Utilizing the soil microbiome for Sustainable Agriculture in the UK: Drawing from rhizobial inoculants and farmer knowledge

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A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

The University of Sheffield

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18 November 2023

Dedicated to my beloved mother, Mrs Raj Rani (1967-2022), who sadly passed away during my PhD.

Acknowledgments

I deeply thank my supervisors Dr Ellie Harrison and Dr Anna Krzywoszynska for giving me the opportunity to do this fascinating PhD. I would like to thank Dr Ellie Harrison who has been a constant support throughout my PhD, for her advice and feedback. I would also like to express my deep gratitude to Dr Anna Krzywoszynka who helped me with her continuous feedback, suggestions, and motivation. Additionally, I would like to thank my supervisors for their support and understanding during my bereavement. I would also like to express my deep thanks to Dr Duncan Cameron for his valuable advice, encouragement, and motivation to keep pursuing my research and to believe in myself. I would also express my gratitude to Dr Ian Lidbury who has helped me in the lab and provided research advice. I would like to thank Helen Hipperson who helped me with RNA data analysis despite being very busy. Thanks to Foster lab, especially Dr Bartolomiej Salamaga for helping me use their Gel imager. I would also like to say thank you to my work friends and lab mates, Grace, Sam, Sue, and Charly for engaging in lively discussions about my research and helping me with lab experiments. I greatly appreciate, Grace and Charly being there emotionally for me when I lost my mom. Moreover, I would also like to thank the Institute for Sustainable Food for funding my PhD and say thanks to the Grantham Centre for Sustainable Futures for funding my research, for providing me with all the training and outreach platforms to present my research work that helped me to become a confident speaker.

I wouldn't be here today without the support of my family. My mother, Mrs Raj Rani, was an immense support, a great inspiration, and a motivation for me to keep excelling in life. She always made me sail through all my hardships with her words and unconditional love, and always inspired me to become an independent woman. Thank you, Mom. I wish you were here to see me complete my PhD. My father, Mr Bakhshish Masih, always inspired me to stay positive and not be discouraged by any setbacks. I would also like to thank my brother, Ebe, who has been there for me always and believed in me at my worst and best.

Last but not least, I am forever grateful to my husband, Yannick, for his immense support, encouragement, and motivation throughout my PhD. Without him I wouldn't have been able to get through, especially, during COVID and when I lost my mother. Thank you for being by my side through everything and always believing in me when I lost faith in myself. Thank you for being a part of my life.

Abstract

The soil microbial community provides many soil functions such as plant growth stimulation and resistance. Modern intensive agricultural practices depend on industrial inputs such as inorganic fertilisers, which weaken the soil microbe-plant interactions. In the light of multiple challenges associated with the use of chemicals in agriculture, it is thus, crucial to make a transition to more sustainable practices and techniques by using and enhancing the functions of the soil microbiome itself. In this thesis, I investigate the use of rhizobial inoculants – nitrogen-fixing bacteria that form symbiotic associations with legumes, used as biofertilizers/inoculants in agriculture - to make this transition.

My thesis approaches this question from two very different approaches – firstly, through classical hypothesis - driven biological research, and secondly, through hypothesis - generating social science.

Temperate phages are naturally occurring symbiotic viruses that can provide beneficial traits to their bacteria including increased competitiveness. I explore the relationship between phages and rhizobia to see if this could 'naturally' enhance potential inoculant strains. In chapter 2, I analyzed the impact of temperate phage carriage on rhizobia performance by measuring gene expression data from within clover nodules and host plant growth. RNA sequencing results show that the same phage has different effects on the gene expression of different rhizobial strains. However, this does not affect the symbiotic efficiency of rhizobia-clover symbiosis i.e. the dry weight of plants and total nitrogen of eight week plants. In chapter 3, I investigated if phage carriage increases the fitness of host rhizobia in competition with other diverse rhizobia. No competitive fitness benefits were observed *in vitro* and *in plantae* in different rhizobia strains, regardless of their susceptibility to phage infection and killing, except a susceptible strain that couldn't be lysogenized by the phage. This non-lysogenized strain increased its competitive fitness by 72% *in vitro* and by 21% in the plant root environment. These results show that phage mediated competitive fitness effects depend on the spatial structure and competitor's susceptibility to phage infection and killing. In chapter 4, I took an entirely different approach to this same question. I interviewed UK legume farmers and urban growers to explore the existing soil microbiome and soil inoculant perceptions and practices. The social science chapter shows that farmers had different perceptions about the soil microbiome and soil inoculants. They followed different practices and soil assessment techniques to take care of the soil microbiome and assess their presence as well as functioning. There is thus diverse

and fragmentary knowledge of soil inoculants in the growing community. The importance of experiential knowledge to people's perceptions of soil inoculants and understanding of the soil microbiome suggests that future research needs to involve the growing community more strongly in product development and testing.

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Declaration

I, Mary Eliza, confirm that the Thesis is my own work. I am aware of the University's Guidance on the Use of Unfair Means (www.sheffield.ac.uk/ssid/unfair-means). This work has not previously been presented for an award at this, or any other, university.

Intended Publications

Chapter 2 is intended to be published in Microbiology Society.

Chapter 3 is intended to be published in Evolutionary Applications.

Chapter 4 is intended to be published in Soil Use and Management.

Chapter 1 - Introduction

1.1 Soil microbiome

The soil microbiome underpins the functioning of all terrestrial ecosystems by providing many services, such as nutrient cycling (Hayat *et al.*, 2010). However, these functions have been weakened by excessive use of fertilisers (Kibblewhite, Ritz and Swift, 2008; Meena *et al.*, 2017). Nitrogen fertilizer use minimizes and diminishes the plant-microbe interaction and symbiotic associations leading to a loss of the microbes and the functions that they provide (Weese *et al.*, 2015; Huang *et al.*, 2019). In addition, nitrogen fertilizers damage the environment due to nitrate and nitrite leaching in the soil and water. It is estimated that 70-90% of fertilizer is lost through runoff resulting in eutrophication (Environmental Audit Committee, 2018). Similarly, fertilizers exacerbate the production of Nitrous Oxide from agricultural systems, a greenhouse gas 280 times more potent at causing global warming than carbon dioxide (Environmental Audit Committee, 2018; Beltran-Garcia *et al.*, 2021). Thus, in order to ensure that the soil microbiome and its functions are maintained and that the environmental impacts of agriculture are reduced, there is an urgent need to switch away from dependence on industrial fertilisers towards sustainable practices. There are many different techniques and technologies that can help in this transition. But it is clear that these approaches must work with, rather than against, the soil microbes whose role in food production has been largely overlooked until now. Microbial inoculants - that is directly enriching soil communities with highly effective microbes - are likely to be a major component of these changes.

1.2 Microbial inoculants

Microbial inoculants are formulations that contain one or more elite - highly effective for desirable traits - microbial strains (Ambrosini, de Souza and Passaglia, 2016; Kaminsky *et al.*, 2019; Santos, Nogueira and Hungria, 2019). Microbial inoculants are generally used as plant growth stimulators - providing bio-available nutrients - or as bio-controls against plant diseases (Babalola and Glick, 2012). They are thus a potential technology, which can reduce fertilizer and pesticide use. Rhizobia, which fix nitrogen in symbiosis with plant hosts, are widely used as microbial inoculants to provide nitrogen to plants, specifically legumes.

1.3 Rhizobial inoculants

Rhizobia are a polyphyletic group of gram-negative bacteria belonging to the *Alphaproteobacteria* and *Betaproteobacteria* classes, defined by their ability to 1) form symbiotic nodules and 2) fix nitrogen for their host plants (Wang *et al.*, 2019). As facultative symbionts, they can live freely as saprophytic bacteria but can also form symbiotic, nitrogen fixing associations with leguminous plants (Poole, Ramachandran and Terpolilli, 2018; Wang *et al.*, 2019). Rhizobia enter legume roots and form globular root organs called root nodules where they fix atmospheric nitrogen into plant usable forms in exchange for carbon from the plants (Wang *et al.*, 2019). This symbiotic nitrogen fixation is estimated to provide nearly 80% of biologically fixed nitrogen in agricultural systems annually (Mendoza-Suárez *et al.*, 2020). Moreover, legumes can also help in transitioning to sustainable diets by replacing meat-based protein (major contributors of greenhouse gas emissions) as they are nutrient-dense in protein, fibre, and minerals, like iron, zinc, and potassium and various vitamins (Grela *et al.*, 2017; Ferreira, Pinto and Vasconcelos, 2021; Semba *et al.*, 2021). Consequently, the Rhizobium-legume symbiosis is thought to play a major role in the transition to sustainable agriculture (Gitz *et al.*, 2016; Semba *et al.*, 2021).

Rhizobia carry symbiosis genes which help them to form symbiosis and fix nitrogen in legumes (Black *et al.*, 2012). These symbiosis genes are present either on plasmids, genetic islands, Integrative and Conjugative Elements (ICEs) or chromosomes depending on the rhizobia species (Black *et al.*, 2012; Wang *et al.*, 2019). The core symbiosis genes involve genes required for initiation of nodule formation, the nodulation (*nod*) gene clusters which are often found in close proximity to nitrogen fixing - *nif* and *fix* genes (Kaneko *et al.*, 2000; Wang *et al.*, 2019). Symbiosis starts when the host plant secretes signalling molecules called flavonoids which induce the production of rhizobial Nodulating Factors (NFs) via *nodABC* genes (Grundy *et al.*, 2023). NFs stimulate root hair deformation around the rhizobial cell, facilitating infection and trigger the nodule formation (Cangioli *et al.*, 2022; Grundy *et al.*, 2023). Nodules are initiated by just one or at most a handful of individual bacteria, which grow through the host root hair in an intracellular infection thread to infect the developing nodule. Once inside the root nodule, the rhizobia form symbiosomes with plant membranes and differentiate into nitrogen fixing bacteroids (Poole, Ramachandran and Terpolilli, 2018; Cangioli *et al.*, 2022; Yun *et al.*, 2023). The root nodules have reduced oxygen concentrations providing the perfect

environment for the rhizobial enzyme nitrogenase to convert dinitrogen into ammonia (Poole, Ramachandran and Terpolilli, 2018). The pathway for symbiosis is shown in Figure 1.1.

