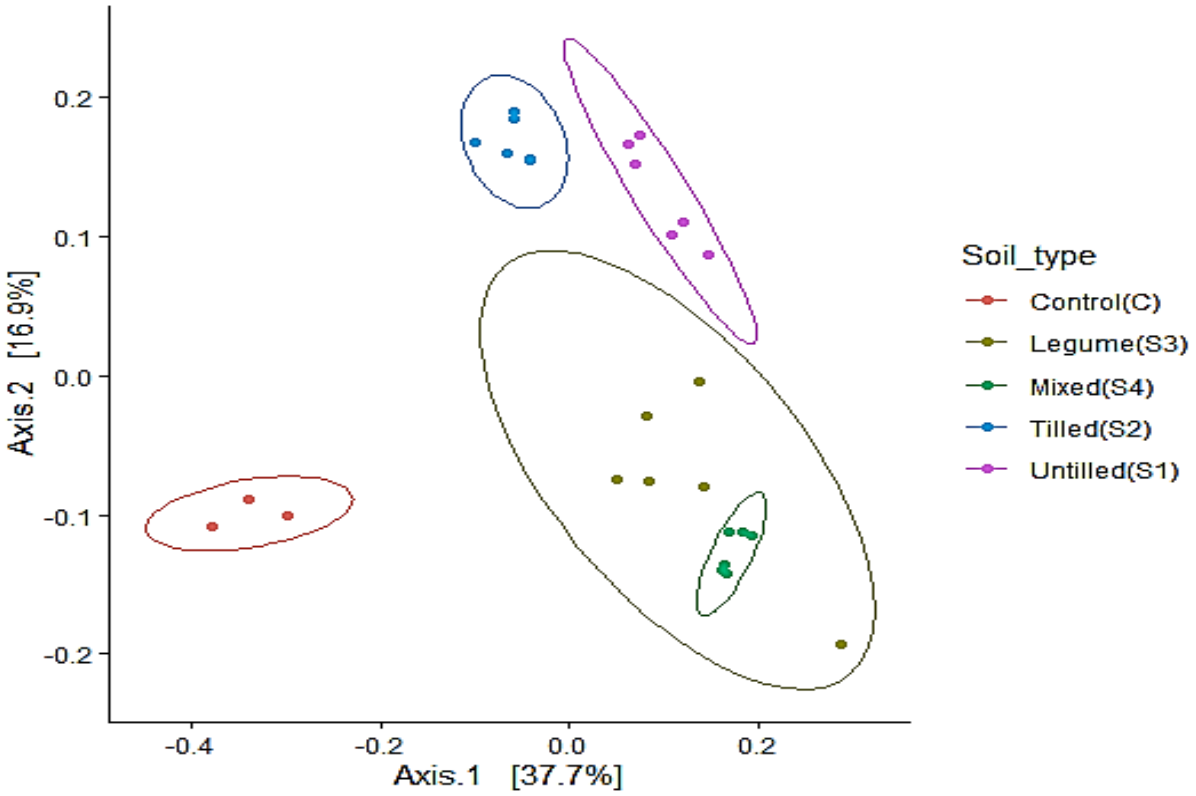


Fig. 6.16 **PCoA plot of diversity made with scores computed using the Bray-Curtis index, representing dissimilarities in the rarefied bacterial species (OTU) community in fallow soils of (Untilled(S1), tilled(S2), legume (S3), mixed (S4), control (C)).** The ellipsis represents a 95% confidence interval.

6.3.5 Change in species diversity and composition between initial microbiome inoculum and rhizosphere sample after host mediated microbiome selection

6.3.5.1 Species richness and diversity

There was a significant difference in both species richness ($F(5) = 22.846$, $p<0.001$) and Shannon diversity indexes ($F(5) = 61.029$, $p < 0.001$) between initial field microbiome and rhizosphere microbiome. Both richness and diversity were significantly higher in the initial soil samples (Fig. 6.17). The difference in relative abundance of top ten class divisions is shown in Fig. 6.18. The top five phyla identified in the soils

were in the order, Proteobacteria > Firmicutes > Actinobacteriota > Verrucomicrobiota > Acidobacteriota. The relative abundance of Acidobacteriota and Actinobacteriota were high in field samples than rhizosphere samples.

6.3.5.2 Species composition

There was significant difference in beta diversity between soil types (F (5) = 28.073, p =0.001). PERMANOVA results showed that TC (F (1) = 20.26, p= 0.001) and pH (F (1) = 55.003, p =0.001) significantly affected the difference in bacterial composition between initial field soil microbiome and rhizosphere soil samples.

The plot based on distance-based redundancy analysis (dbRDA) showed the grouping of soils based on soil variables (Fig. 6.19). The first and second axes explained 93.41% and 6.58% of the variance respectively. The rhizosphere soil samples were high in pH and TC. The bacterial composition in the rhizosphere soils of plants grown in legume soil is significantly correlated to total carbon content. The rhizosphere soils from untilled (S1), tilled (S2) and legume (S3) are oriented in the three different quadrants indicating their difference in bacterial composition after host mediated selection. Objects ordinated close together are expected to have similar variable values. Here, initial soil samples from tilled and legume soils are closer to each other and untilled (initial) is clustered separately. The difference in top fifteen genera identified in initial soil and rhizosphere samples are in Fig. 6.20. The relative abundance of most of the genera were high in rhizosphere samples.

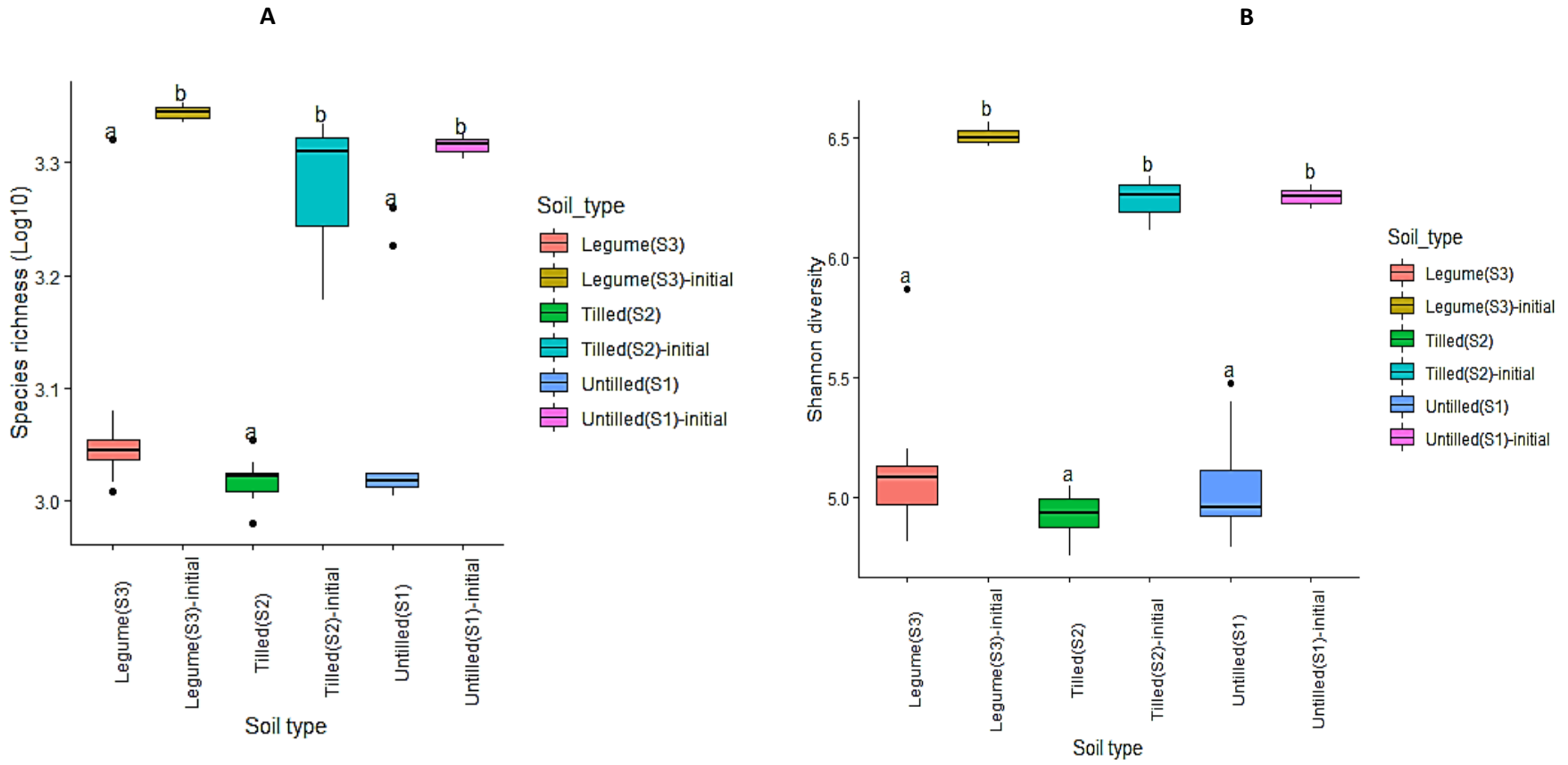


Fig. 6.17 **Difference in A. Species richness and B. Shannon diversity in initial field sample (microbiome inoculum for first generation) and rhizosphere soil samples of plants after microbiome selection in the second generation.** Untilled(S1), Tilled(S2), Legume(S3), Mixed (S4), and Control (C). Different letters show significant influences ($p < 0.05$) using Tukey's multiple range tests. n=3

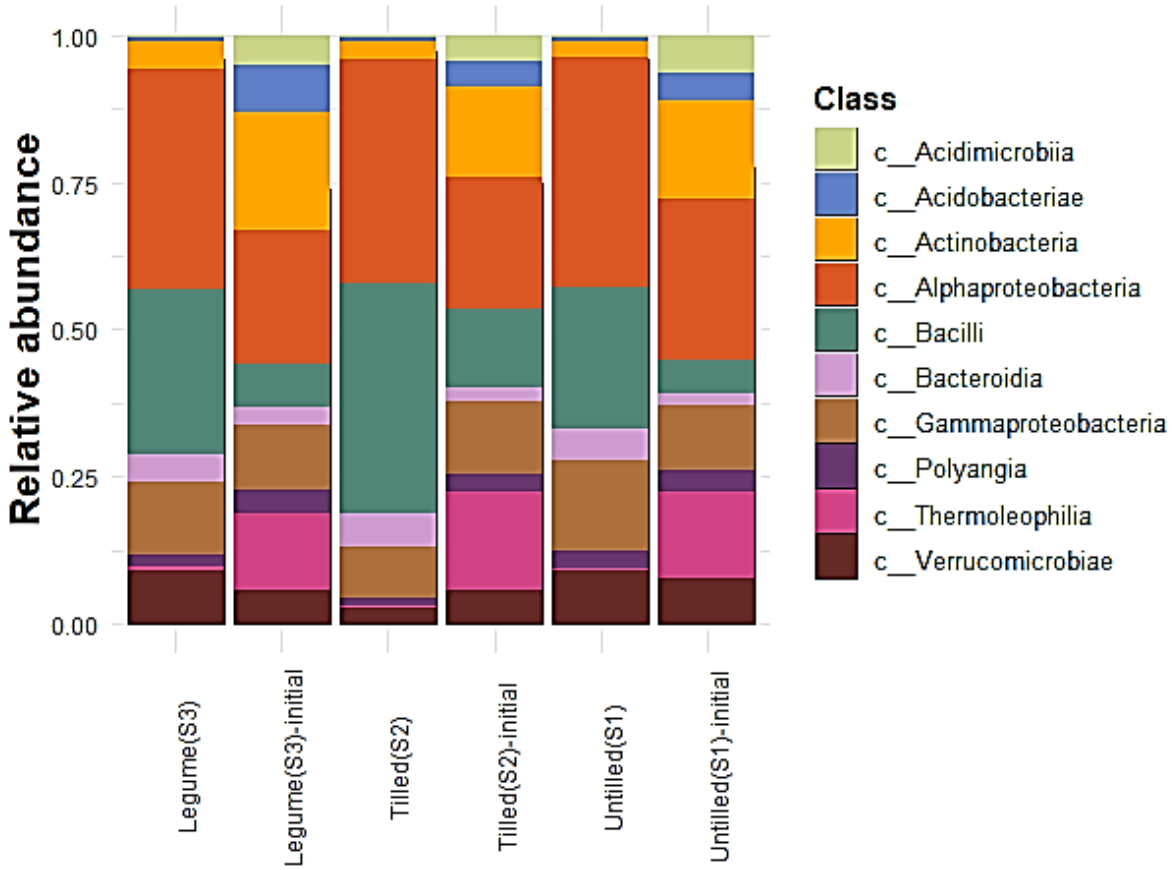


Fig. 6.18 **Relative abundance of top 10 class divisions for bacterial OTUs in initial field sample (microbiome inoculum for first generation) of and rhizosphere soil samples of plants after microbiome selection in the second generation.** Different soil types are Untilled(S1), Tilled(S2) and Legume(S3).