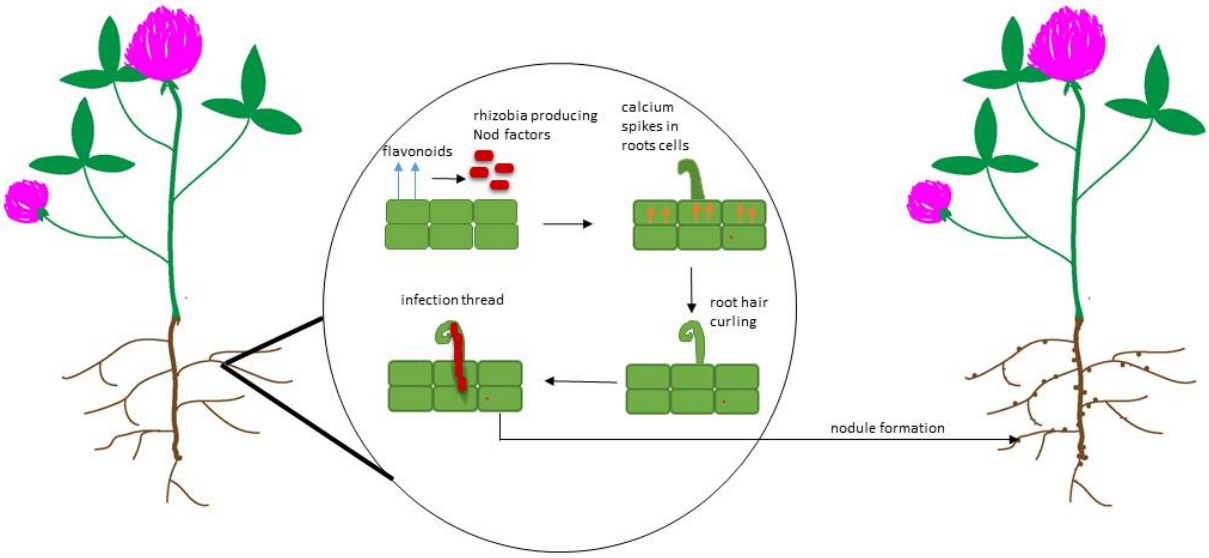


Figure 1. 1 : Nodulation of legumes by Rhizobium.
1) Flavonoids are released by the legume plant root which signal rhizobia to produce nodulation factors (Nod factors). 2) Nod factors are perceived by the legumes, activating the symbiosis signalling pathway and leading to a spike in calcium levels in root epidermal and cortical cells. 3) Rhizobia enter the plant root by the root hair cells and divide causing the root hair to curl. 4) Infection thread is formed at the site of root hair curls and rhizobia enter the root tissue through the infection thread and nodule formation is initiated below the site of infection simultaneously. 5) The infection thread grows towards the emergent nodules and fuses with the nodules. Rhizobia are released into membrane - bound compartments called symbiosomes where they differentiate into nitrogen-fixing bacteroids.

1.3.1 Benefits of rhizobial inoculants

Rhizobial inoculants have been used as growth promoters for more than a century (Zeng *et al.*, 2016; Finks and Martiny, 2023) and in addition to fixing nitrogen, help in phosphorous solubilisation and release growth hormones such as auxins (Marks *et al.*, 2013; Wang *et al.*, 2019). Rhizobial inoculants are especially useful in areas where the native rhizobia populations are either absent or low in number due to no history of legume cultivation (Catroux, Hartmann and Revellin, 2001; Cummings, 2005; Babalola and Glick, 2012). Brazil is an excellent example of rhizobial inoculant success (de Souza *et al.*, 2019). Soybean is not native to Brazil but yet Brazil is now the largest producer of soybean (de Souza *et al.*, 2019). Most of the soybean plants are inoculated with rhizobial inoculants to supply them with nitrogen. It is estimated that the nitrogen provided by these rhizobial inoculants is equivalent to $7 billion worth of fertilisers (Hungria *et al.*, 2006; Hungria, Campo and Mendes, 2007; Marks *et al.*, 2013). Inoculants can also be beneficial to neighbouring non-target plants. For instance,

Rhizobium leguminosarum bv. *viciae* can form effective nodules in different *Lathyrus* species, *Lens culinaris*, *Pisum sativum* and *Vicia* spp (Andrews and Andrews, 2017). This ability to form interactions with multiple partners can help in adaptation of legumes and rhizobia to a wide range of habitats and partners by increasing the probability to find at least one effective partner (Harrison *et al.*, 2018). However, this also means that there is variability in efficiency associated with multiple interactions in different environments leading to inconsistent outcomes of inoculation on crops (Harrison *et al.*, 2018). Inconsistency associated with rhizobial inoculants has been shown in different parts of the world (Catroux, Hartmann and Revellin, 2001; Goddard, Srinivasan and Girard, 2003).

1.3.2 Challenges to rhizobial inoculant consistency

Rhizobial inoculants face many challenges when introduced in fields (Catroux, Hartmann and Revellin, 2001). These challenges include adapting to the local environment and competing with the native or naturalised rhizobial species (Bashan, 1998; Catroux, Hartmann and Revellin, 2001; Cummings, 2005; Babalola and Glick, 2012). Local rhizobia can be highly variable at nodulating and nitrogen fixing efficiency - ranging from highly effective symbionts, to symbiotic 'cheats' which provide little to no benefit to the host (Babalola and Glick, 2012; Thilakarathna and Raizada, 2017; Cangioli *et al.*, 2022) - but are likely to be well adapted to the local conditions. This means that although native rhizobia may be inefficient at fixing nitrogen, they may be highly competitive at nodulating (Batista *et al.*, 2015; Cangioli *et al.*, 2022). Consequently, native rhizobia typically occupy the majority of nodules (Batista *et al.*, 2015). As inoculant performance is dependent on getting elite inoculants to dominate plant nodule populations (evidence suggests that at least >66% of the nodules (Thies, Singleton and Bohlool, 1991; Thilakarathna and Raizada, 2017)), competition from native strains is a major challenge to the effectiveness of inoculants (Mendoza-Suárez *et al.*, 2021; Burghardt and diCenzo, 2023).

Inconsistency in evidence of rhizobial inoculant efficiency and plant yields after use of rhizobial inoculants is a major reason for the low uptake of rhizobial inoculants by people involved in the food growing sector (Cummings, 2005; Babalola and Glick, 2012). However, the lack of uptake can be due to other factors such as incompatibility with agrochemicals, agricultural practices and budget of food growers (Cummings, 2005; Thilakarathna and Raizada, 2017). In this thesis, I use two very different approaches to explore ways of enhancing the effectiveness of rhizobial inoculants in agriculture. The first approach uses temperate

bacteriophages - symbiotic viruses that infect bacteria without (necessarily) killing them - to increase the competitiveness of rhizobia in the lab and in the greenhouse (Gama *et al.*, 2013). The second approach is to use public participation to explore end-users' current understandings and farming practices around soil microbiome and soil inoculants in order to inform better integration of rhizobial inoculants into existing farming practices and cultures.

1.3.3 Using temperate bacteriophages to improve rhizobial inoculants

Bacteriophages (phages) are viruses that infect bacteria. They can replicate either through the lytic or the lysogenic cycle (Kang *et al.*, 2022). In the lytic cycle, phages infect the bacteria, multiply and kill them through the process of lysis (Batinovic *et al.*, 2019; Kang *et al.*, *2022*). The phages which strictly follow the lytic life cycle are called virulent or lytic phages (Williamson *et al.*, 2008; Gama *et al.*, 2013). In the lysogenic cycle, phages enter a symbiotic state where they infect the bacteria, integrate into the bacterial genome and are replicated as the bacteria multiplies (Bobay, Rocha and Touchon, 2013). The integrated phage is known as a prophage. The prophage can enter the lytic cycle in individual bacterial cells either spontaneously or under certain environmental conditions such as UV light or toxins such as mitomycin C, killing the individual cell instantly (Gandon, 2016; Harrison and Brockhurst, 2017; Howard-Varona *et al.*, 2017). The phages which follow the lysogenic life cycle are called temperate phages and their bacterial hosts are known as lysogens (Gandon, 2016; Harrison and Brockhurst, 2017).

The soil is estimated to have 10^9 phages per g of dry soil (Williamson *et al.*, 2017). These soil phages mediate bacterial growth rates through lytic and lysogenic cycles. The replication strategies of bacteriophages affect their hosts in different ways and play a very important role in determining their host's total abundance, population dynamics and the diversity of bacterial soil communities (Williamson *et al.*, 2017; Batinovic *et al.*, 2019). The role played by temperate phages in bacterial populations has been well explored in pathogenic bacteria but their impact on terrestrial bacteria - such as rhizobia - is underexplored.

Temperate phages have been found in genomes of different rhizobia (A Schwinghamer and Reinrardt, 1963; Uchiumi *et al.*, 1989; Engelhardt *et al.*, 2013; Santamaría *et al.*, 2014; Halmillawewa *et al.*, 2016). This includes the phage - vTRX32-1 - that I have used in this thesis (Ford *et al.*, 2021). vTRX32-1 was isolated in York, United Kingdom, by lysogenic induction of *Rhizobium leguminosarum* bv. *trifolii (Rlt)* strain TRX32, isolated from root nodules of clover in York, belonging to genospecies B (Kumar *et al.*, 2015). vTRX32-1 is from a wider

group of temperate phages belonging to the *Autographiviridae* family of bacteriophages. These phages have been found in different *Rhizobium* spp. across Denmark, France and the UK (Ford *et al.*, 2021). It has a large host range, including strains that it can infect but not lysogenize and those it can form novel lysogenic relationships with (Ford *et al.*, 2021). The phage has a conserved 12 bp motif ("CAGATTTAGGTT") within its genomes which it uses to attach itself within the *Rhizobium leguminosarum* hosts. In the phage-free hosts, this motif is present in a tRNA Leucine where the phage integrates, disrupting the bacterial tRNA and replaces its function with the phage tRNA Leu. The phage genome is 45 kb long and carries genes mainly involved in tackling host defenses, DNA processing and phage structure (Figure 1.2) (Ford *et al.*, 2021). The genome also carries insertion elements such as IS1 family transposase suggesting that it may be acting as a payload for these IS elements which have been shown to play a role in evolution of rhizobia (Hernandez-Lucas *et al.*, 2006). Although no known bacterial accessory genes are carried on the phage, it does contain highly variable region containing ORFs of unknown function (Ford *et al.*, 2021). vTRX32-1 could thus, potentially have some effect on its hosts and their symbiotic interactions with legumes, which needs further exploration.

In chapter 2, I investigate the effect of phage carriage on rhizobia-plant symbiosis. Temperate phages can alter bacterial function in several ways. They can disrupt gene functions or cause mutagenesis by direct integration into bacterial genomes driving variation and evolutionary divergence (Harshey, 2012). Phages can also increase fitness of its host by encoding beneficial traits. For instance, many pathogenic bacteria can encode virulence factors due to the presence of phages in their genomes (Faruque and Mekalanos, 2012). In rhizobia, lysogeny has been shown to have varying effects on nodulation and symbiotic effectiveness (Uchiumi *et al.*, 1989; Dhar *et al.*, 2013; Hatem, El -Sabbagh and El -Didamony, 2017), which may be due to many underlying phage-bacterial interactions at the genetic level which have not been fully understood yet.

In chapter 3, I investigate the impact of lysogeny on competitiveness of rhizobial inoculants. Induction i.e. switching of lysogenic-lytic life can affect the competitiveness of phage hosts (Obeng, Pratama and Elsas, 2016; Howard-Varona *et al.*, 2017). Phages can induce in an individual host either due to stressful environmental conditions such as UV light or chemicals such as hydrogen peroxide or spontaneously without any external factors (Nanda, Thormann and Frunzke, 2015; Howard-Varona *et al.*, 2017). In the soil environment, changes in nutrients, pH or temperature may induce the phages (Williamson *et al.*, 2008; Howard-Varona *et al.*,

2017). This leads to release of infective phage particles in the environment which can infect and kill phage-susceptible (likely to be unrelated i.e. they are not clones of the host population) bacteria (Harrison and Brockhurst, 2017). This gives a competitive advantage to host bacteria as they can occupy new niches and utilise nutrients liberated from lysed cells (Obeng, Pratama and Elsas, 2016). This has been observed in many bacteria, for instance, *Bordetella* (Joo *et al.*, 2006; Mahmood *et al.*, 2016), *Pseudomonas* (Davies, James, Kukavica-Ibrulj, *et al.*, 2016) and *Curvibacter* sp. which infect *Hydra vulgaris* (Li *et al.*, 2017).

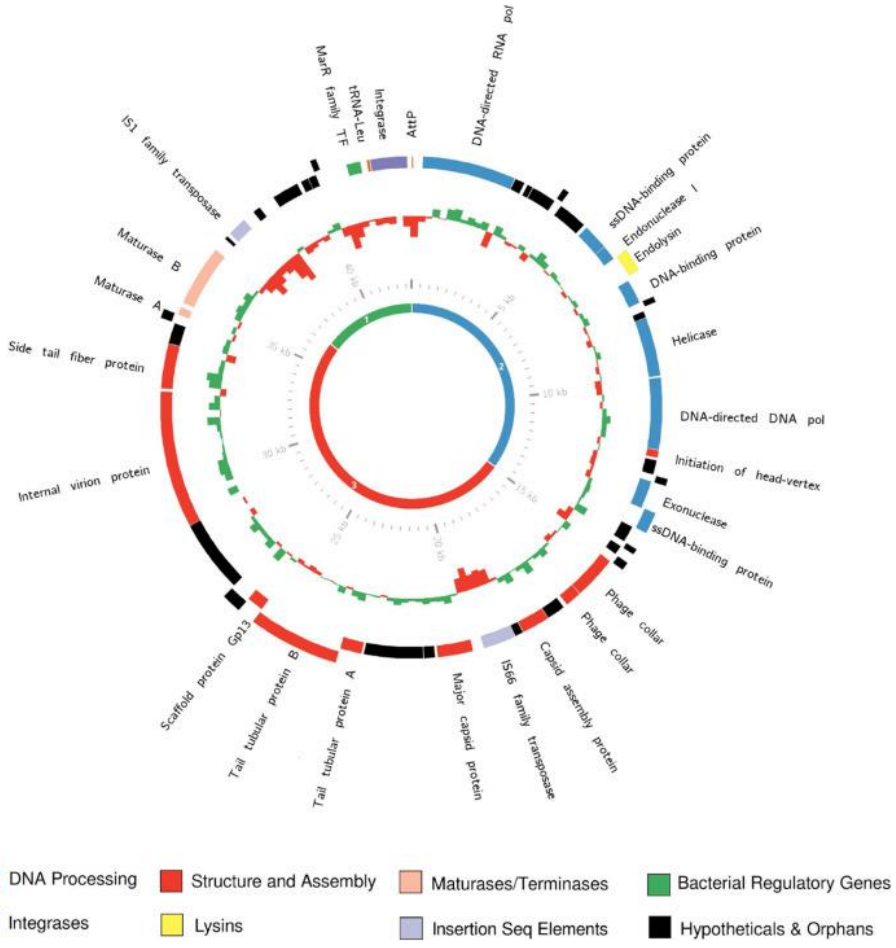


Figure 1. 2 : Annotated genome of vTRX32-1.
The outer ring shows ORFs predicted by RAST (Rapid Annotation using Subsystem Technology), colors correspond to their predicted functions. The inner histogram represents GC%, GC% above the genome average is green and GC% below the average is red. Histogram sizes indicate the difference between the GC% at that locus (250 bp bins) and the genome average GC%. The central ring shows the location of the 3 gene classes in the vTRX32-1 genome (green, blue, and red corresponding to early, middle, and late, respectively. These classes are names on the basis of their transcription order) (Ford et al., 2021).

1.4 Participatory approaches to improve rhizobia inoculants

My second approach to improve rhizobial inoculant and soil microbiome technology use is participatory science. Public participation can be defined as anything that involves or represents

the public (Cornwall, 2008). In this project, the aim of including the public i.e. specifically legume farmers and urban growers, is to use their experience and local knowledge to gain a deeper understanding about the potential for increasing the uptake of rhizobial inoculants.

Previous research has found that farmers' and growers' needs are not reflected in inoculant development. For example, one study found that an ideal inoculant according to a farmer is the one that has maximum efficacy and is easy to use along with a long shelf-life (Catroux, Hartmann and Revellin, 2001). However, inoculants produced according to the best scientific practices have shown variable results when applied to fields (Santos, Nogueira and Hungria, 2019). This variability can be due to a diversity of factors that can affect the efficacy of rhizobial inoculants, for instance, competitiveness against natural/naturalised populations (Thilakarathna and Raizada, 2017). Despite the low uptake of rhizobial inoculants (Santos, Nogueira and Hungria, 2019), there is sparse to no research that would attempt to understand the perspectives of the end users on inoculant use and the role that these perspectives can play in inoculant uptake. In other fields, end user perceptions have been found to have a huge impact on technology transfer and the implementation of practices and policies that affect them (Liebig and Doran, 1999; Petrescu-Mag, Petrescu and Azadi, 2020). For transfer of technologies such as rhizobial inoculants, understanding end user perceptions determines the success and failure of technologies in the market (Bruges *et al.*, 2008). Moreover, non-engagement of end users from the start of the technology design and development leaves out the perceptions and expertise of the end users which usually translates into mistrust and conflicting opinions due to non-transparency (Felt and Fochler, 2008). Inclusion of public through participatory approaches thus plays a very important role in ensuring trust, robustness and transparency as well as the formulation of ideas and concepts which are much more diverse, user-friendly and more readily accepted (Felt and Fochler, 2008; Stitzlein *et al.*, 2020). In addition, participatory approaches have been shown to be one of the best tools to ensure transition to sustainable agriculture (Bruges *et al.*, 2008) .

In chapter 4, I enable the incorporation of end users in the development of rhizobial inoculants by interviewing legume farmers and urban growers about their perceptions and practices around the soil microbiome and soil inoculants. Legume growers were selected as participants in this study as I assumed that legumes growers are most likely to be aware of the symbiotic association between legumes and rhizobia. The benefits of legume-rhizobia symbiosis likely encourage them to use legumes, which impacts their perceptions about soil inoculants and the soil microbiome. In addition to exploring these perceptions, I sought to understand and list

farmer and urban grower practices around plant growth and soil assessment techniques that have the potential to improve rhizobial inoculants and future soil microbiome technology use.

1.5 Aims and research questions

The aim of this PhD is to explore potential ways to improve rhizobial inoculants and their use in agriculture. The two approaches – temperate phages and public participation – form the basis of my research questions. In the temperate phages based research, I ask the following questions

1. Can phages be used to improve the effectiveness of rhizobial inoculants?

The social science research in my project focuses on the following broad research question

2. How can farmer and urban grower perceptions and practices around soil microbiome and soil inoculants help us to improve the uptake of rhizobial inoculants?

1.6 Thesis Outline

The thesis is divided into five chapters. I have outlined each data chapter briefly according to the research question and objectives. Figure 1.3 shows these objectives and the different scales of life i.e. viruses, rhizobia, clover and people used in the thesis.

1.6.1 Chapter 2

What is the impact of lysogeny on the rhizobia-plant interaction?

<u>*Research Question-*</u> What is the impact of temperate phage vTRX32-1 and *Rhizobium leguminosarum* bv. *trifolli* (*Rlt*) interaction on

a) Differential gene expression of two strains – *Rlt* TRX19 and *Rlt* TRX4 in *Trifolium repens* (clover) nodules?

b) *Trifolium repens* biomass and total nitrogen?

<u>*Objectives*</u>

- To compare genes differentially expressed in lysogenic and non-lysogenic rhizobia

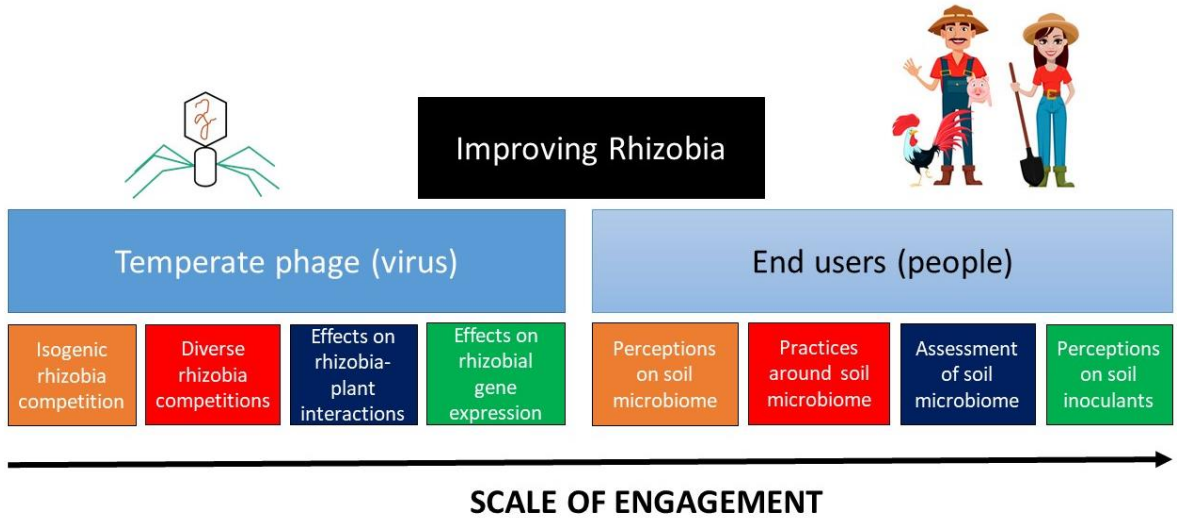


Figure 1. 3 : **The scale of engagement in the thesis: phages, rhizobia, clover and the food grower community.**
The boxes at the lower level (orange, red, blue and green) show the objectives of the thesis. The boxes at the second level (blue) show the study organisms that are the subject of the thesis i.e. temperate phages and grower community (people). The box at the first level show the model organism - rhizobia used, with and sometimes without clover.

- To measure and compare the biomass of clover plants inoculated with rhizobial lysogens and non-lysogens.
- To measure and compare the total nitrogen and Carbon: Nitrogen ratio in plants inoculated with rhizobial lysogens and non-lysogens.

1.6.2 Chapter 3

Do temperate phages give a competitive advantage to rhizobia?

Research Question- Does temperate phage vTRX32-1 give a competitive advantage to *Rlt* TRX19 against isogenic and non-isogenic competitors?