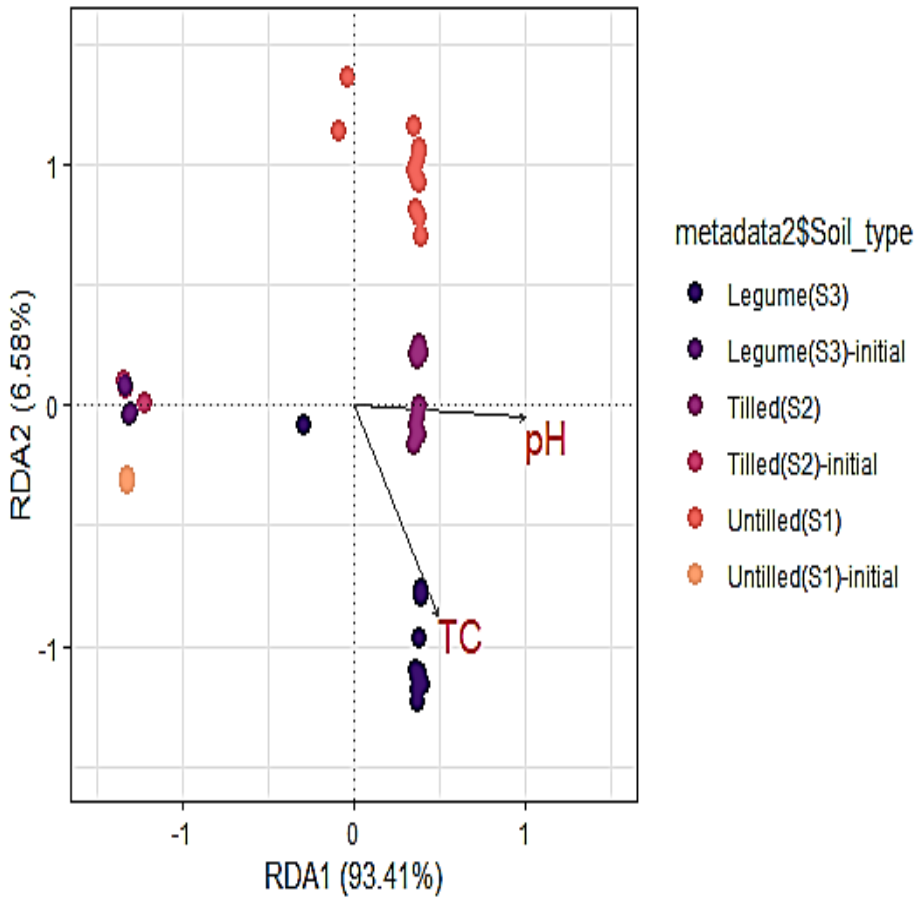


Fig. 6.19 **dbRDA plot based on dissimilarity or distance matrix, representing dissimilarities in the rarefied bacterial species (OTU) community between initial field sample (microbiome inoculum for first generation) and rhizosphere soil samples of plants after microbiome selection in the second generation in (Untilled(S1), tilled(S2) and legume (S3)soils and their relationship with soil properties, Total carbon (TC) and pH.** Samples are represented as points and explanatory variables (soil properties) as vectors. Objects ordinated close together are expected to have similar variable values.

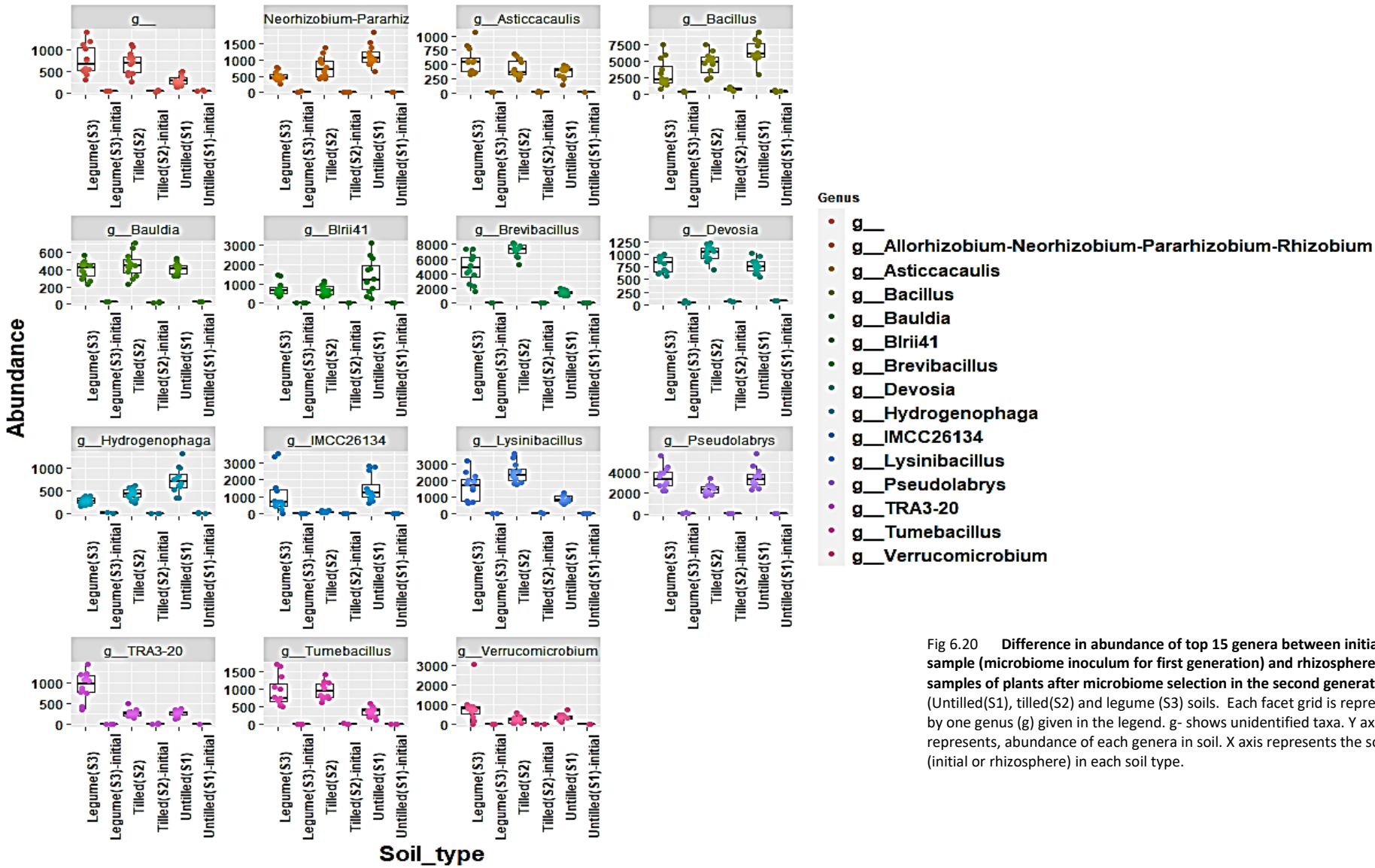


Fig 6.20 **Difference in abundance of top 15 genera between initial field sample (microbiome inoculum for first generation) and rhizosphere soil samples of plants after microbiome selection in the second generation** in (Untilled(S1), tilled(S2) and legume (S3) soils. Each facet grid is represented by one genus (g) given in the legend. g- shows unidentified taxa. Y axis represents, abundance of each genera in soil. X axis represents the soil type (initial or rhizosphere) in each soil type.

6.4 Discussion

The soil is a major reservoir of microbial diversity. Soil biota are responsible for regulating nutrient cycling, energy flow and ultimately plant and ecosystem productivity. Soils have very distinct microbial communities which are influenced by soil physical and chemical characteristics like pH, soil texture, organic matter, and nutrient content (Garbeva *et al*., 2004). Rhizosphere microbial communities can be regarded as a subset of the soil microbial community and equally influenced by soil properties (Marschner *et al*., 2004). In terms of species composition and abundance, microbial communities of the rhizosphere are known to differ from those found in buik soil (Sasse *et al*., 2018) suggesting a strong influence of the presence of plants and their activities on root microbial population. There are also studies in which different plant species growing in the same soil, under the same conditions had similar rhizosphere microbial communities, indicating that the influence of the soil may be greater than that of the plant (Chen *et al*., 2019; Nallanchakravarthula *et al*., 2014).

In this study, the aim was to understand the influence of soil type, plant growth, and varieties on bacterial species richness, diversity, and composition in the rhizosphere of soybean plants when subjected to microbiome selection based on host phenotype and how soil properties affect bacterial community structure. In host mediated microbiome selection, there is indirect selection for beneficial microbiome-host interactions over multiple generations based on host phenotype (Mueller and Sachs, 2015; Jochum *et al*., 2019). This microbiome selection method allows microbiomes to change through both ecological (e.g. diversity, relative abundance) and evolutionary (e.g. extinction events, alterations in allele frequency, mutation, horizontal gene transfer) processes (Mueller and Sachs, 2015). In this study the plants were grown under nutrient limiting conditions and microbiomes were selected based on plant height and it was proposed that there will be differences in bacterial community structure between initial field samples and rhizosphere samples and the changes will depend on soil type, plant growth and plant varieties. The initial inoculum for the study was taken from different areas of a field, differing in tillage, and cropping history.

6.4.1 Effect of soil management practices on bacterial community structure

In this study, the difference in bacterial species richness was not significantly different between field soil samples. Even though not significant, bacterial richness in legume soil was higher than in both untilled and tilled soils. Shannon diversity was significantly higher in legume soils as compared to untilled and tilled soils. The soils designated as untilled soils were from areas of farmland under grass cultivation where grass is cut and left undisturbed on the soil surface. Tilled soils were taken from areas subjected to tillage and legume soils were taken from areas with legume cultivation (*Trifolium pratense*). Zhou *et al*., (2017) found that bacterial diversity and richness was different from that associated with grass upon comparison with legume, with the authors suggesting different root exudation profiles being the primary driver for this difference. Isobe *et al*., (2001) compared five legume species and four grass species and found that legumes secreted significantly more amino acids, sugar, and flavonoids than grass lending support to reasonings for the high bacterial diversity found in our legume soils. In the current study, the root exudate analysis from plants raised in legume soils revealed noticeably higher concentrations of phenols and more amino acids were found in the exudates.

The most dominant bacterial group in soil samples belonged to the phylum Actinobacteria and Proteobacteria which is consistent with other studies related to agricultural ecosystems (Mhete *et al*., 2020, Smit *et al*., 2001). Actinobacteria are a phylum that consist of many Gram-positive bacteria that play a vital role in the cycling of organic compounds (Shivlata and Satyanarayana, 2015). Proteobacteria are a phylum of Gram-negative bacteria, very common in soil environments and are related to a wide range of functions involved in carbon, nitrogen, and sulphur cycling (Mhete *et al*., 2020). Communities of Firmicutes were found to be high in tilled soil. Schmidt *et al*., (2018) reported an increase in the relative proportion of Firmicutes under standard till conditions. At class level, untilled soils were high in the relative abundance of Verrucomicrobiae and Alphaproteobacteria. Legume soils enriched Acidobacteriae, Actinobacteria and Clostridia. The class Desulfuromonadia was also found in high relative abundance in legume soils as compared to other soils. Low abundance of Acidobacteria was found in tilled soils. Members of

Acidobacteriae phylum are often affected by soil physico chemical properties and were found to be negatively correlated to soil nitrogen content (Zheng *et al*., 2021).

The untilled soils were characterized by high organic matter content, nitrate, phosphorus, and ammonium content. The carbon:nitrogen ratio (C:N) was also high in untilled soils (11:1). Decomposition is limited at high C:N (van den Berg *et al*., 2012) which may have contributed to the low diversity in untilled soil upon comparison to legume soil. In the absence of tillage, most of the carbon from the organic matter is tied up in the topsoil layer (Schmidt *et al*., 2018). In our study, sampling was conducted at a depth of 20cm, which may be one of the reasons for the low bacterial diversity found in untilled soils. Consequences of tillage include equal distribution of nutrients and microhabitat disturbance which are known to impact and ultimately reduce microbial diversity (Sengupta and Dick, 2015). Tillage regimes disrupt soil bacterial communities and their densities effectively redistributing these to differing depths. The results showed no significant difference in species richness between untilled and tilled soils. In the absence of tillage nutrient transport in soil is limited and therefore there is limited nutrient availability to microbes at lower depths which explains the lack of significant difference in bacterial richness between these two soils.

Cover crops like legume were shown to improve the microbial diversity in soils (Venter *et al*., 2016). The result from this study shows that growing legumes in the field can increase the bacterial diversity in soils. The beta diversity analysis in this study showed a significant effect of soil type on bacterial composition and the three soils had distinct bacterial composition showing the impact of tillage and cropping history on soil bacterial communities. The unique OTUs found in each soil confirms this fact that even within the same area, changes in cultural practices and cover crops contributes to bacterial structure in soil.

6.4.2 Effect of interaction between soil type, plant growth and variety on bacterial community structure

6.4.2.1 Interaction effect of soil type, plant growth and variety

In this study, the interaction between soil type, plant growth and variety had no significant effect on bacterial richness and diversity after the second generation of microbiome selection. Bacterial richness was significantly affected by soil type and soybean plant cultivation whereas diversity was mainly affected by

soil type. Both richness and diversity were low in control (autoclaved coir: sand) soil. Rhizosphere soil communities were shown to be influenced by plant genotype, plant age and development and soil type. In a study using two soils with similar cropping history, Grayston *et al*., (1998) found significant clustering of potential microbial activities by plant type. In another study, to assess the variation of dominant bacterial populations in respect of soil type (silty sand and loamy sand), plant type (clover, bean, and alfalfa) and developmental stage of the plant, plant species had the greatest effect on rhizosphere communities and plant development stage had the least effect (Wieland *et al*., 2001). The authors also found that the effect of the plant was dependant on the soil type sampled. Marschner *et al*., 2001) found that the effects exerted by different plants on bacterial communities were controlled by soil type. In this study, soil type was found to be a major factor contributing to the variation in bacterial community structure. NMDS analysis showed clustering of bacterial community based on soil type.

Plant roots release a wide variety of compounds including amino acids, sugars, organic acids, enzymes which create a unique environment for the growth of microorganisms in the rhizosphere (Garbeva *et al*., 2004). Bacteria respond differently to the compounds released by plant roots and so, rhizosphere communities are expected to be different with different root exudate compositions. The results from this study showed a difference in relative abundance of bacterial genera in different soil types which varied with growth and variety. The important orders with plant growth promoting bacteria found in this study were Azospirillales, Bacillales, Chitinophagales, Opitutales, Polyangales, Rhizobiales and Sphingomonadales (Cecagno *et al*., 2015; Akinrinlola *et al*., 2018; You *et al*., 2021). Amongst these orders specific groups of Opitutales (IMCC26134), Sphingomonadales, Chitinophagales and genera like *Devosia* and *Asticcacaulis* were found to play active roles in nitrogen cycling (You *et al*., 2021). Our results which show the dominant effect of soil type in defining bacterial communities is supported by a few other related studies (Chiarini *et al*., 1998., Latour *et al*., 1999).