Objectives

- To measure competitive fitness of TRX19 lysogens against lysogeny susceptible and lysogeny resistant isogenic strains.
- To measure competitive fitness of TRX19 lysogens against strongly lysogenic, weakly lysogenic and lysogeny resistant non-isogenic strains.

1.6.3 Chapter 4

Soil microbiome and soil inoculant perceptions in the UK farmers and urban grower community.

Research Question- What are the different understandings, practices and assessment techniques related to the soil microbiome and soil inoculants in the legume farmer and urban grower community?

Objectives-

- To list the different understandings of the soil microbiome and rhizobial inoculants in the farmer and urban grower community
- To list the practices and quantitative and qualitative assessments used by farmers and urban growers to interact with care for the soil microbiome which can help in the success of rhizobial inoculants

Chapter 2 - What is the impact of lysogeny on the rhizobia-plant interaction?

2.1 Introduction

Rhizobia are essential components of the soil microbiome that interact with legumes to form symbioses in the form of root nodules where they fix nitrogen in exchange for carbon compounds. Rhizobia interact with many Mobile Genetic Elements (MGEs) - DNA segments which can move between genomes (Frost *et al.*, 2005) - in their environment. These MGEs form an essential part of the rhizobial genome and play an important role in rhizobial ecology and evolution (Wang *et al.*, 2019; Heath *et al.*, 2022). Of all the rhizobial MGEs, plasmids have been studied extensively (Mercado-Blanco and Toro, 1996; Zahran, 1999; Young *et al.*, 2006; López-guerrero *et al.*, 2012) and have been shown to make up 30%-50% of the *Rhizobium* genome (López-guerrero *et al.*, 2012). In *Rhizobium leguminosarum*, this can amount up to 11 plasmids (López-guerrero *et al.*, 2012; Romero and Brom, 2004). Some of the rhizobial plasmids – pSyms - and Integrative and Conjugative Elements – ICEsyms – carry many genes that underlie the nitrogen-fixing symbiosis with legumes, which is a major characteristic of the Rhizobium clade (Wang *et al.*, 2019). Beyond carrying these key symbiotic genes, rhizobial plasmids play a major role in the survival, symbiosis and competitive fitness of *Rhizobium*: enhancing nodule formation (Mercado-Blanco and Toro, 1996b; Brom *et al.*, 2000; Abdel-Salam *et al.*, 2013), competitiveness and nitrogen fixation (Martinez-Romero and Rosenblueth, 1990), metabolic functions (Ding *et al.*, 2012) and stress resistance (Cytryn *et al.*, 2008). Plasmids can also reduce rhizobial fitness, for instance, by inhibiting nitrogen fixation (O'Connell *et al.*, 1998) and competitiveness (Pankhurst, Macdonald and Reeves, 1986). While many studies have focused on plasmids and conjugative elements within rhizobial genomes, the impact of other mobile elements such as temperate phages are less well understood.

2.1.1 Temperate phages as rhizobial MGEs

Temperate phages – viruses that infect and integrate in bacteria – have been found in many rhizobia (Takahashi and Quadling, 1961; A Schwinghamer and Reinrardt, 1963; Abebe *et al.*, 1992; Dhar *et al.*, 2013; Engelhardt *et al.*, 2013; Halmillawewa *et al.*, 2016; Sharma *et al.*, 2019; Ford *et al.*, 2021). In Rhizobia, as yet, no beneficial accessory traits have been identified on prophages. However, many prophages contain hypothetical genes and few studies have tested the impact of rhizobial lysogens on symbiosis. This is because rhizobial prophages are

in some ways similar to prophages of pathogens in their traits such as host infection and providing benefits to their hosts (Paul, 2008; Pfeifer *et al.*, 2022). These benefits vary widely and depend on the phage life stages such as integration or excision. As integrated prophages, they can increase host fitness by causing phenotypic changes due to the gain of novel accessory genes, which may increase adaptive traits in certain environments. For instance, the pathogenicity of Enterohemorrhagic *Escherichia coli* stems from the production of shiga toxins encoded on prophages (Bonanno and Loukiadis, 2016). In the marine environment, prophages provide cyanobacterium *Prochlorococcus* the ability to use nitrogen and phosphorous (Gazitúa *et al.*, 2021; Wang *et al.*, 2022). Prophage integration can also drive mutagenesis leading to a rapid adaptation to new environments. For instance, in *Pseudomonas aeruginosa* strains that infect the lungs of cystic fibrosis patients, transposable temperate phage LESϕ4 drove mutations in type IV pilus and Quorum Sensing genes which accelerated the pathogenic host's adaptation to the lung (Davies, James, Williams, *et al.*, 2016). Superinfection exclusion i.e. resistance against infection by additional related phages is another benefit provided by prophage integration to their hosts (Domingo-Calap, Mora-Quilis and Sanjuán, 2020). Host benefits due to prophage excision are often due to reconstitution of gene function, when the prophage integration site is within a gene. In *Bacillus subtilis*, for example, excision of prophage SPβ reconstitutes the function of a spore envelope gene (Abe *et al.*, 2014). Finally, prophages can provide a competitive advantage to their hosts by acting as biological weapons. This occurs when prophage induces, i.e. enters the lytic cycle, to release infectious phage particles. These phage particles kill phage-sensitive competitors giving a competitive advantage to hosts that increase in population and can occupy niches occupied previously by competitors (Harrison and Brockhurst, 2017; Howard-Varona *et al.*, 2017). The effects of competitive fitness will be discussed in detail in chapter 3.

While temperate phages provide many benefits, they often can also incur a cost to their bacterial hosts. Gene disruption due to integration of temperate phages can be the first inherent cost to host cell as observed in *Staphylococcus aureus* where ϕ13 insertion leads to disruption of the β toxin gene (Coleman *et al.*, 1991). In other cases, they can lead to insertion activation of genes, such as the insertion of phage D3119 leads to a 45-60 fold increase in acyl amidase production in *Pseudomonas aeruginosa*, conferring resistance to fluoroacetamide (Rehmat and Shapiro, 1983). Induction of prophages can provide benefits against phage-susceptible competitors but it is a lethal cost to individual host cells in whom induction happens (Harrison and Brockhurst, 2017). In other cases, the vulnerability of prophage infected cells can be

exploited by its competitors. For example, induction of prophages in *Staphylococcus aureus* is triggered by its competitor *Streptococcus pneumonia* by production of hydrogen peroxide, which induces the SOS response (Pericone *et al.*, 2003; Selva *et al.*, 2009). Temperate phages can also affect the intrinsic fitness of hosts by being a metabolic cost as seen in *Pseudomonas aeruginosa*. The phage PaP1 in *P.aeruginosa* downregulated certain amino acid and energy metabolism pathways due to increased production of phage-encoded proteins. It also negatively affected the betaine-choline pathway which is involved in antimicrobial resistance, leading to accumulation of toxic products in its host which can lead to death (Zhao *et al.*, 2017).

The costs of acquiring MGEs can have a wide range of impacts including interference with transcription, translation, and downstream cellular interactions of MGE encoded genes and, like previously seen, have been widely studied in plasmids (Baltrus, 2013; San Millan and Maclean, 2019). Transcription of MGE encoded genes is usually not considered a huge cost compared to translation of MGE encoded genes (Baltrus, 2013). These translation costs can be either due to high expression of MGE genes which extensively use host resources to produce proteins (Shachrai *et al.*, 2010) or due to blocking or sequestering of host enzymes by MGE genes which are often AT-rich. AT-rich genes carry many intragenic promoters, which lead to binding of histone-like nucleoid structuring (H-NS) proteins. These regions can capture RNA polymerase reducing transcription of their own genes, but also, leading to lower expression of host genes (Lamberte *et al.*, 2017). However, when these H-NS proteins are absent, AT-rich genes can lead to cell death (Lamberte *et al.*, 2017). A mismatch in codon usage by MGEs can be another huge cost due to translation. Bacteria have evolved to use certain tRNA codons in their genomes, which are best fit for their cellular processes. Many studies show that a mismatch between the tRNA codon usage by MGE encoded genes and those present in the host can lead to translation inefficiency, mistranslation, allocation of wrong ribosomes and ribosomal stalling (Plotkin and Kudla, 2011; San Millan and Maclean, 2019). A lack of additional proteins and enzymes along with tRNA mismatch can further lead to protein misfolding which can cause cytotoxicity and disrupt cellular processes (Allan and Wilke, 2009; Geiler-Samerotte *et al.*, 2011). An example of this is plasmid pQBR103 in *Pseudomonas fluorescens* (Harrison *et al.*, 2015). pQBR103 provides a fitness benefit to host cells in high mercury environments due to carriage of mercury resistance genes. The plasmid, however, imposes a cost on host cells, especially in low mercury environments, due to high expression of host genes, out of which 90% were involved in translation, protein folding, tRNA aminoacylation, rRNA binding, and ribosome functioning (Harrison *et al.*, 2015). However,

these costs were shown to be ameliorated in evolved host cells due to the loss of the *gacA/gacS* regulatory system which reduced translational demand (Harrison *et al.*, 2015).

2.1.2 Temperate phage impact on rhizobia symbiosis

In rhizobia, the impact of phages on their hosts and symbiosis is unclear. Studies on different temperate phages show contradicting effects on the symbiosis, nodulation and nitrogen fixing interactions of different rhizobia with their respective host plants (Uchiumi *et al.*, 1989; Abebe *et al.*, 1992; Dhar *et al.*, 2013; Hatem, El -Sabbagh and El -Didamony, 2017). For instance, Uchiumi et al., 1989 showed that lysogeny negatively affects nodule formation in *Rhizobium leguminosarum* bv. *trifolii* (Uchiumi *et al.*, 1989). However, other studies on soybean infecting *Bradyrhizobium japonicum* lysogens found that there is no effect of phage presence on nodulation and symbiosis of rhizobia (Abebe *et al.*, 1992; Dhar *et al.*, 2013). Similarly, Hatem et.al, 2017 did not observe any effect of phage presence on nodulation, nitrogenase activity, plant dry weight and total nitrogen in *Rhizobium* on *Sesbania aegyptica* (Hatem, El -Sabbagh and El -Didamony, 2017). While these studies show the effects of phage presence on plant growth, no prophage-encoded genes have been associated with these effects. Thus, the effect of phage presence on rhizobial gene expression when in symbiosis with host plants is still unknown. The aim of this chapter thus, is to explore the effects of phage used on the gene expression of the focal rhizobia as well as plant growth when it is in symbiosis with plants. This is especially essential to know as in chapter 3, I investigate phage-mediated competitive effects in these rhizobia.

In this chapter, I investigate the impact of one rhizobial temperate phage, vTRX32-1 on rhizobial symbionts. Phage vTRX32-1 is inducible and widely infectious. It was isolated from a rhizobial symbiont – *Rhizobium leguminosarum* bv *trifolii* strain (*Rlt)* TRX32 found in a clover (*Trifolium repens*) nodule in York (Ford *et al.*, 2021). This phage was found across Europe in different *Rhizobium* spp. at varying frequencies showing its long evolutionary history with *Rhizobium* (Ford *et al.*, 2021). I predicted that given its long history in rhizobia, vTRX32-1 carriage might have some effects on symbiotic effectiveness of two different *Rlt* strains. To investigate these effects on the 2 strains – *Rlt* TRX19 and *Rlt* TRX4 – within clover symbiosis, I 1) measured the transcriptional impact of prophages in symbiotic nodules *in plantae*, and 2) measured plant growth. I find that phage impact on rhizobial gene expression varies for each rhizobial strain. However, phage presence does not affect rhizobia-clover symbiosis.

2.2 Methodology

2.2.1 Bacterial and phage strains

All experiments were conducted on *Rhizobium leguminosarum* bv. *trifolii* (*Rlt*) host strains TRX19 and TRX4. These strains were isolated from the root nodules of *Trifolium repens* i.e. clover growing in one 1m sq site in York and belong to the genospecies C and D, respectively (Kumar *et al.*, 2015). Both strains were selected as they do not produce any infectious phage/phage-like particles in an inhibition test and cannot be induced to lysis through exposure to mitomycin C. The phage vTRX32-1 was isolated from *Rlt* strain TRX32, isolated from the same site and belongs to genospecies B (Ford *et al.*, 2021). Three incomplete and one questionable phage (incomplete and questionable are score categories assigned by PHASTER based on phage features and region where it is present) were found in TRX19 using the PHASTER tool (Zhou *et al.*, 2011; Arndt *et al.*, 2016). The number of prophages in TRX4 is however, unknown as I did not have the genomic sequence of TRX4.

The strains were labelled with gentamycin resistant markers and either Green Fluorescent Protein (GFP) or mCherry (MC) fluorescent markers using MiniTn7 (Mendoza-Suárez *et al.*, 2020, 2021). The strains were labelled and provided by my supervisor, they were thus not constructed by myself as part of this work. Temperate phage vTRX32-1 was introduced into the bacteria by applying 20 µL of 10^7 PFU/mL concentrated phage cultures (isolated from growing cultures of phage-infected bacteria) in the middle of the bacterial culture and spreading on agar plates. The plates were incubated for 72 hours at 28ºC. Phage-integrated strains (lysogens) were then isolated by streaking from regrowth within the zone of clearing. Subsequent lysogenic clones were confirmed by PCR using the following phage specific primers - 1) GTCGAGTGCTTGACCTCCTC (forward), 2) ACCTCTTCTTGGTCGCTTCA (reverse) targeting the conserved maturase B gene and, 3) CAGTCCTGCCACCTCAATGT (forward), 4) ACGAAGAAATCCGTTGCCCT (reverse) targeting a highly variable phage region. These primers were designed and provided by my supervisor, Dr. Ellie Harrison. Cultures were grown in 6 mL TY (Tryptone - 6 g/L, Yeast - 3 g/L) liquid medium in 30 mL glass universals at 28ºC in an incubator shaken at 180 rpm for 72 hours. Plaques were measured on the original host (TRX32), TRX19 and TRX4 after 72 hours, using phage spotting (as a measure of spontaneous induction). In TRX32, there were no plaques observed while in TRX19 and TRX4, the number of plaques produced were the same i.e. $1x10^9$ PFU/mL.

2.2.2 Plant varieties and culture conditions

White clover plants (variety = Avoca, DLF seeds Ltd.) were grown in 1 L tricorn pots containing 900 g autoclaved vermiculite and sand mix (1:4 ratio). Seeds were sterilised by immersing and shaking in 3% bleach for 30 minutes at room temperature. The seeds were washed with sterile water to remove any traces of bleach, spread on sterile filter paper and left to germinate for 5 days at room temperature. Single seedlings were randomly selected and placed in the tricorn pots. The seedlings were then inoculated with 200 µl of $1x10^7$ CFU/mL bacterial culture, 10 mL sterile nitrogen free Jensen media (Howieson and Dilworth, 2016) and 10 mL sterile distilled water. The rhizobia free nitrogen (N+) positive controls were additionally supplied with 0.01 mL of 0.83 mM/L ammonium sulphate solution every week. This concentration is similar to the volume of fertiliser applied to 1 m^3 of topsoil at the rate of 0.5 kg/hectare and it has been shown to increase clover production (Burchill *et al.*, 2014). The tricorn pots were covered with autoclaved sunbags, which contain a filter for gas exchange, and secured tightly to prevent cross contamination. A sterilised silicon watering tube capped with a .45µm sterile filter was used for watering and feeding. Treatment pots were placed in a controlled environment chamber (16/8 hour day/night cycle at 22 ºC/20 ºC, 500 PPFD).

2.2.3 RNA sequencing experiment

Pots were set up with either non-lysogenic strains TRX4 or TRX19, or lysogenic strains, TRX4v32 (TRX4+vTRX32-1), or TRX19v32 (TRX19+vTRX32-1). Three replicate pot microcosms were prepared for each treatment and each technical replicate was planted with three clover seedlings, making 9 replicate plants in total as clover seeds are known to have a high genetic variability in natural populations (Wu *et al.*, 2021). All the strains were GFP labelled. Clover seedlings in the pot microcosms were inoculated with $1x10^7$ CFU/mL. The plants were grown as described and harvested after eight weeks. Four and eight weeks were used as the two time points as clover nodules usually develop 3-5 days after inoculation (Pankhurst and Gibson, 1973; Uchiumi *et al.*, 1989). Similar studies in rhizobia and pea (which is a bigger seed) symbiosis use 4-5 week old plants for symbiosis based experiments (O'Connell *et al.*, 1998; Westhoek *et al.*, 2017). Roots were separated from shoots and washed in distilled water. All the root nodules from each replicate plant were collected separately and transferred to a sterile 2 ml eppendorf.

RNA was extracted from nodules using Qiagen RNeasy mini kit following methods outlined in Green et al., 2019. Nodules from each replicate were macerated using sterile pestles. The

nodule suspensions were further lysed in Tissue lyser II using 300 mg 0.1 mm silica and 100 mg 0.1 mm glass beads and centrifuged at 6000 rpm for a minute to remove cell debris. RNA from samples was obtained using RNeasy columns. The RNA samples were sent for sequencing to the Centre for Genomic Research, University of Liverpool.

Raw sequence data was run through FastQC (Andrews, 2010) and multiQC (Ewels *et al.*, 2016) to visualise data quality. The data was further trimmed and filtered using Trimmomatic (Bolger, Lohse and Usadel, 2014). The trimmed and filtered data was mapped to the reference genome using HISAT2 (Kim, Langmead and Salzberg, 2015; Pertea *et al.*, 2016). TRX19 (with vTRX32-1) was used as the reference genome for both TRX4 and TRX19. TRX4 was not used as a reference genome for TRX4 as a complete annotated genome of TRX4 was not ready to use. HTSeq 2.0 (Putri *et al.*, 2022) was used for absolute expression, these values were used to generate transcripts per million scores for each gene and assemble all the gene transcripts. Normalized differential expression of genes was calculated using DESeq2 (Love, Huber and Anders, 2014) in R.

2.2.4 Plant dry weight and total fixed nitrogen

Clover seedlings in the pot microscosms were inoculated with 200 µl of $1x10^7$ CFU/mL TRX4, TRX19, TRX4v32 or TRX19v32 treatments. The negative control and positive nitrogen control were applied with 200 µl of sterile water. Each treatment had 12 replicates. The plants were grown as described above. Out of the 12 replicates of each treatment, 6 were harvested after four weeks and 6 after eight weeks. Roots were separated from shoots and washed in distilled water. Root nodules were carefully removed from the roots, washed and counted. Bulk soil from each replicate was transferred to falcon tubes containing root wash solution i.e.10 mM $MgSO_4$ and 0.1% Tween and mixed with 4 g of sterile glass bead mix (diameters – 2 mm and 4 mm). The falcon tubes with the soil was vortexed for a minute, left for 30 minutes and vortexed again for a minute. The soil wash supernatant was diluted and plated onto TY agar plates supplemented with 3 ng/mL gentamycin and 1µg/mL cycloheximide and kept in incubator at 28°C. Cycloheximide was added to prevent fungi from growing on the plates. All the root nodules from each replicate were collected and transferred to a 2 ml eppendorf labelled with respective replicate name and number. The nodules were sterilised by filling the eppendorfs with 2 ml of 3% bleach solution and shaking for 30 mins at room temperature. The nodules were washed 6 times with sterile distilled water to remove any traces of bleach. The eppendorfs with nodules were filled with 0.5 ml of TY. The nodules were crushed, supernatant

diluted and plated onto TY agar plates supplemented with 3 ng/mL gentamycin and 1 μg/mL cycloheximide. The plates were kept in incubator at 28°C. The roots and shoots were kept in drying oven at 60°C for 48 hours and weighed.

2.2.5 Sample preparation for Isotope Ratio Mass Spectroscopy (IRMS)

Natural abundance of ^{15}N method was used to calculate the fixed nitrogen by rhizobia (He *et al.*, 2009; mariotti, 1963). To measure the total nitrogen and carbon: nitrogen ratio, dried shoot material from each treatment was used for Isotope Ratio Mass spectroscopy (IRMS). Each sample was powdered in Qiagen tissue lyser II using 5 mm tungsten beads. The powdered sample was weighed and packed in a 51 mm X 51 mm tin foil. The tin foil was closed tightly into a pellet and sent for IRMS sampling to Faculty of Science Mass Spectrometry Centre, University of Sheffield. The samples were tested using an ANCA GSL 20-20 Mass Spectrometer made by Sercon PDZ Europa (Cheshire).

2.2.6 Statistical Analyses

The RNA data was analysed using DESeq2 in R. Normalisation of data was done using DeSeq2. DeSeq2 normalises the data by computing a scaling factor for each sample. This scaling factor is a median of the ratio of the read count of each gene in a sample and its geometric mean across all samples (Anders and Wolfgang, 2010; Dillies *et al.*, 2013). DESeq2 uses negative binomial distribution to calculate the means and variance of the two groups. It further used Wald t-test to calculate the p-values.

GO functional analysis was used to screen for enrichment of genes involved in specific roles in the differentially expressed data set (Tomczak *et al.*, 2018).

The plant dry weight, total shoot nitrogen, and carbon to nitrogen ratio data were analysed using linear models i.e. lm() and groups were compared using fit.contrast().