The mixing of microbiomes resulted in new combinations of taxa in mixed soil (S4) which proved favourable for plant growth. Bacterial orders found to be in high abundance in the rhizospheres of plants in mixed (S4) soil compared to untilled (S1), tilled (S2) and legume (S3) were Rhizobiales, Azospirillales, Caulobacterales, Polyangales, Sphingomonadales and Xanthomonadales. Similarly, communities of Rhizobiales,

Micrococcales, Verrucomicrobiales and Chitinophagales were high in legume (S3) soils. Some of the members found in abundance in these soils included *Asticcacaulis*, *Devosia* and IMCC26134 are involved in nutrient cycling (You *et al*., 2021). Other genera found in high adundance in mixed and legume soils were *Pseudolabrys* which are known to metabolize organic acids (Kampfer *et al*., 2006) with populations increasing in response to nitrogen application (Truu *et al*., 2020). Unique OTUs found in mixed soils further indicates that mixing of microbiomes may have resulted in novel combinations of microbes with novel beneficial effects on growth (Mueller and Sachs, 2015). The interaction between plants and bacterial composition varies depending on soil type, as evidenced by these findings.

6.4.2.2 Effect of soil properties on bacterial composition in rhizosphere

Distance based redundancy analysis (dbRDA) showed clear grouping of soil bacterial communities based on soil properties. The activity of soil enzymes NAG and PHOS, and TC in the soils were the main factors that were correlated to the bacterial community composition in soils. The community in legume rhizosphere soils were found to be high in NAG activity. High enzyme activity in the rhizosphere enhanced nitrogen cycling and enhanced plant growth in these soils. We previously showed in Chapter 4 that plant height was positively correlated to NAG activity in soil. In this study, plants were grown under limiting nutrient conditions forcing reliance on nutrient mineralization for their growth and increasing NAG activity which contributed to an improvement in plant growth. A similar result was obtained by Weidner *et al*., (2015) in *Arabidopsis thaliana* where they observed a strong link between nutrient mineralization and plant growth. The bacterial community composition in mixed soils (S4) was positively correlated to total carbon content in soil. Total soil carbon provides both an energy source for microbial growth, and a high surface area substrate for microbial colonization (Allison *et al*., 2007) helping explain the high relative abundance of some of the taxa in this soil. In this study, soil parameters could explain only 67% of the difference in community composition between soils. Untilled soils remained as a separate cluster in dbRDA analysis indicating that there are other factors contributing to control of the microbial composition.

The relative abundance of Bacilli was high in control soils whereas all other major classes of bacteria were in low abundance in this soil. *Bacillus asahii* was found in high abundance in control soils. These strains

were previously detected in alkaline soils exposed to a large amount of organic matter (Yadav *et al*., 2011). Feng *et al*., (2015) reported increased activity of alkaline phosphatase and phosphate hydrolase with *Bacillus asahii*. In our study, the PHOS activity reported in control rhizosphere soils can be explained by the presence of *Bacillus asahii*. The growth of plants in control can be assigned to the nutrient cycling abilities of this strain.

In this study, bacterial richness was not significantly different between soil types except for control. NMDS analysis showed clear clustering of soils based on bacterial community. Mean pairwise distance calculated in the plot is independent of number of bacterial strains in the community (Weidner *et al*., 2015) and reflects the community metabolic potential. Based on our results it can be said that the source of inoculum (soil type) and various soil properties, especially total carbon had a greater influence on bacterial community structure. The unique OTUs found in each soil again shows the distinct characteristic of each soil with respect to their bacterial composition. The difference in bacterial community structure in the rhizosphere should be viewed as their difference in metabolic potential which has a significant effect on nutrient recycling and thus plant growth.

6.4.3 Effect of host mediated selection on bacterial composition

The difference in relative abundance of some of the phyla between fallow and planted soils shows the importance of root exudates in selecting bacterial communities in the rhizosphere. Other investigations have found a clear contrast between bulk and rhizosphere soil microbiomes (Bulgarelli *et al*., (2015); Mendes *et al*., (2014); Kavamura *et al*., (2019)). Similarly, the difference in bacterial abundance between initial field soil and rhizosphere samples shows the changes in the taxa after plant growth. In both cases, selection was done by the plants. The difference in species richness and diversity was not significantly different between control fallow and other fallow soils (untilled, tilled, legume and mixed), whereas there was a significant difference in diversity between rhizosphere soils of control and other treatments. It is important to note that even though the control soil was autoclaved before starting the study, storage conditions of this soil might have resulted in contamination from airborne microbes. Similarly, the study was not conducted under sterile conditions. Even so, the reduction in diversity and richness in control soils

suggests that the field microbiome has an impact on other treatments. Both species richness and Shannon diversity were significantly lower in rhizosphere soil samples as compared to the initial field samples. In this study, soil suspensions from field samples were used as microbiome inoculum in the first generation. Plant height was discovered to be a key factor determining yield in a study done to determine particular plant features contributing to soybean output (Li *et al*., 2019). In this study, plant height was chosen as the trait for selection of microbiome. The rhizosphere soils from high growing plants from the first generation was used as the inoculum for the second-generation plants. The field microbiome sample was subjected to two rounds of selection. Sare *et al*., (2020) found that microbiome transfer significantly influences the quantity and bacterial diversity. Swenson *et al*., (2000) found that the use of soil slurries limits the transfer of soil derived nutrients but also impact function in the inoculated soil. When Swenson *et al*., (2000) altered the amount of soil slurry by a factor of ten, they noticed a variation in *Arabidopsis* plant biomass. In the current study, the soil slurry was inoculated into a 1:1 potting mixture (coir: sand) which significantly differed in soil properties. Here, microbes were introduced to a novel environment, with fewer soil compounds which may have caused adverse effects on the microbial communities as their structure has been shown to be dependent on the properties of soil (Howard *et al*., 2017). Howard *et al*., (2017) also suggested that during the transfer of microbiomes, there is a substantial reorganization of the assemblages which is affected by soil compounds and presence or absence of other soil organisms. In our study we transferred microbiomes through two generations ultimately selecting for and creating new communities of microbes. Lawrence *et al*., (2012) have shown that novel trans-generational microbial communities function differently and more productively than those that are grown alone (monocultures) or in lower diversity combinations. This proved to be true in this study especially in the case of mixed soils (S4), where the combination of bacterial populations from three separate field soils were able to boost overall plant productivity. Lawrence *et al*., (2012) thought that this may be due, in part, to microbes adapting to use metabolites produced by their neighbouring microbes and the absence of either beneficial or suppressive metabolites resulted in the success of certain abundant organisms. The nodulation patterns of plants grown in mixed soils suggests that the plants were able to select for a favourable combination of bacterial population to suit their needs.

In this study, there was an increased abundance of Bacilli and Verrucomicrobiae in planted soils of all soil types. An increased abundance of Verrucomicrobiae in the presence of legume plants was reported by Zhou *et al*., (2017). The phylum Verrucomicrobiae is a globally distributed, abundant, and active group of soil and water bacteria (Bergmann *et al*., 2011). Their possible roles in degrading contaminants and as indicator of soil fertility have been reported by Navarrete *et al*., (2015). Similarly, many of the members of Bacilli are identified as plant growth promoters. In this study, many of the OTUs were not resolved to species level, but one of the species identified was *Bacillus asaahi* which were found to be involved in nutrient recycling (Feng *et al*., 2015). On analysing the top fifteen genera in the initial field soil and final rhizosphere soil, the relative abundance of most of the genera were found high in the rhizosphere which again lends support to selection pressure exerted by plants. Plants in each soil displayed significantly varied relative abundances of distinct species in their rhizosphere, according to the results of linear discriminant analysis (LDA) effect sizes (LEfSe, Fig. 6.8).

We showed, in our study, that when subjected to nutrient limiting conditions plants were able to successfully select for bacterial taxa involved in nutrient cycling. In both the generations, plants in legume soil and mixed soils had better growth than plants in control, untilled and tilled soils and we noticed a reduction in mean differences in plant height between varieties within the same treatment in the second generation. As found in host mediated selection of drought tolerance in wheat by Jochum *et al*., (2019), this study also found reduction in alpha diversity between selection rounds. Changes in beta diversity shows host mediated changes in the rhizosphere bacterial population. Similar results in species community structure associated with dilution were reported by Yan *et al*., (2017). The authors suggested that the plant exerts selection on the microbial community in the rhizosphere based on functional traits than taxonomies, ie, the plants enriched their rhizosphere by selecting microbes with specific functional genes (eg: transporters, Embden–Meyerhof–Parnas pathway and hydrogen metabolism). In this study, it is possible that plants were selecting for species that can help the plants overcome nutrient deficit. Some of the genera identified in this study include *Asticcacaulis, Devosia*, and *Pseudolabrys* which were found to be involved in nutrient cycling (You *et al*., 2021, Kampfer *et al*., 2006). According to the findings of this study, using the host phenotype of higher plant height (high growth) under nutrient shortage conditions, it is

feasible to select advantageous host-bacterial interactions through multiple generations, and soil type is the key factor determining the interactions.

6.4.3.1 Influence of soil properties on bacterial community composition during microbiome selection

The dbRDA analysis (Fig. 6.19) of initial soil sample and final rhizosphere soil samples showed that pH and total carbon were the most important factors that contributed to the change in bacterial community composition. Bacterial cells are pH sensitive, and bacterial species have a rather restricted pH tolerance for growth (Rousk *et al*., 2010). In an experiment with barley and cucumber in three different Californian soils, Marschner *et al*., (2004) found that even a change of one unit in pH may significantly affect the bacterial community structure. In our study, the relative abundance of Acidobacteria was less in rhizosphere soils as compared to the initial field soils which can be attributed to the difference in pH between these soils (Mhete *et al*., 2020). Total carbon in the rhizosphere, the main source derived from plant derived root exudates (Chapparo *et al*., 2013) was another important factor that affected the difference in bacterial composition between initial field samples and rhizosphere samples. The plants grown in legume soils had better growth compared to ones in untilled and tilled soils. The results from the root exudate analysis (Chapter 5) showed that the exudation of carbohydrates was high in plants grown in legume soil. The increase in carbon content in these soils can be attributed to the increased growth (increased plant height) of plants in these soils which led to increased root exudation in these soils which in turn affected the bacterial composition in the rhizosphere.

6.4.4 Conclusions

In this study, our aim was to understand 1. differences in diversity and composition of bacterial communities between field samples taken from areas subjected to different management practices and cropping history and 2. how an inoculum of the microbiome isolated from these soils would select for and ultimately influence the rhizosphere bacterial community composition. Our results demonstrated that the bacterial diversity in legume soils were significantly different from untilled and tilled soils. PERMANOVA

analysis showed a significant effect of soil type on bacterial composition and showed clustering of soils based on bacterial composition. Unique OTUs found in each soil further suggests that these soils vary in their bacterial structure and composition. This difference was likewise seen in the rhizosphere samples of plants grown in coir: sand media inoculated with these field samples and this difference in bacterial composition was shown to be significantly affected by TC content in the rhizosphere as well the activities of soil enzyme. Mixing of field soils increased relative abundance of some of the beneficial taxa in the soil which helped in plant growth and development.

In addition, we observed significant difference in alpha and beta diversity between initial field soil and rhizosphere samples. Some of the genera including *Asticcacaulis, Devosia*, and *Pseudolabrys* which were reported earlier to be involved in nutrient cycling were found in higher abundance in rhizosphere soil samples than initial field soils. Similarly, populations of bacteria belonging to classes Bacilli and Verrucomicrobiae were found in higher abundance in rhizosphere soil samples as compared to the fallow soils supporting the notion of selection pressure exerted by plants. From our study, it can be said that when plants are subjected to nutrient limiting conditions, they are able to successfully select for bacterial taxa involved in nutrient cycling and this selection is significantly affected by soil type and soil properties and most especially the total carbon content.

Chapter 7. Conclusions and Future works

The purpose of the research presented in this thesis was 1. to determine the differences in soil characteristics and bacterial composition between samples taken from a field that differed in management approaches and cropping history, and 2. to investigate changes in soil enzyme activity, root exudation, and bacterial community composition in the rhizosphere soil of soybean plants under nutritional stress utilising a technique known as host driven microbiome selection (Mueller and Sachs, 2015). The results from this study revealed differences in soil properties between the field soils and showed significant increases in bacterial diversity in legume grown soils. Rhizosphere microbiome selection based on plant height resulted in more diverse bacterial communities and the selection of bacterial communities in the rhizosphere by plants was significantly affected by soil type.

7.1 Initial field soil microbiome differed with management practices and cropping history

The work presented in Chapter 3 found significant differences in physical, chemical, and biological properties between soils taken from areas of field that differed in management practices and cropping history. In this study, absence of tillage for extended periods of time (more than 5 years) resulted in high organic matter content in untilled soils. The activity of both the soil enzymes (NAG and PHOS) measured in this study were high in untilled soils. Similar results were reported by He *et al.*, (2007) who found that no till practices with crop residue retention increased the soil microbial community and which in turn improved soil nutrient cycling. The high organic matter accumulation and high activity of nutrient cycling enzymes like NAG and PHOS contributed to the high available nutrient content measured in untilled soils in this study. Activities of both enzymes investigated in this study showed significant positive correlation with soil organic matter content.

The increased soil enzyme activities recorded in untilled system may be associated with the production of enhanced enzyme levels by the active microbial communities that decompose organic matter (Muruganandam *et al.*, 2009). Murugananandam *et al.*, (2009) conducted a phospholipid fatty acid analysis

(PLFA) to study the microbial community composition in no till and tilled soils and found a greater relative abundance of the fungal biomarkers, 18:2ω6c and 16:1ω5c in untilled soils. They suggested the involvement of fungal organisms in enhancing the activities of enzymes, especially nitrogen mineralizing enzymes, in no-till conditions. In our study, NGS analysis was used to understand and to compare and contrast soil bacterial communities. Analysis of our results has shown there to be significant differences in bacterial composition between soils. These differences may be driven by differential organic matter content, since distance-based redundancy analysis revealed that organic matter content in soil had a significant impact on bacterial composition.