2.3 Results

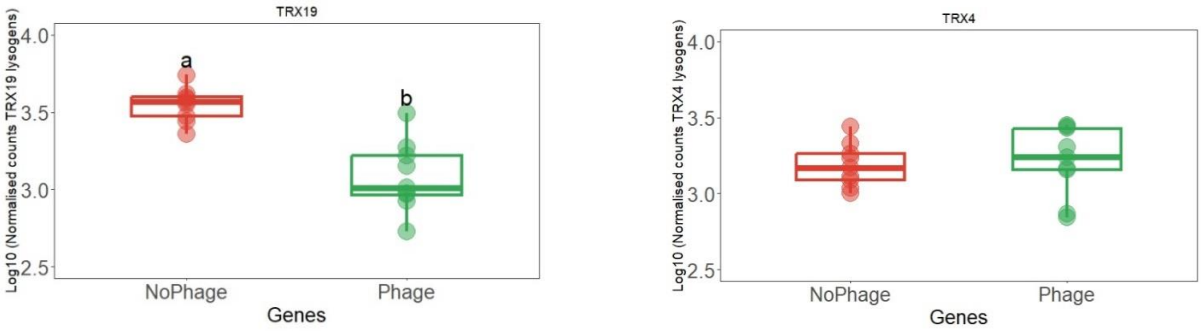
Prophage presence had different effects on *Rlt* TRX19 and *Rlt* TRX4 symbionts. While there was no difference in gene expression of *Rlt* TRX19 driven by the phage, in *Rlt* TRX4, some differences were observed. Comparison of phage genes to chromosomal genes suggested that the phage genes in TRX19 lysogens have much lower expression relative to non-phage genes,

while phage genes were more highly transcribed in TRX4 (Figure 2.1). Despite this, plant dry weight and nitrogen fixed by rhizobia were not affected due to phage carriage.

2.3.1 Transcriptional response to prophage in plant bacteroids

To assess the impact of prophage carriage on the symbiotic performance of *Rlt*, I estimated the transcriptional response of 2 distinct *Rlt* strains - TRX19 and TRX4 - carrying phage vTRX32-1 isolated from nodules.

First, the mean expression of phage genes and non-phage genes were compared from the *Rlt* lysogens of TRX19v32 and TRX4v32. In TRX19 lysogens, a significant difference was found in the log of normalised counts (refers to ratios of raw gene counts and size factor of each sample) of phage and non-phage genes (Figure 2.1: (a), $F_{1,16} = 30.48$, $p = 4.64e\text{-}05$). The mean log10 normalised count of non-phage genes was 3.54 ± 0.03 and mean log10 normalised count of phage genes = 3.08 ± 0.07). In TRX4 lysogens, no such difference was found (Figure 2.1: (b), $F_{1,16} = 0.07$, $p = 0.78$), the mean log10 normalised count of non-phage genes was 3.18 ± 0.04 and mean log10 normalised count of phage genes was 3.21 ± 0.07.



(a) (b

Figure 2. 1 : Phage and non-phage genes compared from *Rlt* (a) TRX19 lysogens and (b) TRX4 lysogens.
The x-axis shows the different gene groups (phage and non-phage) while the y-axis shows the log 10 of normalised counts where normalised counts refer to ratio of raw gene counts and size factor.

PCA analysis of both the groups and their all genes showed that 99% of the variation was due to the two *Rlt* strains (Figure 2.2)

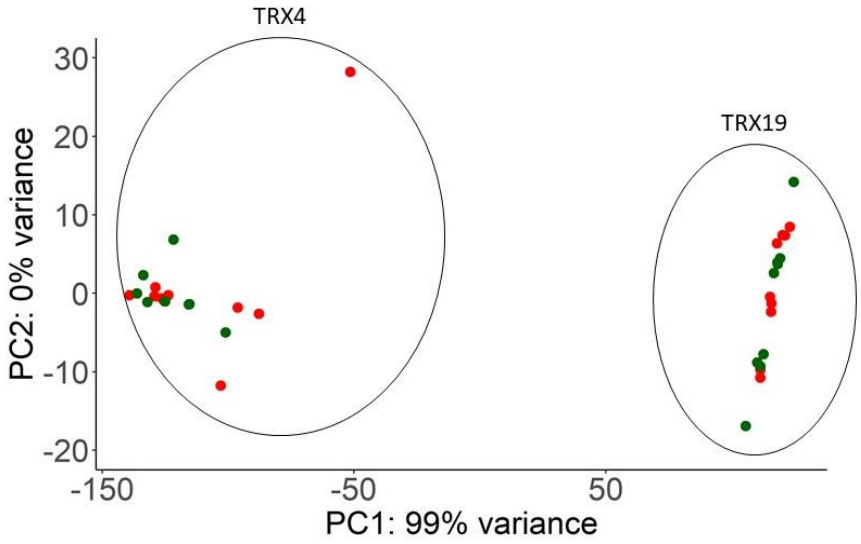
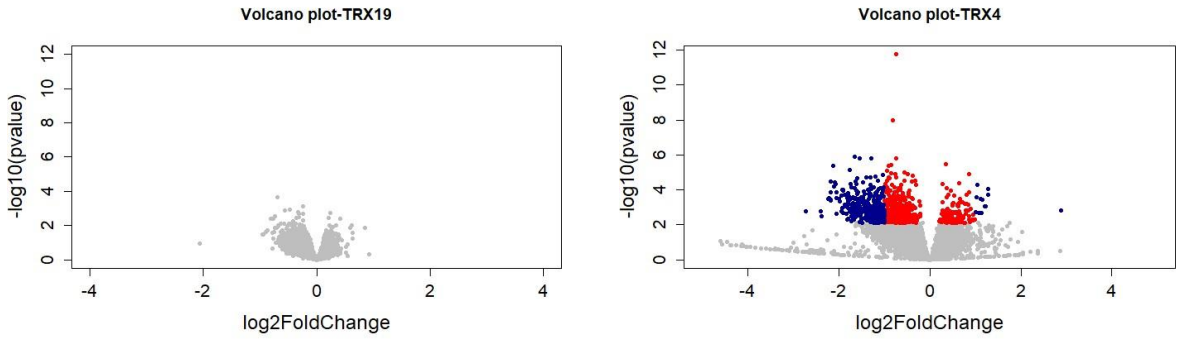


Figure 2. 2 : PCA plot showing variance between individual replicates based on the expression of non-phage genes in both the *Rlt* groups - TRX4 and TRX19.
The figure shows phage presence and absence (where NP are replicates without phage and P are replicates with phage). The plot shows that the two *Rlt* clusters are responsible for 99% variation.

Differential gene expression of TRX19 and TRX4 genes showed very different responses driven by phage carriage. The genomes of the strains were aligned to the reference TRX19 with phage vTRX32-1. After alignment and normalisation, all the gene counts below 10 were removed. After filtering for low gene counts, 7473 genes were left in TRX19 and 6004 genes in TRX4. In TRX19, out of the 7473 genes, no genes were found to be upregulated or downregulated in phage carrying rhizobia as compared to the phage-free rhizobia (Figure 2.3(a)). In TRX4, however, out of the 6004 genes, 777 genes were found to be downregulated and 152 genes were found to be upregulated in phage-carrying rhizobia as compared to the phage-free rhizobia (Figure 2.3(b)). However, because most of these genes were hypothetical/unannotated, I further analysed the data that had annotated genes. To further investigate the functional effects of phage-associated transcription response, I repeated the analysis focusing only on annotated genes. After unannotated genes were removed, I was left with 3205 genes (53% of 6004 genes) in TRX4 and 3560 genes (47% of 7473 genes) in TRX19. As in unannotated genes, none of the genes were upregulated or downregulated in TRX19 lysogens. In TRX4 lysogens, however, out of the 3205 genes, 60 genes were upregulated while 480 genes were downregulated relative to phage free TRX4.



(a) (b)

Figure 2. 3 : Transcriptional response to prophage carriage in two Rlt strains isolated from nodules - (a) TRX19, (b) TRX4.

Points represent individual genes with colour denoting significance: grey shows non-significant genes, red shows significant genes (p>0.05) where log2fold change $\leqslant$ 1 and blue shows significant genes where log2fold change $\geqslant$ 1.

2.3.1.1 Functional analysis

398 genes were found to be significantly different in TRX4 and had a Log Fold Change $\geqslant$ 1. GO functional enrichment analysis was used to identify significant functions likely to be altered in response to the phages. The biological processes, terms for protein catabolism and lipid A biosynthesis process were most significant while in the cellular component, proton transportation was found to be enriched. In addition to these functional groupings, many individual genes of note were altered.

2.3.1.1.1 Genes involved in symbiosis

Many genes involved in nodulation, nitrogen fixation and symbiosis were significantly affected by phage presence in TRX4. *nodD2_4*, a part of *nodD2* which is involved in nodulation was significantly downregulated with Log Fold Change (LFC) = -3.25. However, other sub-units of *nodD2* such as *nodD2_1* and *nodD2_2* were not significantly different. *nodD2* represents the first level of host-rhizobia interaction. It binds to the *nod* box which is present before the *nod* operons and negatively regulates the rest of the *nod* operon (Schultze and Kondorosi, 1998). Other *nod* genes - *nodA*, *nodM* and *nodF* were not differentially expressed, however, *ompR* which plays an important role in curl induction and invasion of host roots (Rodríguez *et al.*, 2020) was also found to be downregulated with LFC = -2.10 .

Nitrogen fixation genes - *fix, nif* and *rpoN2_2* - were also significantly downregulated. Genes associated with production of the nitrogenase enzyme, *nifA*, *nifB* and *nifS* were significantly downregulated with LFC= -4.32 in *nifA*, LFC =-3.55 *nifB* and LFC = -3.45 in *nifS*. Gene *nifA* is a positive regulator of *fix* and *nif* genes, while *nifS* and *nifB are* involved in adding metal compounds to nitrogenase (Cebolla and Palomares, 1994). Gene *lon* (LFC = -2.57) produces an endopeptidase which hydrolyses proteins in the presence of ATP. *lon* has been shown to maintain active NifA in cells by degrading inactive NifA in microaerobic environments leading to increase of active NifA (Huala, Moon and Ausubel, 1991).

fixP and *fixX* genes are part of larger nitrogenase-associated complexes that are essential for nitrogenase functioning. *fixX* is similar to ferodoxins and *fixP* is part of the membrane bound cytochrome oxidase (Cebolla and Palomares, 1994). Both *fixP* and *fixX* were downregulated with LFC = -2.66 and LFC = -3.2, respectively and other *fix* gene - *fixK_2* which is a positive regulator of another nitrogenase complex fixNOQP was also downregulated (LFC = -0.96) (Cebolla and Palomares, 1994). *rpoN2_2*, an essential gene required for nitrogen fixation (Niner and Hirsch, 1998) was also downregulated at LFC = -3.19.

2.3.1.1.2 A Gene involved in motility and chemotaxis

Chemotaxis genes, *cheB,* are part of the chemotaxis operon which regulates the movement of bacteria through methylation (Harighi, 2009). *cheB_1* was significantly different with LFC = -0.94.

2.3.1.1.3 Genes involved in phage regulation and SOS response

Many SOS response genes were also downregulated in TRX4 lysogens. This includes the *ftsH_2* (LFC = -2.46), *clpP_1* (LFC = -2.10), *clpP_2* (LFC = -2.25), *clpS* (LFC =12.49), *clpPS2* (LFC = -1.96) and *clpX* (LFC = -2.71) genes. *ftsH_2* and *clp* genes encode proteases which are part of the heat shock protein family and are important for protein folding, denaturation and degradation of proteins (Nocker *et al.*, 2001). The *degP_2* gene, which is a periplasmic serine endoprotease essential for survival at high temperatures, was also downregulated (LFC= -2.33) (Narberhaus *et al.*, 1998). *hspQ* gene produces heat shock protein hspQ. hspQ regulates expression of another gene *dnaA* which produces DNA primase essential for DNA replication (Shimuta *et al.*, 2004) *hspQ* was heavily downregulated with LFC = -4.18.

recA (LFC= -3.42), *lexA_1* (LFC= -3.18) and *lexA_2* (LFC= -2.46) are central components of the SOS response and responsible for DNA damage repair. RecA is an inducer of LexA, which represses the expression of SOS response genes. Both, gene *recA* and gene *lexA* are known to

play a major role in maintaining lysogeny in pathogenic bacteria (Waldor and Friedman, 2005). Other genes involved in the SOS response i.e. *ssb* (LFC = -2.98), *uvrA* (LFC = -1.54), *uvrB* (LFC = -1.84) and *ftsZ_2* (LFC = -1.77) were also downregulated.

2.3.1.1.4 Genes involved in carbohydrate and ammonia assimilation

Genes *rbsB*_9 (LFC = -3.26), *rbsC_13* (LFC = -0.64), *rbsC_16* (LFC = -1.40) and *rbsC_21* (LFC = -2.21), all important for ribose utilisation were downregulated. Genes involved in glutamine biosynthesis and ammonia assimilation were also downregulated (Niner and Hirsch, 1998). The genes involved in glutamine synthesis that were downregulated were *glnA* (LFC = -1.77), *glnB* (LFC = -3.19), *glnH_1* (LFC = -2.91) and *glnQ_9* (LFC = -1.70). The gene *glnB* produces a nitrogen regulatory protein P-II which has been shown to be essential for nitrogen assimilation as well as nodule development (Arcondéguy *et al.*, 1997).

2.3.1.1.5 Protein catabolism and anabolism

GO analysis revealed a significant enrichment of genes targeting the biological process 'protein catabolism' among the differentially expressed gene set. In contrast to the majority of differentially expressed genes, several genes in this category were upregulated in response to phages. This includes *aat* which is a Leucyl/phenylalanyl-tRNA protein and one of the 6 annotated genes to be upregulated (LFC = 2.04). *aat* has been found to be involved in carbon and nitrogen metabolism in rhizobia as it allows growth on aspartate (Rastogi and Watson, 1991). Gene *arcB_2* (LFC = 2.32), which produces a carboxylate reductase and is involved in arginine biosynthesis, was also upregulated. There is evidence that *arcB* has a possible role in nitrogen fixation (D'Hooghe *et al.*, 1997). Other genes involved in protein catabolism such as *ftsH_2* and the *clp* gene family have been described above.

Other genes of note, *dapH and dapH_2* which produce enzymes essential for lysine biosynthesis (Niner and Hirsch, 1998) were both upregulated. *dapH* had an LFC of 2.08 and *dapH_2* of 2.28. The *pcaJ* gene which produces a putative 3-oxoadipate CoA-transferase subunit B was also upregulated (LFC = 2.59). A gene similar to *pcaJ, pcaM,* which is involved in the catabolism of arabinose and protocatechuate (an intermediate aromatic compound produced due to degradation of substances such as lignin) has been shown to improve *R. leguminosarum* bv. *viciae* growth in the rhizosphere of non-host pea plants (Paula *et al.*, 2015).

Genes that are required for amino acid synthesis and transport were all downregulated. These involved gene *mdh_1* (LFC = -3.48), essential for synthesis and transport of aspartate, genes *dctB_1* (LFC = -2.26), *dctB_2* (LFC = -2.52) and *dctD_2* (LFC = -1.83), essential for transport

of dicarboxylic acids such as aspartate, malate and succinate.and gene *dme* (LFC = -3.08) containing NaD+, which is required in high amounts as TCA intermediates, were all downregulated. In addition, *hemA_1* (LFC = -2.68), *hemA_2* (LFC = -3.16) and *hemN_2* (LFC = -1.76), all required for aminoleuvenic acid synthesis in nodules, were also downregulated (McGinnis and O'Brian, 1995). *rpoE1* is a cytoplasmic sigma factor which has been shown to activate when sulphites are oxidised in the stationary phase (Bastiat *et al.*, 2012). *rpoE1* has a potential role in either sulphite catabolism to provide energy to bacterial cells or prevent cell toxicity due to sulphites. *rpoE1* was also downregulated with LFC of -4.41.

To validate the effects of the above mentioned up and downregulated genes, functional assays such as those to measure enzymatic activity can be used. For instance – many genes involved in glutamine biosynthesis are downregulated in this study, which means if these genes have a functional effect, the amount of glutamine produced by lysogens would be less than non-lysogens. Glutamine can be quantified by an assay which would first involve conversion of glutamine to α-ketoglutarate as the end product along with reduction of nicotinamide adenine dinucleotide i.e. NAD+ to NADH (Keast *et al.*, 1998). This conversion can be measured using a spectrophotometer or a colorimeter to quantify the amount of glutamine present in the samples. However, as the RNA was isolated from rhizobia in the root nodules, metabolomics (i.e. analysing the metabolites directly from root nodules) would be a better approach to validate the effects of significant genes.

2.3.1.1.6 Lipid A biosynthesis

The gene *glmU* and some genes of the *acp* family identified for lipid A biosynthesis, an important component of the bacterial cell wall were found to be downregulated. *glmU* (LFC = -2.81) catalyses a reaction in the de novo biosynthetic pathway for UDP-N-acetylglucosamine which is essential for glutamine synthesis (Mengin-Lecreulx and Van Heijenoort, 1994). *acpP* had a LFC of -2.74, *acpS* of -1.4 and *acpXL* of -2.5. *acpXL* encodes the AcpXL protein, which is involved in production of the very-long-fatty acid chain for the lipid A of the cell wall. Moreover, mutations in *acpXL* have been shown to delay nodule formation in *R. leguminosarum bv. trifolli* (Brown *et al.*, 2011). Other proteins which were downregulated and help in lipid A biosynthesis were *lpxA* (LFC = -2.71) and *lpxD* (LFC = -2.10), both of which encode acyl transferases (Becker *et al.*, 2004).

2.3.1.1.7 Proton and molecule transporters

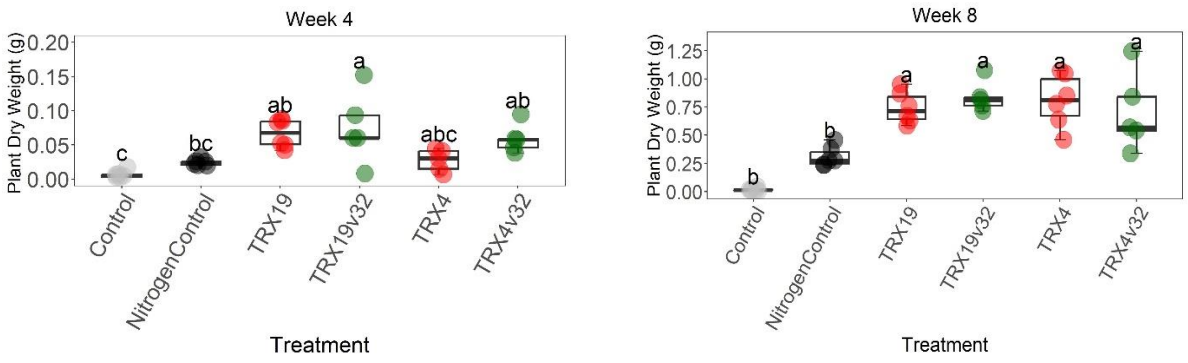
In molecular functioning, most significant genes were involved in proton transporting and ATP-peptidase and leucyltransferase activity. For proton transporting, major *atp* gene subunits such as *atpC* (LFC = -2.60), *atpG* (LFC = -3.06), *atpD* (LFC = -2.63), *atpF_2* (LFC = -2.28) and *atpB_2* (LFC = -3.41) were downregulated. Other transporter genes such as *rhtC* whose product is a L-threonine exporter and *xylG* which produces xylose ABC transporter ATP binding subunit of xylose ABC transporter were however, upregulated with LFC of 2.44 and LFC of 2.30, respectively.

2.3.2 Plant dry weight and total nitrogen fixed

2.3.2.1 Plant dry weight

I compared the biomass of four week and eight week old clover plants of the following treatments – phage free TRX19 and TRX4, phage carrying TRX19v32 and TRX4v32, negative control and rhizobia free nitrogen positive control using a linear model. The total biomass i.e. shoot and root biomass of all treatments was significantly different after 4 weeks, driven primarily by the negative control (Figure 2.4: (a), $p = 0.0003251$, $F_{5,c27} = 6.78$). All rhizobial treatments were significantly different to the negative control, except TRX4 and the nitrogen controls ($p < 0.23$). When the treatments were compared to the nitrogen control, TRX19 ($t = -2.9$, $p = 0.006$), TRX19v32 ($t = -3.37$, $p = 0.002$) and TRX4v32 ($t = -2.32$, $p = 0.02$) were significantly different. In pairwise comparisons, the total biomass of TRX19 was not significantly different to TRX19v32 ($t = -0.54$, $p = 0.59$). Similarly, there was no difference in TRX4 and TRX4v32 ($t = -2.01$, $p = 0.054$).

After 8 weeks, the negative control and the nitrogen control were significantly different to all the rhizobial treatments (Figure 2.4: (b), $F_{5, 28} = 18.11$, $p = 5.18e-08$). The dry weight of negative and nitrogen positive controls was 56% and 45% less than all the rhizobial treatments.



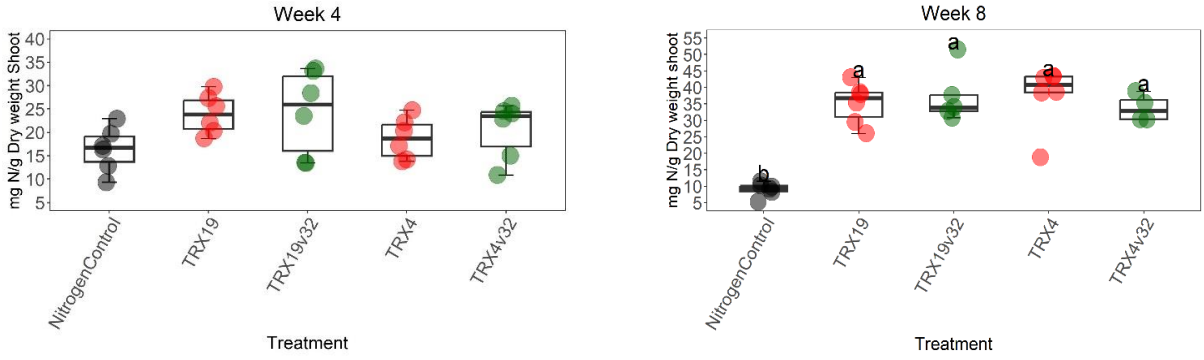
(a) (b)

Figure 2. 4 : The plant dry weight of control, nitrogen control, TRX19, TRX19v32, TRX4 and TRX4v32 after a) 4 weeks and b) 8 weeks.