The bacterial diversity (richness and diversity) measured in this study was higher in legume soils than in both untilled and tilled soils. Initial soil analysis of legume soils showed low organic matter and nutrient content in these soils as compared to the untilled and tilled soils. The untilled soils were from areas under grass cultivation where grass is cut and left undisturbed on the soil surface to decompose. Tilled soils were taken from areas subjected to tillage and legume soils were taken from areas with legume cultivation (*Trifolium pratense*). The higher bacterial diversity found in legume soils can be attributed to the root exudation profiles of legume plants in these soils during sampling time. In a study by Zou *et al*. (2017), legumes and grass were shown to differentially affect both soil chemical properties and microbial community structure. In the present study, the initial soil analysis results showed low nutrient content in legume soils which might be due to the uptake of nutrients by the plants growing in this soil. Plant nutritional strategy is an important factor involved in shaping microbial community structure in soil (Guyonnet *et al*., 2018). The different nutritional strategies of grass (in untilled soil) and legumes (in legume soil) seem to have affected the nutrient cycling and bacterial diversity in these soils. We did not measure the plant biomass and nutritional traits of different species of plants occupied in the sampling areas. This should be considered in future studies to understand the influence of nutritional strategies of different plant species on microbial community composition in the soil.

Our findings reveal that significant differences in bacterial composition can be found even in adjacent soils and that these differences are impacted by soil conditions, cropping, and management approaches. The inclusion of legumes in crop rotation enriches the soil not only due to the presence of nitrogen fixing

bacteria, but the root exudates of legume plants might also be contributing to enrich other root microbiota which resulted in the increased diversity of bacteria in legume soil.

7.2 Plant growth is correlated to rhizosphere soil enzyme activity

The results presented in Chapter 4 showed that the difference in plant growth is significantly influenced by the difference in enzyme activities in the rhizosphere. This study found significant positive correlation between extracellular enzyme, N-acetyl beta glucosaminidase (NAG) activity and plant height.

The production of extra cellular enzymes is a major mechanism by which microorganisms gain access to the organic bound nutrients and make the nutrients accessible to plants (Sinsabaugh, 2010). Plant root exudates are considered as potential catalysts in this process of breaking down complex organic polymers to release mineralized nitrogen and phosphorus (Haichar *et al.*, 2008). This represents a mutual relationship between plants and microbes, and it can be said that the production of extra cellular enzymes is an important mechanism that helps microbes establish a beneficial association with plants. This creates a selection pressure in the rhizosphere where plants strive to stimulate better coordination with microbes. In this study, the plants were graded based on plant height and the microbiomes from tallest plants were mixed and transferred to the plants in the second generation, with control plants receiving no microbial inoculum. The difference in plant height between two generations of plants in treated soils (with microbiome from either untilled, tilled, legume, mixed) were significant and the plants in control (with no field microbiome) were not significantly different in plant height between two generations. The difference in plant height across generations in microbiome inoculated soils strongly suggests an effect of microbiome selection.

The two extracellular enzymes monitored in this study were N-acetyl beta glucosaminidase (NAG) and Phosphatase (PHOS). These are reported to be involved in nitrogen and phosphorus recycling in soil respectively. The results from this study showed high NAG activity in legume soils after two generations of microbiome selection. Unfortunately, the available forms of nitrogen (NH_3 and NO_3) were below the level of detection in all the soils and so it was not possible to estimate the increase in nitrogen level in soils after microbiome selection. Increase in root exudation of carbohydrates by plants in legume soil might have

stimulated a larger proportion of soil microbes which might have contributed to the increased NAG activity in their rhizosphere. Landi *et al*. (2006) reported that an increased root exudation of glucose resulted in increased nitrogen immobilization in the rhizosphere. The presence of nodules in the plants of legume soils also might have contributed to the increase in NAG activity in the rhizosphere. Roldan *et al*. (2003) reported that the pace at which nitrogen is added to the soil by leguminous plants affects not only the N-cycle but also enzyme activity. Leguminous plants like soybean are known to produce NAG under nutrient deficit conditions. The method of soil enzyme analysis used in this study does not distinguish the sources of enzyme in soil. The enzyme sources can be plant roots, soil colloids, and microbes (Nannipieri *et al*., 2003). The increase in NAG enzyme activity in plant rhizosphere as compared to fallow soils in this study clearly indicates the selection pressure exerted by plants. There is a need for method development to differentiate the contribution of enzymes by different sources (plants, microbes, soil colloids) in soil.

There is also a necessity to measure the activity of a group of nitrogen acquiring enzymes in soil rather than individual ones. NAG activity recorded in mixed soils were significantly lower than in legume soils but slightly higher than the activity recorded in other soils (untilled, tilled and control) and the plant growth in mixed soils was not significantly different from plants in legume soils. This raises the question if an increase in NAG activity alone can explain the better growth of plants in this study. Other nitrogen acquiring enzymes reported to be found in rhizosphere are proteases (PR), ureases (UR) and L-asparaginases (LA) (Fujita *et al*., 2018) and the activity of these enzymes might also have contributed to the nutrient cycling in the rhizosphere of plants in this study. Synthesis of LA and UR in *Bacillus subtilis* were reported to increase in the presence of compounds that are poor sources of ammonium like aspartate, proline, and glutamate but they did not increase under carbon limiting conditions or in the presence of arginine, aspartate and glutamate (Atkinson and Fischer, 1991). Contrary to this, PR and NAG are more dependent on carbon demand and soil properties like pH and they are also considered as carbon acquiring enzymes in addition to nitrogen acquiring enzymes (Fujita *et al*., 2018). It therefore seems likely that the observed increase in NAG activity in the rhizosphere of plants in legume soils appears to be attributable to enhanced carbon exudation in these soils. Once again, PCoA analysis showed grouping of soils based on soil properties and mixed soils were mainly characterised by high carbon content. It is possible that carbon and nitrogen

acquiring enzyme PR might have been responsible for the nitrogen recycling in mixed soils. To gain a clearer picture of nutrient cycling in the rhizosphere, measurements of PR, LA, and UR, as well as NAG, are required. Our results also confirms that activity of NAG is more dependent on soil properties. We found strong positive correlation of NAG activity with total carbon and pH in soils.

The activity of phosphatase (PHOS) enzyme was not significantly correlated to plant height or biomass in this study. An increase in phosphatase activity was recorded in rhizosphere soils of plants in tilled soil and legume soils. It is postulated that in legume crops (nitrogen fixing crops), nitrogen fixation entails high phosphorus activity to enhance phosphorus acquisition from organic sources (Houlton *et al*., 2008). Png *et al*., (2017) found that the increased phosphatase activity in legume soils is not directly linked to nitrogen fixation. The low phosphatase activity measured in mixed soils, therefore does not follow the hypothesis that nitrogen fixation directly allows greater investment in phosphatases. Nitrogen fixation was not measured in this study, but presence of nodules suggests nitrogen fixation in these soils. Symbiotic ectomycorrhizal fungi were found to release phosphatase enzymes for organic phosphorus acquisition and future research should include measurement of fungal activities including ectomycorrhizal fungi (Png *et al*., 2017) while studying soil enzyme activities. The increase in phosphatase activity in tilled soils can be attributed to the taxa class Bacilli found in high relative abundance in these soils. Ramesh *et al*., (2011) reported increased rhizosphere phosphatase activity in the rhizosphere of soybean plants inoculated with *Bacillus* isolates.

Overall, our results suggest that soil enzyme activities are good indicators of nutrient changes happening in the rhizosphere. We found that the enzyme NAG was positively correlated to plant growth. Host mediated microbiome selection is a good platform to study various interactions happening in the rhizosphere of plants and plants can selectively recruit microbes necessary for nutrient cycling when grown under nutrient deficient conditions which is reflected in the soil enzyme activities in the rhizosphere. Both the enzyme activities were significantly influenced by soil type and the difference in soil type is reflected in their bacterial composition.

7.3 Root exudation is affected by soil – plant interaction

This is the first time an attempt was made to study the difference in root exudation in plants during a multigenerational microbiome selection. The results revealed that there was a significant effect of soil-plant interaction on root exudation with soil type having more significant effect. The study on root microbiome of *Arabidopsis thaliana* under controlled conditions revealed soil type as the major source of variation in root microbiome and genotype effect was found to be less significant (Lundberg *et al*., 2012). Similar observations were made in mustard (*Boechera stricta*) (Wagner *et al*., 2016) and maize (Chen *et al*., 2019).

This study failed to find evidence to suggest that the concentration of root exudates changes during multigenerational microbiome selection. The hypothesis was that since during microbiome selection in each generation, there will be a decrease in diversity of bacteria (Jochum *et al*., 2019), this decrease in richness and diversity might affect root exudation in plants. The concentration of both phenols and carbohydrates in root exudates did not differ significantly between generations in all soils. Root exudation is an important phenomenon by which plants enrich their rhizosphere microbiota. In a recent study to understand the modulation of root exudation by rhizosphere microbiome in tomato, Korenblum *et al*., (2020) found that tomato plants exude a complex of metabolites, including acyl sugars, steroidal glycoalkaloids, hydroxycinnamic acid derivatives, and the chemical makeup of the exudation is impacted by specific microbial taxa established on local roots. They found that the colonization of *Bacillus subtilis* in tomato root increased exudation of acyl sucrose. This suggests that the absence of significant difference in concentration of root exudation of carbohydrates and phenols between generations may not necessarily mean that there was no effect of selection on root exudation. The analysis method used in this study does not allow identification of different types of sugars and phenols exuded. Identification of different sugar and phenolic compounds will help to understand the difference due to microbiome selection better.

The concentration of carbohydrates and phenols were significantly high in the rhizosphere of plants grown in legume soil and mixed soil. There was nodulation in plants grown in these soils. Legume (S3) soil was

from an area in the field where red clovers (a legume crop, *Trifolium pratense*) were grown during sampling time and this explains the presence of rhizobia in these soils. Mixed (S4) soil was prepared by mixing the field soils (untilled (S1), tilled (S2), and legume (S3)) in equal proportions. Mixing of microbiomes might generate novel combinations of microbes with novel beneficial effects on a host (Mueller and Sachs, 2015), or merge previously separate networks of microbes into a superior compound network (called community-network coalescence; Rillig *et al*., 2016) or generate novel competitive interactions between microbes that increase microbiome stability (Coyte *et al*., 2015). In this study, the presence of nodules in plants in mixed soil and better growth of plants in these soils suggests that mixing of field microbiomes resulted in a novel network of microbes in mixed soil and plants with the help of exudates were able to select their rhizosphere microbiome beneficial to their growth and development. During nitrogen scarcity in soil, legume crops were reported to exude phenolic compounds (flavonoids and isoflavanoids) into the rhizosphere which act as signalling molecules that triggers a group of bacteria (*Rhizobia*) to enter a symbiotic association with plants and formation of nodules (D'Haeze *et al*., 2002). The analysis of amino acids in these soils in the second generation showed presence of tryptophan, ornithine, tyrosine, methionine, lysine, and asparagine which were not detected in other soils (untilled and tilled). Asparagine and tryptophan are reported to be chemotactic to plant-associated- bacteria including *Rhizobium leguminosarum*, *Bacillus subtilis* and *Azospirillum brasilence* (Carvalhais *et al*., 2015). Presence of these compounds in the rhizosphere of legume and mixed soils suggest that they acted as signalling molecules and helped in the formation of nodules in plants in these soils. Besides, tryptophan is also reported as a precursor to produce important flavonoid compounds involved in rhizobia-legume symbiosis (Mandal *et al*., 2010). The increased phenol concentration in the rhizosphere of the plants in legume and mixed establishes that the increased exudation of flavonoids was a means by plants to establish symbiotic relationship with rhizobia present in soil to survive nutrient deficient condition.

The results on root exudation confirms that the soil type is a major factor influencing the microbiome of rhizosphere soils and that plants can select for specific microbes to establish a mutual relationship with the help of their root exudates. This study shows the importance of studying various components of root exudates in understanding the changes happening in the rhizosphere during microbiome selection. Root

exudates can be used to create specific environment around roots to encourage growth of beneficial root microbiota (Wang *et al*., 2017) and this has great potential in sustainable agriculture.

7.4 Plants select distinct bacterial taxa to satisfy the nutrient demand in rhizosphere and it depends on soil type

Next generation sequencing methods were used to study the difference in bacterial taxa in the rhizosphere of plants grown in different soils. The results showed that soil or source of inoculum was the most important factor that determined the bacterial diversity and composition in the rhizosphere of soybean plants in this study. Wieland *et al*., (2001) and Marschner, *et al*. (2001) reported that the effects exerted by different plants on bacterial communities were controlled by soil type. Distinct OTUs were identified in each soil in this study and grouping of soils in NMDS plot based on bacterial composition establishes that soil type was the important factor that contributed to the variation in bacterial composition. The most important soil properties that affected the bacterial community composition were total carbon content in the rhizosphere soils and soil enzyme (NAG and PHOS) activities. Earlier while discussing the results of soil enzymes, we found that NAG activity was significantly correlated to growth of plants and increased root exudation in legume plants positively contributed to the increased NAG activity measured in the rhizosphere of plants grown in legume soils. This holds true for mixed soils as well which had high levels of carbohydrate exudation in their rhizosphere. Other enzymes involved in nutrient cycling, such as protease, and Rhizobia nodule activity, may have also contributed to improved plant growth in mixed soils. The bacterial taxa identified in the rhizosphere of mixed soils vary from that found in legume soils. The relative abundance of Proteobacteria (Rhizobiales, Burkholderiales) and Myxococcotta (Polyangiales) were significantly high in mixed soils. Among this Burkholderiales were found to be capable of promoting plant growth through degradation of pollutants (Siciliano *et al*., 2001), fixing N (Estrada-De los Santos *et al*., 2001) and synthesizing phytohormones (Suarez-Moreno *et al*., 2012). Legume soils were found significantly high in Actinobacteriota, Rhizobiales (Xanthobacteraceae including unknown genus and *Pseudolabrys*) and Verrucomicrobiota. Xanthomonadales members are known hydrocarbon decomposers (Lueders *et al*., 2006). Many members of Actinobacteria were found to play an important role in nitrogen cycling in desert and cultivated farm ecosystems (Zhang *et al*., 2019). The possible roles of members of Verrucomicrobiota

in degrading contaminants and as indicator of soil fertility have been reported by Navarrete *et al*., (2015). Similarly, many of the members of Bacilli are identified as plant growth promoters (Akinrinlola *et al*., 2018). The rhizosphere soils of plants in other soils (untilled, tilled and control) also had distinct taxa associated with them. Rhizosphere of untilled soils were more dominated by Gammaproteobacteria (many unidentified order and genus) and Verrucomicrobiota (order Opitutales). Tilled soil rhizospheres were colonised by Bacteroidota, Bacilli (*Paenibacillus*, *Lysinibacillus*) and Rhizobiales (family Devosiaceae). The rhizosphere of plants in control showed high abundance of Bacilli (*Bacillus asahii*) and Rhizobiales (unidentified genus). Even though many of these taxa were not identified to species level in this study, the difference in their OTUs and the unique OTUs identified in each soil shows that under nutrient limiting conditions plants enrich their rhizosphere with growth promoting microbial taxa from the existing microbial pool.

Host mediated selection of microbiome decreased the bacterial diversity and richness in the rhizosphere soils of plants after second generation of selection. Bacterial composition was also significantly affected by selection process. The distance-based redundancy analysis (dbRDA) revealed that pH and total carbon were the most important factors that contributed to the change in bacterial composition. This result is in confirmation with other studies where a significant correlation was noticed between soil pH and bacterial composition (Rousk *et al*., 2010, Marschner *et al*., 2004). Total carbon content in the rhizosphere soils were high which can be attributed to the root exudation in the rhizosphere. The main source of carbon in the rhizosphere is from plant derived root exudates (Chapparo *et al*., 2013). This increase in carbon content helped in the increase of relative abundance of many taxa in the rhizosphere soil as compared to the initial soil samples giving further evidence for the role of root exudates in microbiome selection. The result from our study shows that the reduction in microbial diversity by selection forced plants to select from a restricted community. In a recent study in barley, Saghai *et al*., (2022) found that reduction of microbial diversity by dilution constrained plant microbiome selection and this constrained recruitment was associated with changes in functional traits within the microbial community. The different taxa identified in the rhizosphere of plants in different soil types in this study and their role in plant growth promotion shows that under nutrient stress plants recruit specific taxa in their rhizosphere which help the plants to

grow under nutrient deficit conditions and their selection is significantly affected by the source of inoculum. The significant correlation of soil enzymes, NAG (involved in nitrogen recycling) further confirms this result.

The results in this study are based on amplicon sequencing of 16S rRNA gene fragments and many of the biological functions (eg: nutrient cycling, disease resistance) do not correspond with 16S rRNA gene data (Berg *et al*., 2014). More theoretical and applied research is required to explore the mechanisms underlying community assembly and function in plants. Shotgun metagenomic analysis provides the entire genomic information in the environmental sample (unlike 16S where it concentrates on specific part of the gene) and helps in studying the functional aspect of the microbiome in the sample. New phylogenetic and functional understanding will result from the comparison of plant-associated communities and metagenomic studies. Functional analysis will show whether the presence of specific microbes can help the plants.

7.5 Final conclusions

This research adds to our knowledge of soil-plant-microbe interactions, which is critical in sustainable agriculture because it allows us to employ the plant microbiome to alleviate nutrient stress in agricultural environments. Plant microbiome is a direct function of microbial community found in the soil and agricultural practices have got a significant impact on soil microbial community. Our results confirmed that the difference in management practices significantly influenced the bacterial community in soils and this difference was noticed throughout during microbiome selection where the greatest factor found significant in the difference in diversity of bacteria in the rhizosphere was soil type. The results from this study have enough evidence to suggest that the rhizosphere enzyme activity is a strong indicator of biological activity in the rhizosphere and is responsible for nutrient content in the rhizosphere. The enzyme NAG showed significant positive correlation with plant height and above ground dry mass in this study. The results showed that, even with two generations of selection, we can get a microbiome rich in plant beneficial bacteria capable of promoting growth in plants under nutrient stress. Further selection rounds might be

able to provide us with a stable microbiome with beneficial properties (Panke-Buisse *et al*., 2015). Identification of different root exudate components will further help us to unravel the mechanisms controlling plant- microbe interactions (Mueller and Sachs, 2015). This study only looked at the bacterial components of the microbiome. The microbiome components found in the rhizosphere also include fungi, protozoa, nematodes, algae and microarthropods (Raaijmakers *et al*., 2008). Understanding the processes that alter the makeup, dynamics, and activity of the rhizosphere microflora, as well as their reliance on plant root exudates, is critical for the development of innovative techniques to enhance plant growth and health. Future studies on plant microbiome should include these various components along with bacteria to understand their role in soil- plant interactions.

7.6 Future perspective

Microbiome engineering is still in its infancy and has great potential application (Foo *et al*., 2017). The emerging field of rhizosphere and microbiome engineering offers an exciting and powerful opportunity to fill critical gaps in knowledge and provide solutions. However, many unanswered and important questions remain - what methods of microbiome engineering is most efficient? Does mixing of evolving microbiomes between hosts accelerate or decelerate the response to selection? The metabolic changes happening during this interaction might give us an insight into how plants recruit specific microbial communities for defence (Mueller *et al*., 2016). There are still knowledge gaps in our understanding of dynamic feedbacks between plant physiology and microbial functions that drive rhizosphere colonization and maintenance of plant microbe interactions (Busby *et al*., 2017). There is a need to characterize the role of different components of root exudates in shaping the microbial communities and their functions (Lebeis *et al*., 2015). The assemblage of microbiome depends on the response to the multiple signals from the plant and more data on these interactions will contribute to the development of strategies to manipulate plant health (Rosier *et al*., 2016).

The plant-associated microbiome has the ability to increase or decrease species coexistence and, as a result, affect not only a single plant but entire ecosystems and it does this by offering novel

nutritional and defensive pathways and by altering metabolic pathways (Berg et al., 2014). Numerous bioinoculant formulations are built on a single, carefully chosen cultivable microbe that is intended to boost plant growth and control disease. Root exudates play a major role in facilitating beneficial plant-microbe interactions and research should focus on understanding how root exudates can be used to manage the soil microbiome. Characterizing the numerous chemicals involved for the network communication between plants and microorganisms with the technologies now in use is a significant issue in this research. To characterise the microbiome signalling molecule, it is required to apply molecular methods like metabolomics and metatranscriptomics as well as current instruments with greater sensitivity, such as spectroscopies. For the purpose of improving research on plant microbiomes, multidisciplinary approaches that combine novel and conventional instruments are crucial.

Soil is considered as the main source of microbial diversity in the rhizosphere (Berg *et al*., 2009). Recent research on the seed microbiome revealed that during the germination period, endophytic bacteria in seeds are liberated and contribute to the microbial composition in the rhizosphere (Johnston-Monje *et al*., 2016; Nelson, 2018). The relationship between soil microbiome and seed endophytes is still unclear. More research is required to comprehend how seed endophytes travel during plant growth and development and how this affects the soil microbiota.

Microbiome engineering gives us an opportunity to study the plant- microbe interactions in its complexity involving different communities. This will lead to a more complete understanding of these interactions and could lead to potential mechanisms for improving crop growth (Christian *et al*., 2015). The healthy microbiome will depend on the environmental challenges faced by the plant (Busby *et al*., 2017). Artificial microbiome selection performed by manipulating various stress factors will help in understanding the microbiome properties under different contexts (Mueller and Sachs, 2015). It is important to understand the communication between plant and microbiota associated with it and their role in controlling the plant immune system. A healthy microbiome might shield

plants against pathogen infection and can biologically control illnesses has been widely demonstrated (Mendes *et al*., 2011; Berendsen *et al*., 2012). Uncovering the processes by which plants manage their microbiome, and the microbiome controls plant health, will offer up new possibilities for improving crop quality and yield. This is critical to meet the growing need for food production due to global population expansion. By manipulating the microbiome to choose more effective microbial groups for plant development, or using it as an inoculant, it may be possible to utilise the microbiome in agriculture. To advance sustainable farming practises, it is crucial to reduce the usage of pesticides and artificial fertilisers based on an understanding of the potential of the plant microbiome.

Discoveries about plant microbiomes could lead to improvements in sustainable agriculture, such as the creation of microbial inoculants as biofertilizers, biocontrol agents, or stress-relieving products (Berg, 2009; Schreiter *et al*., 2014). A component of the beneficial microbiome should be included as a biomarker during the plant breeding process. In order to stop the spread of plant diseases and the dangerous interactions between human pathogens and plants, it may be crucial to have a deeper understanding of the entire plant microbiome (Schreiter *et al*., 2014). Overall, we are beginning to understand the complex and intimate relationship between the microbiome of the plant and the effects it has on plant fitness and productivity. The ecological, evolutionary, biochemical, and molecular responses of these interactions to climate change, however, are still poorly understood and, in some cases, altogether unknown (Trivedi *et al*., 2022). Understanding this will make it easier to forecast how climate change will affect the microbiome associated with plants and open up new opportunities for applied research to take advantage of interactions between plants and microbes to increase the climate resiliency of plant communities.

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Appendix

A. Pilot study for root exudate analysis

A.1 Planting

Surface sterilized soybean seeds (with ethanol (70%), Section 2.4.1.4) were planted in 5-inch plastic pots (Terracotta plastic pots, 1 litre volume, Teku brand, LBS, UK). The pots were washed with water and dried before use. All the pots were wiped clean with ethanol (70%) prior to use. The pots were filled with 1:1 mixture of field soil: autoclaved compost (1:1 volume/ volume, 3 litres of field soil with 3 litres of compost mixed and autoclaved). This was repeated for all the three soils (untilled (S1), tilled (S2), legume (S3)). The control pots were filled with autoclaved compost (John Innes potting compost, with 7:3:2 v/v/v of loam: peat substitute:sand and 2:2:1 w/w/w of horn meal, superphosphate and sulphate of potash). The seeds were planted in the middle of the pots (one seed per pot, five pots per soil type) at a depth of 5cm. The temperature recorded during the growth of plants varied between 20 to 25°C. The pots were irrigated with tap water regularly making sure there is no excess run off from the pots.

A.2 Root exudate collection

Plants were harvested after 5 weeks of growth. Root exudates were collected from each plant immediately after harvest. The washed roots along with the whole plant was immersed in 300ml of sterile water in cylindrical flasks and kept on a shaker for 3hours at 25°C to collect root exudates (modified from Kawasaki *et al*., 2018). 50 ml of the collected root exudates were filter sterilised by passing through 0.22um filters (sterile modified acrylic polyethersulfone membrane, Millex®, Sigma, UK) and stored in 50ml falcon tubes at -20°C until analysis.

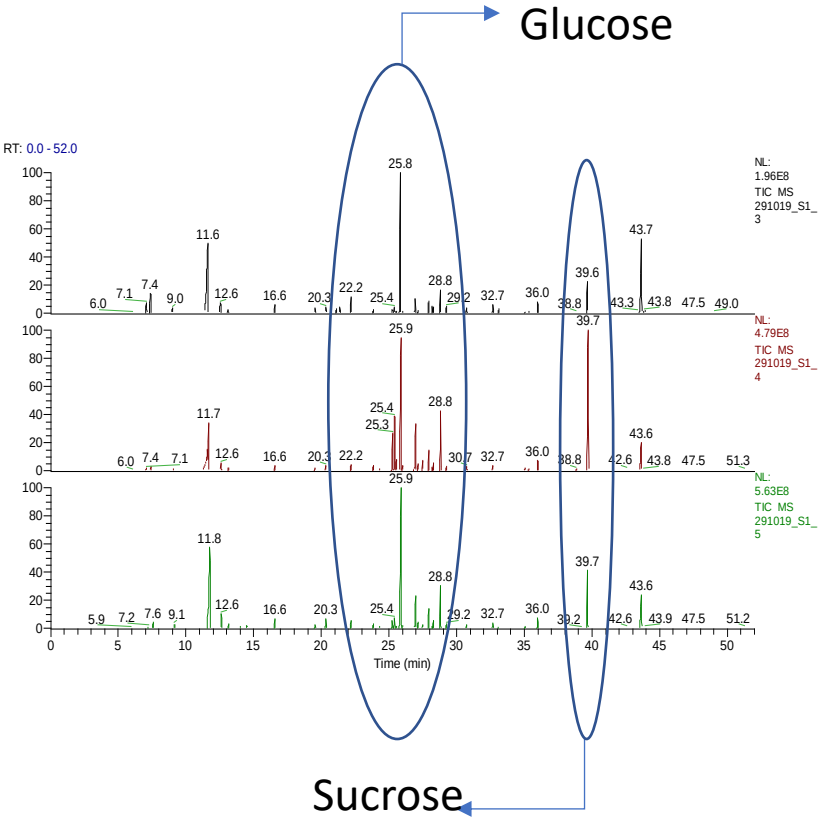
A.3 Derivatization and GCMS analysis of root exudates

50 ml aliquots of root exudates collected as described in sampling section were lyophilised (Heto PowerDry Thermo scientific PL3000) and resuspended in 200ul of methanol and transferred to Eppendorf tubes and the solvent was evaporated to dryness in a speed vac evaporator at 30°C. The samples were then derivatized by adding 25ul of methoxyhydroxymethylamine (20mg mL^{-1} in pyridine, Sigma Aldrich) and

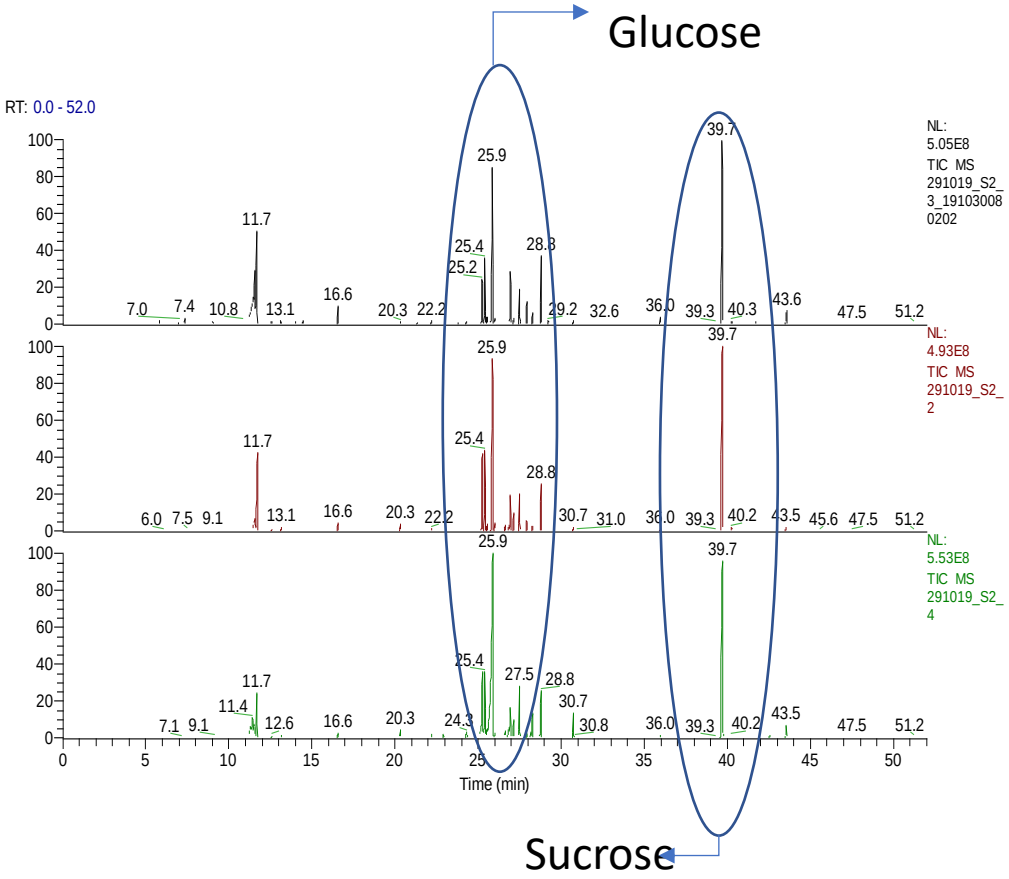
incubated for 2 h at 37°C, 250 rpm followed by the addition of N-Trimethylsilyl-N-methyl trifluoroacetamide, MSTFA (Sigma Aldrich) and incubated at 37°C, 250rpm for 30 minutes (Neumann, G. *et al*, 2014). The samples were then transferred to GC-MS glass vials with insert (0.2 mL, clear glass insert (with plastic bottom spring), O.D. × H 6 mm × 29 mm, Sigma, UK) and analysed.

1 µL aliquots were analyzed using an Thermo Trace GC ultra-gas chromatograph (Thermo scientific, UK) in the splitless mode, coupled to an ion trap mass spectrometer ITQ1100 (Thermo scientific). Separation was performed on a Rxi®5HT MS Integra column (Thermo scientific) with a 0.25 mm inner diameter and 0.25 µm film thickness. Injection temperature was 160°C. The temperature program for GC separation was 3 min at 80°C isothermal followed by a ramp of 5°C min^{-1} to 300°C for 5 min. Mass spectrometric (MS) data was recorded with XCalibur (Thermo Scientific software version 3.0) using electron impact in positive ion mode with a maximum ion time of 25 milliseconds with three micro scans in a range of 50–700 m/z. Data was analysed using Xcalibur (Thermo Scientific).

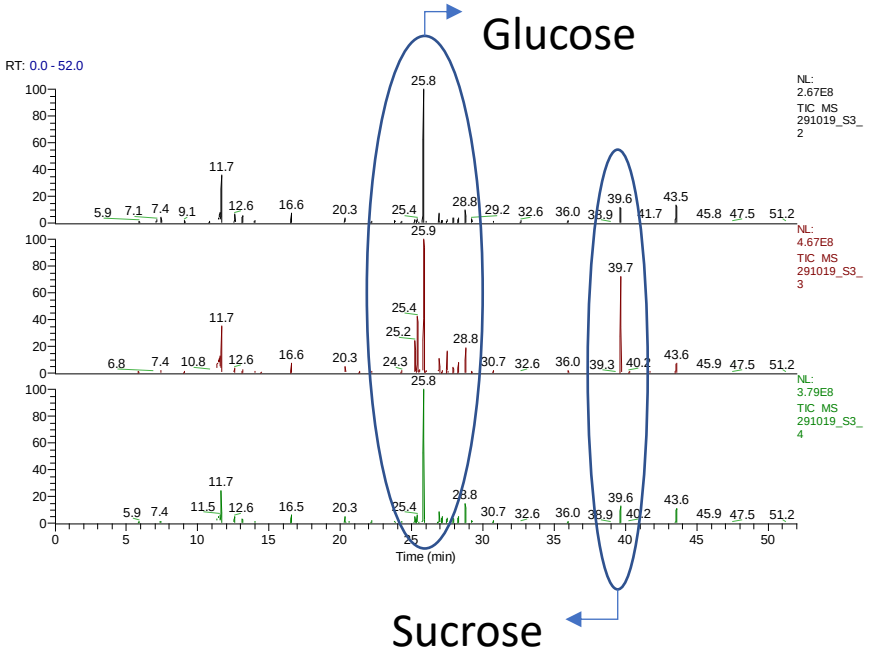
The chromatograms obtained had peaks at different retention times, but it was difficult to identify them due to the lack of a specific compound library. The chromatograms obtained for each soil type can be seen in (Fig. A.1 to A.4). To identify the compounds exuded, the possible compounds found in root exudates were identified from the literature, derivatized and analysed in GCMS in the same way as our samples which were then compared against the sample chromatograms. Only glucose and sucrose have so far identified in the samples. No peak for sucrose was found in the control chromatogram.



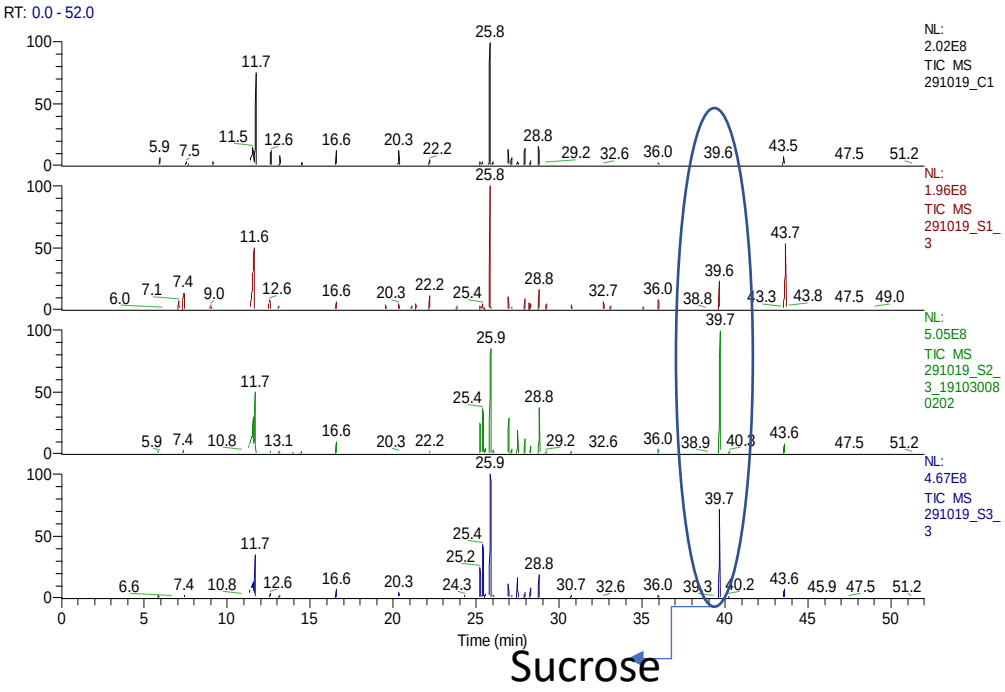
Supplementary Fig. A.1 **Chromatogram showing peaks from GCMS analysis of root exudates of plants in untilled soil (S1).** The retention time at 25.9 indicates glucose and at 39.7, sucrose. S13- plant 3 in S1, S14- plant 4 in S1 and S15- plant 5 in S1.



Supplementary Fig. A.2 **Chromatogram showing peaks from GCMS analysis of root exudates of plants in tilled soil (S2).** The retention time at 25.9 indicates glucose and at 39.7, sucrose. S2 2- plant 2 in S2, S2 3- plant 3 in S2 and S2 4- plant 4 in S2.



Supplementary Fig. A.3 **Chromatogram showing peaks from GCMS analysis of root exudates of plants in legume soil (S3).** The retention time at 25.9 indicates glucose and at 39.7, sucrose. S3 2- plant 2 in S3, S3 3- plant 3 in S3 and S3 4- plant 4 in S3.



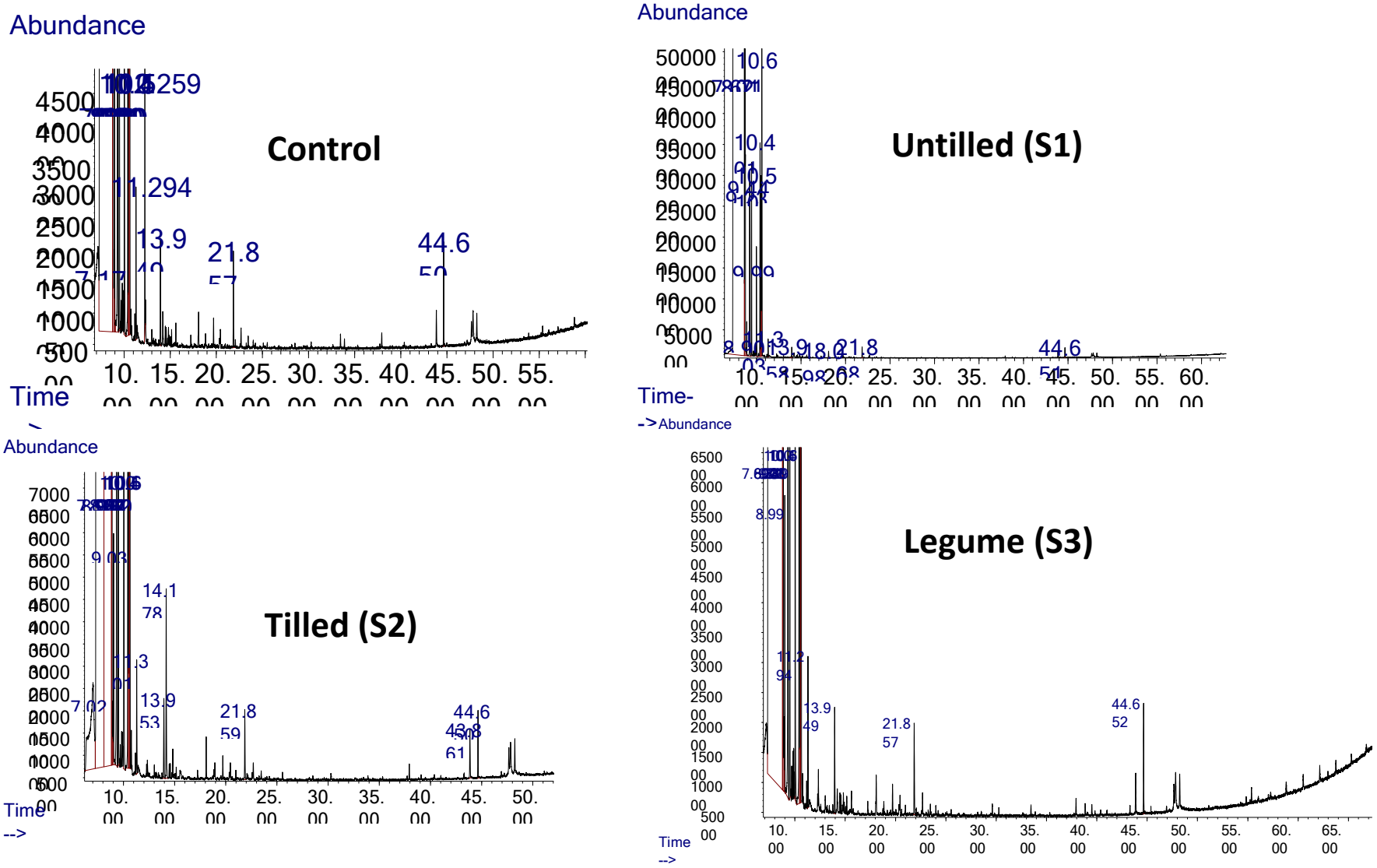
Supplementary Fig. A.4 **Chromatogram showing peaks from GCMS analysis of root exudates of plants in untilled (S1), tilled (S2), legume soil (S3) and autoclaved compost (C1).** The retention time at 25.9 indicates glucose and at 39.7, sucrose.

The method failed to identify other compounds like phenols and amino acids. The study was conducted at the Food and Nutritional Sciences Division, University of Reading, under the guidance of Dr Stephen Elmore. The lyophilized root exudates were redissolved in 250ul of derivatizing agent, N,O- bis (trimethylsilyl) trifluoroacetamide (BSTFA) with Trimethylchlorosilane (TMCS) (both from Thermo scientific, UK, CAS N0. 75-77-4) in a reaction vial (Reacti- Vial™). The mixture was vortexed (MS1 Minishaker IKA®) for 1 minute and incubated at 60°C for 15 minutes and the sample transferred to autosampler vials with inserts and analysed using GCMS.

The chromatograms obtained for each soil is given in Fig. A.5. Exudation profile of soybean plants varied in each soil type. The various compounds identified using NIST library database is given in Table A.1.

Supplementary Table A.1 List of compounds identified in the root exudate of plants grown in different soil types (Untilled (S1), tilled (S2), legume (S3), and Control (C). The similar colours indicate the compounds identified in common in soils.

Untilled (S1)	Tilled (S2)	Legume (S3)	Control (C)
Formamide	2,4-imidazolidinedione,1,5,5-trimethyl	Formamide	N-TMS-acetamide
Ethanamidic acid	N-TMS-acetamide	Ethanamidic acid	Ethanamidic acid
Silanamine,N,N-methanetetraylbis	Formamide	L-alanine-N,isobutoxycarbonyl	N,O-Bis-tms-valine?
N,N-dimethylvaleramide	Ethanimidic acid	Silanamine,N,N-methanetetraylbis	Silanamine
Trimethylsilyl borate	Ethylbis(TMS)amine	N,N-dimethylvaleramide	N,N-dimethylvaleramide
acetamide	Pyrimidine,2,4-difluoro-6-dimethylamino	Glyoxylic acid	Glyoxilic acid
L alanine,N-formyl	N,N-Dimethylvaleramide	Trimethylsilyl borate	Trimethylsilyl borate
trimethylsilylcarbamate	Trimethylsilylborate	2-propanamine	dimethylacetamide
tetrasiloxane decamethyl	acetamide	trimethylsilylcarbamate	trimethylsilylcarbamate
N,N-diethylcarbamate	propanamine	tetrasiloxane decamethyl	N,N-diethylcarbamate
Propanoic acid,tms ester	trimethylsilylcarbamate	N,N-diethylcarbamate	propanoic acid
tetrasiloxane,decamethyl	4,5 dihydro,1H pyrazole	Carbanilide	Tetrasiloxane,decamethyl
trisiloxaneoctamethyl	N,N-diethylcarbamate	Pentanedione	Hexanoic acid
2-Pentanoic acid,TMS ester	Chromene-3-carboxamide	1,3-bisbutane	trisiloxaneoctamethyl
Sulfamic acid,tms ester	1,3-bisbutane	trisiloxaneoctamethyl	Sulfamic acid
Silanol,trimethyl carbonate	trisiloxaneoctamethyl	Ethanedioic acid	Azulene
Naphthalene	Butanoic acid	Silanol	tris methyl borate
tris (TMS)borate	1,2,bis (TMS)ethane	Naphthalene	3,4,methylenedioxycinnamic acid
pentasiloxane-dodecamethyl	Naphthalene	tris methyl borate	p-)dimethyamino)cinnamic acid
3,4,methylenedioxycinnamic acid	2,propenoic acid	Butanoic acid	Naphthalene ester
Silanol-trimethyl-phosphate	Tertiary butyl,6 methyl phenol	4,7-dimethoxyindan-1-one	dodecanoic acid
Naphthalene ester	Dithioerythritol	Naphthalene ester	Azalaic acid
2-isopropyl-3 ketobutyrate	Methylene dioxy cinnamic acid	2-Butenoic acid, ester	hexadecanoic acid
Azalaic acid	2-Butenoic acid, ester	1,3,5,7,9-pentaethylcyclopentasiloxane	trans-9-octadecanoic acid ester
hexadecanoic acid	Serine	Butanedioic acid	11-trans-octadecanoic acid
2-(4-trimethylsiloxyphenyl)2-(2-trimethylsiloxyphenyl)propane	Azalaic acid	Asparagine?	octadecanoic acid
cis-13-octadecanoic acid	hexadecanoic acid	Protrptyline	
trans-13-octadecanoic acid,TMSester	11 cis octadecanoic acid	Azalaic acid	
octadecanoic acid	cis 13 octadecanoic acid	beta-D-galactofuranose	
	octadecanoic acid	Myo-inositol	
		glucopyranose	
		hexadecanoic acid	
		Quinoline,8 chloro-4-(4-chlorophenoxy)	
		trans-13-octadecanoic acid,TMSester	
		11-cis-octadecanoic acid, tms ester	
		octadecanoic acid	



Supplementary Fig. A.5 **Chromaqatograms from Gas chromatography mass spectrometry (GCMS) analysis of root exudates from plants grown in different soil types** (Untilled (S1), Tilled (S2), Legume (S3), Control (autoclaved coir: sand 1:1)

This method identified different compounds in the root exudate of soybean plants. Due to the financial costs involved in large scale analysis, it was decided to use colorimetric analysis for sugars and phenols and GCMS (EZfaast) method for amino acids (Explained in Section 2. 11).

B. Soil enzyme

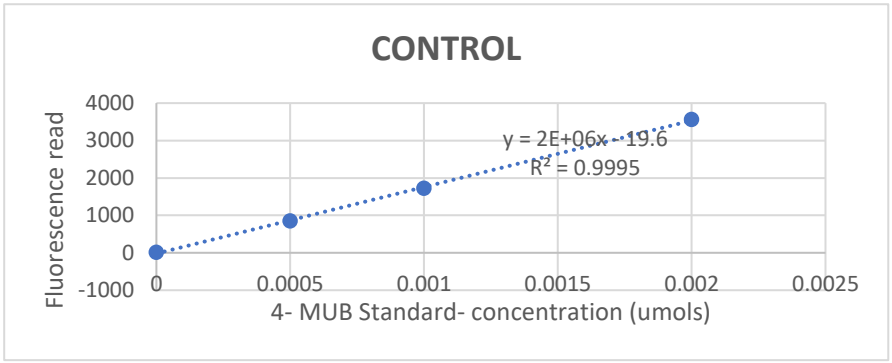
The layout of 96 well plates for soil enzyme assay of rhizosphere soils of untilled (S1) is shown in figures, B.1 and B.2. All the soils (tilled (S2), legume (S3), mixed (S4), and control (C) were laid in the same way. Separate plates prepared for soya bean variety Siverka in each soil in the same way.

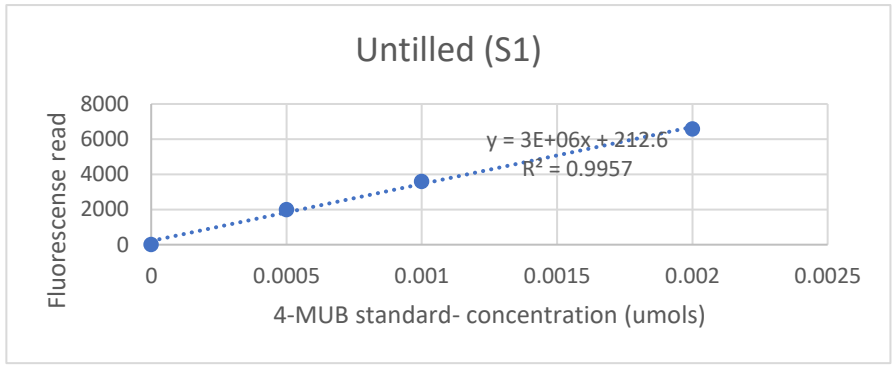
	1	2	3	4	5	6	7	8	9	10	11	12
A	S1KH1+0 std	S1KH2+0 std	S1KH3+0 std	S1KH4+0 std	S1KH5+0 std	S1KH6+0 std	S1KL1+0 std	S1KL2+0 std	S1KL3+0 std	S1KL4+0 std	S1KL5+0 std	S1KL6+0 std
B	S1KH1+2.5uM	S1KH2+2.5uM	S1KH3+2.5uM	S1KH4+2.5uM	S1KH5+2.5uM	S1KH6+2.5uM	S1KL1+2.5uM	S1KL2+2.5uM	S1KL3+2.5uM	S1KL4+2.5uM	S1KL5+2.5 uM	S1KL6+2.5 uM
C	S1KH1+5 uM	S1KH2+5 uM	S1KH3+5 uM	S1KH4+5 uM	S1KH5+5 uM	S1KH6+5 uM	S1KL1+5 uM	S1KL2+5 uM	S1KL3+5 uM	S1KL4+5 uM	S1KL5+5 uM	S1KL6+5 uM
D	S1KH1+10 uM	S1KH2+10 uM	S1KH3+10 uM	S1KH4+10 uM	S1KH5+10 uM	S1KH6+10 uM	S1KL1+10 uM	S1KL2+10 uM	S1KL3+10 uM	S1KL4+10 uM	S1KL5+10 uM	S1KL6+10 uM
E	S1KH1+25 uM	S1KH2+25 uM	S1KH3+25 uM	S1KH4+25 uM	S1KH5+25 uM	S1KH6+25 uM	S1KL1+25 uM	S1KL2+25 uM	S1KL3+25 uM	S1KL4+25 uM	S1KL5+25 uM	S1KL6+25 uM
F	S1KH1+50 uM	S1KH2+50 uM	S1KH3+50 uM	S1KH4+50 uM	S1KH5+50 uM	S1KH6+50 uM	S1KL1+50 uM	S1KL2+50 uM	S1KL3+50 uM	S1KL4+50 uM	S1KL5+50 uM	S1KL6+50 uM
G	S1KH1+100uM	S1KH2+100uM	S1KH3+100uM	S1KH4+100uM	S1KH5+100uM	S1KH6+100uM	S1KL1+100uM	S1KL2+100uM	S1KL3+100uM	S1KL4+100uM	S1KL5+100uM	S1KL6+100uM
H												

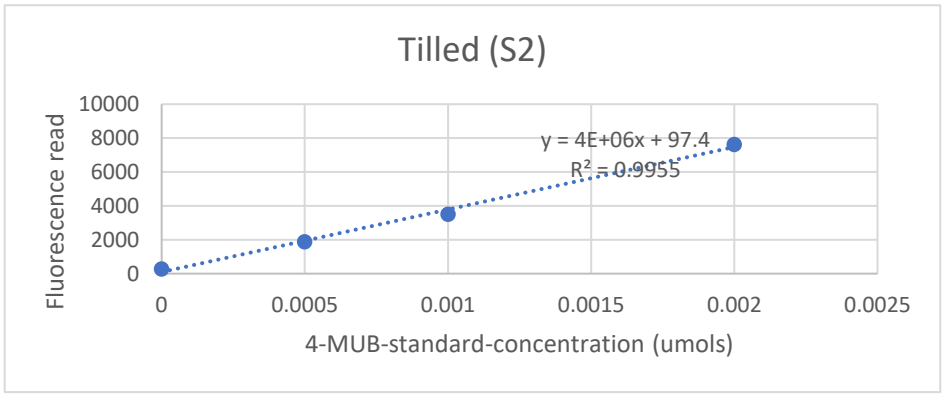
Supplementary Fig. B.1 **Soil enzyme assay layout of standard 4-methylumbelliferone (MUB in uM) in 96 well plate for untilled (S1) rhizosphere soil**, Yellow- Rhizosphere samples of variety Kenchen, High growth plants, Blue- Rhizosphere samples of variety Kenchen, Low growth plants. K- variety Kenchen, H- high growth plants, L- low growth plants

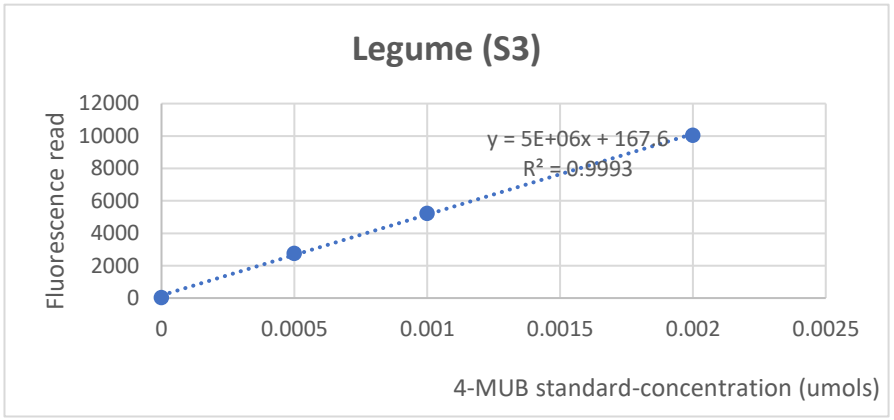
	1	2	3	4	5	6	7	8	9	10	11	12
A	S1KH1+4-MUB-NAG	S1KH1+4-MUB-NAG	S1KH1+4-MUB-NAG	S1KL1+4-MUB-NAG	S1KL1+4-MUB-NAG	S1KL1+4-MUB-NAG	S1KH1+4-MUB-PHOS	S1KH1+4-MUB-PHOS	S1KH1+4-MUB-PHOS	S1KL1+4-MUB-PHOS	S1KL1+4-MUB-PHOS	S1KL1+4-MUB-PHOS
B	S1KH2+ 4-MUB-NAG	S1KH2+4-MUB-NAG	S1KH2+4-MUB-NAG	S1KL2+4-MUB-NAG	S1KL2+4-MUB-NAG	S1KL2+4-MUB-NAG	S1KH2+4-MUB-PHOS	S1KH2+4-MUB-PHOS	S1KH2+4-MUB-PHOS	S1KL2+4-MUB-PHOS	S1KL2+4-MUB-PHOS	S1KL2+4-MUB-PHOS
C	S1KH3+ 4-MUB-NAG	S1KH3+4-MUB-NAG	S1KH3+4-MUB-NAG	S1KL3+4-MUB-NAG	S1KL3+4-MUB-NAG	S1KL3+4-MUB-NAG	S1KH3+4-MUB-PHOS	S1KH3+4-MUB-PHOS	S1KH3+4-MUB-PHOS	S1KL3+4-MUB-PHOS	S1KL3+4-MUB-PHOS	S1KL3+4-MUB-PHOS
D	S1KH4+ 4-MUB-NAG	S1KH4+4-MUB-NAG	S1KH4+4-MUB-NAG	S1KL4+4-MUB-NAG	S1KL4+4-MUB-NAG	S1KL4+4-MUB-NAG	S1KH4+4-MUB-PHOS	S1KH4+4-MUB-PHOS	S1KH4+4-MUB-PHOS	S1KL4+4-MUB-PHOS	S1KL4+4-MUB-PHOS	S1KL4+4-MUB-PHOS
E	S1KH5+ 4-MUB-NAG	S1KH5+4-MUB-NAG	S1KH5+4-MUB-NAG	S1KL5+4-MUB-NAG	S1KL5+4-MUB-NAG	S1KL5+4-MUB-NAG	S1KH5+4-MUB-PHOS	S1KH5+4-MUB-PHOS	S1KH5+4-MUB-PHOS	S1KL5+4-MUB-PHOS	S1KL5+4-MUB-PHOS	S1KL5+4-MUB-PHOS
F	S1KH6+ 4-MUB-NAG	S1KH6+4-MUB-NAG	S1KH6+4-MUB-NAG	S1KL6+4-MUB-NAG	S1KL6+4-MUB-NAG	S1KL6+4-MUB-NAG	S1KH6+4-MUB-PHOS	S1KH6+4-MUB-PHOS	S1KH6+4-MUB-PHOS	S1KL6+4-MUB-PHOS	S1KL6+4-MUB-PHOS	S1KL6+4-MUB-PHOS
G	4-MUB-NAG Blank	4-MUB-NAG Blank	4-MUB-NAG Blank				4-MUB-PHOS Blank	4-MUB-PHOS Blank	4-MUB-PHOS Blank			
H												

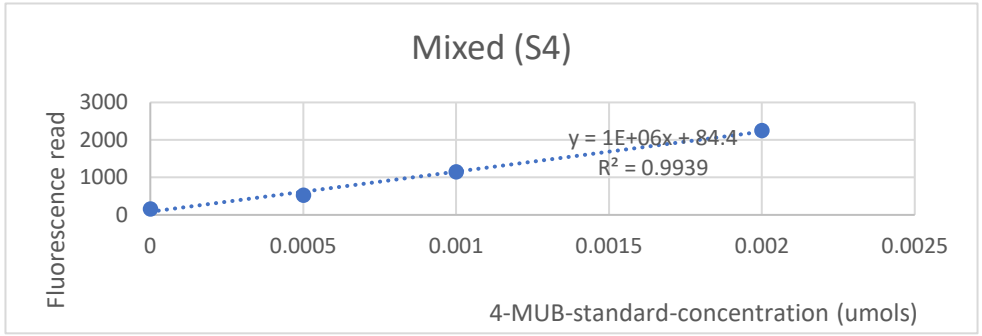
Supplementary Fig. B.2 **Soil enzyme assay layout of samples in 96 well plate for untilled (S1) rhizosphere soil**, Yellow- Rhizosphere samples of variety Kenchen, High growth plants, Blue- Rhizosphere samples of variety Kenchen, Low growth plants. 4-MUB- NAG= 4-MUB-N-acetyl- β -D-glucosaminide, 4-MUB-PHOS= 4-MUB phosphate,NAG Blank- sodium acetate buffer + 4-MUB- NAG, PHOS blank- sodium acetate buffer + 4-MUB-PHOS blank, K- variety Kenchen, H- high growth plants, L- low growth plants











Supplementary Fig B.3. **4-methylumbelliferone (4-MUB) standard curve plot for soil enzyme analysis.** Scatterplot visualization of standard curves with raw fluorescence data as the dependent variable (y-axis) and standard concentration (µmol) as the independent variable (x-axis).

Supplementary Table B.1 The mean concentrations of various soil parameters in control (C) soil (Coir: sand in 1:1 v/olume/volume), NAG- soil enzyme N-acetyl beta glucosaminidase, PHOS- soil enzyme Phosphatase, SE- standard error of mean

Parameter	**Units**	**Mean**±SE
Total Carbon	mg Kg^{-1}	4.2±0.14
Available Phosphorus	mg Kg^{-1}	1.57±0.11
Available Potassium	mg Kg^{-1}	96.42±1.56
NAG activity	nmoles $gsoil^{-1}$ $hour^{-1}$	6.38±0.198
PHOS activity	nmoles $gsoil^{-1}$ $hour^{-1}$	0.19±0.036
Water holding capacity	%	59.86±3.41
pH		8.89±0.06

Supplementary Table B.2. The mean activity of soil enzyme N- acetyl-beta glucosaminidase (NAG) in rhizosphere of soybean plants grown in control (C), untilled (S1), tilled (S2), legume (S3), and mixed (S4) soils. SE- standard error of mean, df- degrees of freedom

Soil_type	**Generation**	**Variety**	**Growth**	**Mean NAG activity (**nmoles $gsoil^{-1}$ $hour^{-1}$**)**	**SE**	**df**
Control(C)	First generation	Kenchen	High growth	0.914	0.569	162
Legume(S3)	First generation	Kenchen	High growth	0.757	0.373	162
Mixed(S4)	First generation	Kenchen	High growth	4.608	0.441	162
Tilled(S2)	First generation	Kenchen	High growth	10.550	0.373	162
Untilled(S1)	First generation	Kenchen	High growth	9.430	0.441	162
Control(C)	Second generation	Kenchen	High growth	0.328	0.569	162
Legume(S3)	Second generation	Kenchen	High growth	11.444	0.373	162
Mixed(S4)	Second generation	Kenchen	High growth	1.158	0.441	162
Tilled(S2)	Second generation	Kenchen	High growth	0.188	0.373	162
Untilled(S1)	Second generation	Kenchen	High growth	0.340	0.441	162
Control(C)	First generation	Siverka	High growth	2.446	0.569	162
Legume(S3)	First generation	Siverka	High growth	0.889	0.402	162
Mixed(S4)	First generation	Siverka	High growth	3.407	0.373	162
Tilled(S2)	First generation	Siverka	High growth	7.505	0.402	162
Untilled(S1)	First generation	Siverka	High growth	15.225	0.402	162

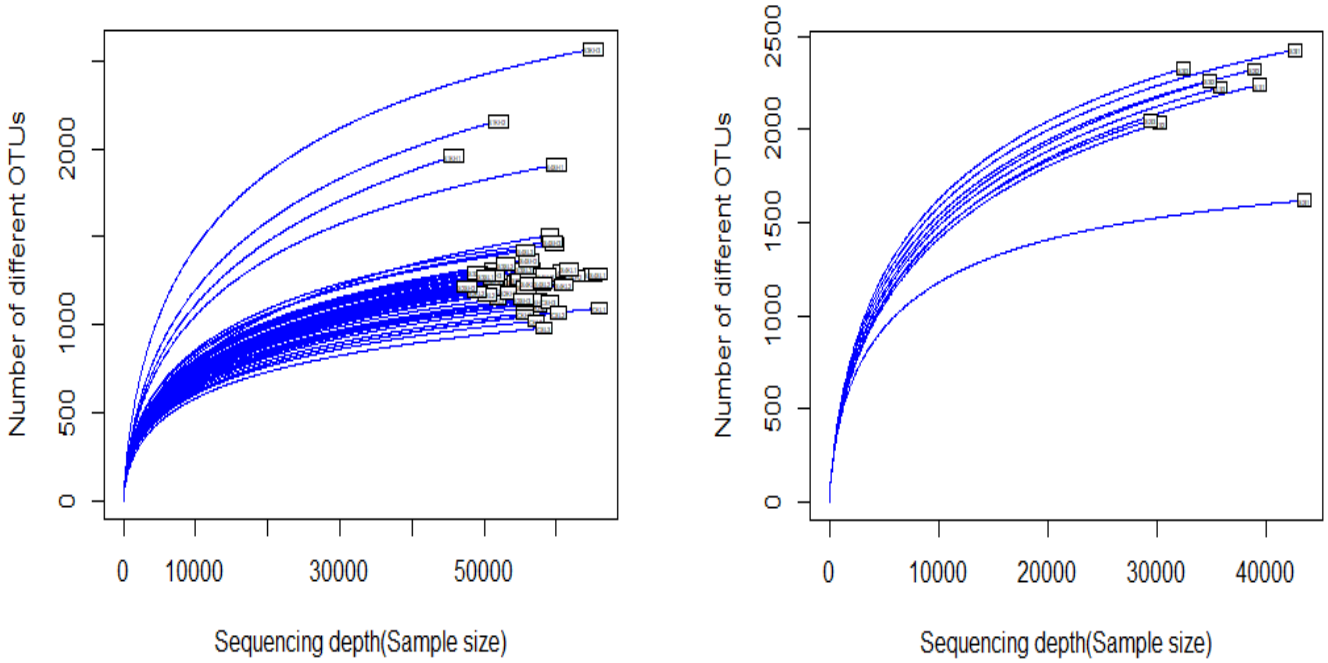
Control(C)	Second generation	Siverka	High growth	0.208	0.569	162
Legume(S3)	Second generation	Siverka	High growth	11.997	0.402	162
Mixed(S4)	Second generation	Siverka	High growth	0.872	0.373	162
Tilled(S2)	Second generation	Siverka	High growth	0.189	0.441	162
Untilled(S1)	Second generation	Siverka	High growth	0.213	0.402	162
Control(C)	First generation	Kenchen	Low growth	2.624	0.569	162
Legume(S3)	First generation	Kenchen	Low growth	0.770	0.493	162
Mixed(S4)	First generation	Kenchen	Low growth	1.587	0.441	162
Tilled(S2)	First generation	Kenchen	Low growth	8.432	0.441	162
Untilled(S1)	First generation	Kenchen	Low growth	16.873	0.402	162
Control(C)	Second generation	Kenchen	Low growth	0.216	0.569	162
Legume(S3)	Second generation	Kenchen	Low growth	9.460	0.493	162
Mixed(S4)	Second generation	Kenchen	Low growth	1.035	0.441	162
Tilled(S2)	Second generation	Kenchen	Low growth	0.255	0.493	162
Untilled(S1)	Second generation	Kenchen	Low growth	0.153	0.493	162
Control(C)	First generation	Siverka	Low growth	2.586	0.569	162
Legume(S3)	First generation	Siverka	Low growth	0.804	0.402	162
Mixed(S4)	First generation	Siverka	Low growth	2.094	0.441	162
Tilled(S2)	First generation	Siverka	Low growth	5.878	0.402	162
Untilled(S1)	First generation	Siverka	Low growth	16.540	0.441	162
Control(C)	Second generation	Siverka	Low growth	0.327	0.569	162
Legume(S3)	Second generation	Siverka	Low growth	11.256	0.402	162
Mixed(S4)	Second generation	Siverka	Low growth	1.331	0.402	162
Tilled(S2)	Second generation	Siverka	Low growth	0.152	0.441	162
Untilled(S1)	Second generation	Siverka	Low growth	0.168	0.441	162

Supplementary Table B.3. The mean activity of soil enzyme Phosphatase (PHOS) in rhizosphere of soybean plants grown in control (C), untilled (S1), tilled (S2), legume (S3), and mixed (S4) soils. e, SE, standard error of mean; df,z degrees of freedom

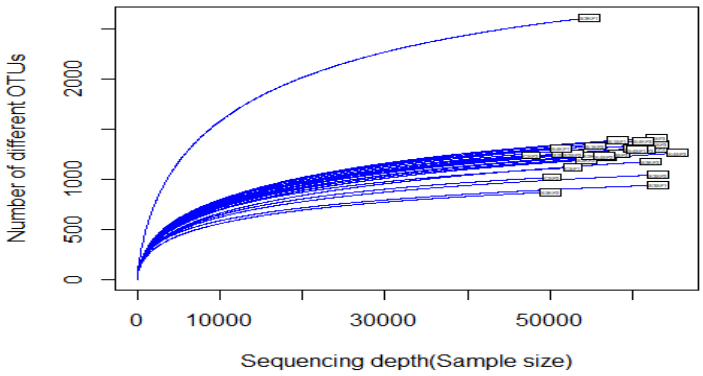
Soil_type	Generation	Variety	Growth	Mean PHOS activity (nmoles/gsoil/hour)	SE	df
Control(C)	First generation	Kenchen	High growth	9.462	0.760	161
Legume(S3)	First generation	Kenchen	High growth	3.582	0.497	161
Mixed(S4)	First generation	Kenchen	High growth	10.792	0.588	161
Tilled(S2)	First generation	Kenchen	High growth	2.809	0.497	161
Untilled(S1)	First generation	Kenchen	High growth	1.287	0.588	161
Control(C)	Second generation	Kenchen	High growth	5.014	0.760	161
Legume(S3)	Second generation	Kenchen	High growth	8.921	0.497	161
Mixed(S4)	Second generation	Kenchen	High growth	3.957	0.588	161
Tilled(S2)	Second generation	Kenchen	High growth	7.901	0.537	161
Untilled(S1)	Second generation	Kenchen	High growth	4.834	0.588	161
Control(C)	First generation	Siverka	High growth	4.346	0.760	161
Legume(S3)	First generation	Siverka	High growth	5.531	0.537	161
Mixed(S4)	First generation	Siverka	High growth	6.373	0.497	161
Tilled(S2)	First generation	Siverka	High growth	4.621	0.537	161
Untilled(S1)	First generation	Siverka	High growth	1.747	0.537	161
Control(C)	Second generation	Siverka	High growth	2.651	0.760	161
Legume(S3)	Second generation	Siverka	High growth	4.308	0.537	161
Mixed(S4)	Second generation	Siverka	High growth	3.083	0.497	161
Tilled(S2)	Second generation	Siverka	High growth	7.577	0.588	161
Untilled(S1)	Second generation	Siverka	High growth	2.976	0.537	161
Control(C)	First generation	Kenchen	Low growth	10.132	0.760	161
Legume(S3)	First generation	Kenchen	Low growth	3.338	0.658	161
Mixed(S4)	First generation	Kenchen	Low growth	5.530	0.588	161

Tilled(S2)	First generation	Kenchen	Low growth	2.962	0.588	161
Untilled(S1)	First generation	Kenchen	Low growth	2.873	0.537	161
Control(C)	Second generation	Kenchen	Low growth	2.502	0.760	161
Legume(S3)	Second generation	Kenchen	Low growth	4.759	0.658	161
Mixed(S4)	Second generation	Kenchen	Low growth	3.778	0.588	161
Tilled(S2)	Second generation	Kenchen	Low growth	8.651	0.658	161
Untilled(S1)	Second generation	Kenchen	Low growth	4.234	0.658	161
Control(C)	First generation	Siverka	Low growth	11.172	0.760	161
Legume(S3)	First generation	Siverka	Low growth	4.209	0.537	161
Mixed(S4)	First generation	Siverka	Low growth	7.367	0.588	161
Tilled(S2)	First generation	Siverka	Low growth	1.449	0.537	161
Untilled(S1)	First generation	Siverka	Low growth	3.970	0.588	161
Control(C)	Second generation	Siverka	Low growth	4.949	0.760	161
Legume(S3)	Second generation	Siverka	Low growth	4.075	0.537	161
Mixed(S4)	Second generation	Siverka	Low growth	4.164	0.537	161
Tilled(S2)	Second generation	Siverka	Low growth	5.894	0.588	161
Untilled(S1)	Second generation	Siverka	Low growth	2.758	0.588	161

C. Next Generation Sequencing data



Supplementary Fig. C.1 – **A. 16S rarefaction curve for Rhizosphere soil samples.** Each curve represents a different sample. **B. 16S rarefaction curve for Initial field soil samples.** Each curve represents a different sample.



Supplementary Fig. C.2 – **16S rarefaction curve for fallow soil samples.** Each curve represents a different sample.