Different colours represent the different replicates within the treatments, grey shows negative control, black shows rhizobia free nitrogen positive control, red show phage free treatments while green show phage treatments.

2.3.2.2 Total nitrogen in plants using ^{15}N abundance method

Total nitrogen in the above ground shoot of plants was measured after 4 weeks and 8 weeks. There was no significant difference in the treatments after 4 weeks (Figure 2.5: (a), $F_{4, 25} = 1.905$, $p = 0.141$). However, after 8 weeks, there was a significant difference between all the rhizobial treatments and the nitrogen control (Figure 2.5: (b), $F_{4, 22} = 19.08$, $p = 6.69e\text{-}07$). The nitrogen added plants had 25% less nitrogen as compared to the rhizobial treatments. There was no significant difference observed in strain specific comparisons amongst lysogens and phage free treatments (Figure 2.5).



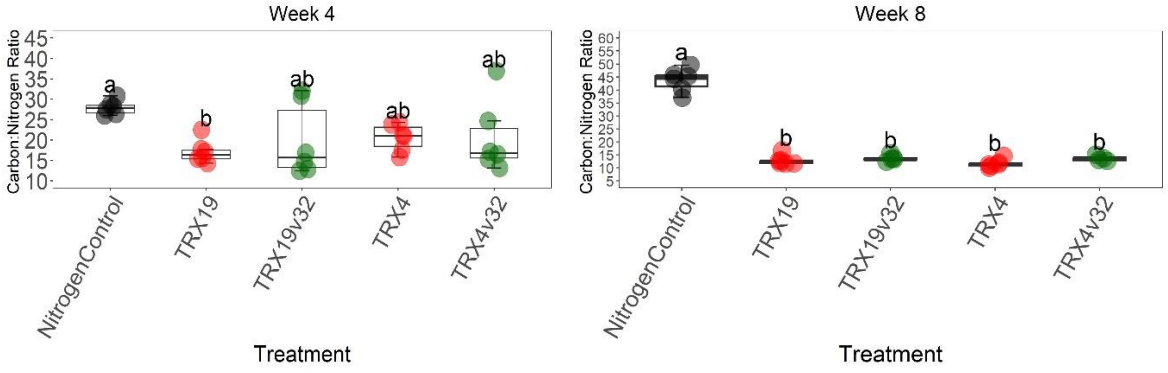
(a) (b)

Figure 2. 5 : Total shoot nitrogen measured in nitrogen control, TRX19, TRX19v32, TRX4 and TRX4v32 as mg N/g dry weight after a) 4 weeks and b) 8 weeks.

Different colours represent the different replicates within the treatments, black shows rhizobia free nitrogen positive control, red show phage-free treatments while green show phage treatments.

2.3.2.3 Carbon: Nitrogen (C:N) Ratio

A significant difference was observed in the carbon and nitrogen ratio of the treatments after 4 weeks (Figure 2.6: (a), $F_{4,25} = 2.62$, p = 0.05). There was however no significant difference between TRX19 and TRX19v32 (Figure 2.6 (a): p = 0.9, t = 0.005). Similarly, there was no significant difference between TRX4 and TRX4v32 (Figure 2.6: (a), p = 0.42, t = -0.81).



(a) (b)

Figure 2. 6 : Carbon nitrogen ratios measured in nitrogen control, TRX19, TRX19v32, TRX4 and TRX4v32 after a) 4 weeks and b) 8 weeks.
Different colours represent the different replicates within the treatments, black shows rhizobia free nitrogen positive control, red show phage-free treatments while green show phage treatments.

The difference in C:N ratio was maintained after 8 weeks (Figure 2.6: (b), $F_{4,22} = 177.52$, p = 0.0001) with rhizobia free nitrogen positive plants having 30% higher ratios than the rhizobial treatments. There was no significant difference between TRX19 and TRX19v32 (Figure 2.6: (b), t = -1.22, p = 1.61), and TRX4 and TRX4v32 (Figure 2.6: (b), t = -0.52, p = 0.60).

2.4 Discussion

Rhizobia can have important interactions with MGEs. While the interactions of rhizobia with ICEs and plasmids (Zahran, 2017; Wardell *et al.*, 2021) are well studied, other MGEs still need further exploration. Here, I show that temperate phage vTRX32-1 can have major transcriptional effects on their host rhizobia as observed in *Rlt* TRX4, yet they can also be entirely silent, as observed in *Rlt* TRX19.

Carriage of phage vTRX32-1 led to downregulation of many different rhizobial genes in the *Rlt* TRX4 strain. These genes were involved in a wide range of functions, including the SOS response, metabolism and, surprisingly, nodulation and nitrogen fixation.

The interaction between lysogeny and the RecA-LexA controlled SOS response has been well described in other phage systems and can both trigger (Sedgwick, Yarranton and Heath, 1981; Quinones, Kimsey and Waldor, 2005) and be triggered by phage lysis (Campoy *et al.*, 2006). Down regulation of both the activator (RecA) and repressor (LexA) are predicted to have contradictory actions, but the downregulation of other SOS related genes suggests that the SOS response may in fact be repressed in *Rlt* TRX4 lysogens. High *recA* expression, through initiation of the SOS response or direct interaction with phage repressors has been show to increase phage induction (Sedgwick, Yarranton and Heath, 1981; Quinones, Kimsey and Waldor, 2005), which might suggest that TRX4 lysogens are more stable. However, comparison of phage gene expression with chromosomal gene expression suggests this is not the case. In strain TRX4, the expression of phage genes was high - average gene expression was similar to chromosomal genes - while in TRX19, which showed no transcriptional response, expression of phage genes was lower than chromosomal genes. As phage genes are tightly repressed during lysogeny and expressed only on entering lysis (Owen *et al.*, 2020), this implies that a higher proportion of TRX4 lysogens are in active lysis than are TRX19.

The significant downregulation of many key symbiosis genes was unexpected. Downregulation of the *nodD2_4* gene, a negative regulator involved in the regulation of *nod* genes might suggest that there is an increase in the expression of the *nod* operon, but no other *nod* genes were affected. However, since genes involved in nitrogenase functioning (*nif and fix*) and nitrogen assimilation (*rpoN2_2)* are positive regulators and are involved in the functioning of the nitrogenase enzyme, their downregulation suggests that the phage may reduce these functions. The genes involved in lipid A production were also repressed by the phage. Repression of lipid A has been shown to delay nodule formation with smaller white, irregularly shaped nodules due to unstable cell membranes (Brown *et al.*, 2011; Bourassa *et al.*, 2017)..

Genes for ribose, ammonia, glutamine, and aspartate biosynthesis and assimilation were also downregulated. However, lysogens showed an increase in the expression of genes involved in the biosynthesis of lysine, asparagine, and catabolism of arabinose and protocatechuate. Thus, phage carriage seems to re-route the carbon and nitrogen metabolism in the bacteroids. Surprisingly, the changes observed are likely to provide a competitive advantage and growth in the rhizosphere as the decrease in nitrogen assimilation and nitrogenase has been shown to support and increase the growth in the rhizosphere or micro-aerobic conditions but not in nodules (Becker *et al.*, 2004). Moreover, protocatechuate and arabinose have been shown to be the preferred alternative carbon sources that help in increasing competitive fitness and growth

in the rhizosphere of non-host plants (Paula *et al.*, 2015). This suggests that the phage might be providing a benefit when the lysogens are in the free-living state. However, why the phage increases the expression of these genes in the nodules is unclear. Studies with other prophages have shown that phages usually upregulate the expression of genes associated with phage-encoded genes (Zhao *et al.*, 2017). The downregulation of proteins involved in catabolism further suggests that active phage lysis might reduce the host proteases to avoid degradation of phage proteins.

I did not see any differential gene expression in *Rlt* TRX19. This maybe because the phage is more stable lysogenically in TRX19 as compared to TRX4 leading to observable differences in gene expression. The large and significant differences in gene expression of *Rlt* TRX4 and *Rlt* TRX19 was, however, not seen in plant growth experiments. The dry weight of plants was found to be significantly different after 8 weeks in the treatments. The weight of nitrogen added control plants was 25% less than both the phage free and phage carrying rhizobia. However, there were no significant differences in the weight of plants inoculated with phage carrying (TRX19v32 and TRX4 v32) and phage free rhizobia (TRX19 and TRX4). These results are in accordance with results that have been observed earlier in *Glycine max* (soybean) and in *Sesbania aegyptica* where phage presence did not affect nodulation, nitrogen fixation, and symbiosis in plants (Abebe *et al.*, 1992; Dhar *et al.*, 2013; Hatem, El -Sabbagh and El -Didamony, 2017). The carbon:nitrogen ratio was higher for nitrogen added controls as compared to the phage free and phage carrying rhizobia. The higher carbon:nitrogen ratio in nitrogen controls may be due to there being more carbon from organic matter but no microbes available to utilise the carbon even though the nitrogen was supplied at a constant rate (Xing *et al.*, 2018). In the rhizobial treatments, however, rhizobia were present to assimilate the carbon and, at the same time, produce ample amount of bioavailable nitrogen for plants to use, leading to a lower carbon:nitrogen ratio (Xue *et al.*, 2020).

To conclude, I have shown two very different responses to lysogeny in two symbiotic *Rlt* strains. Phage presence resulted in differential regulation of many genes : >15% of the genome, in the strain TRX4, including many functions associated with symbiosis. No such downregulation was observed in TRX19. Moreover, I found that regardless of the impact of the phage on bacterial transcription, lysogeny did not affect the rhizobial-clover symbiosis. This finding has important implications for the potential of phages to enhance rhizobial inoculants. Temperate phages could provide other advantages, for instance, in competition with other bacteria, which would make them useful agents for improving the success of inoculant

strains. The absence of negative effects, in a range of bacterial hosts with very different interactions with the phage is key to ensuring that there are no negative impacts on the symbiosis.

Chapter 3 - Do temperate phages give a competitive advantage to rhizobia?

3.1 Introduction

Prophages - temperate phages integrated in the bacterial genome - can confer a competitive fitness advantage to their bacterial hosts (lysogens) by acting as weapons in bacterial conflict (Chao and Levin, 1981; Gama *et al.*, 2013). This happens when a switch to the lytic cycle occurs known as induction in hosts, either spontaneously (Nanda, Thormann and Frunzke, 2015) or due to certain environmental triggers such as UV light or toxins such as Mitomycin C (Brown *et al.*, 2006; Harrison and Brockhurst, 2017). Induction of prophages leads to the lysis of host cells and release of infective phage particles which spread in the local environment and kill bacterial populations that are susceptible to phage infection and killing. As these susceptible cells are likely unrelated competitors to the lysogenic host, this gives a competitive advantage to surviving host cells (Harrison and Brockhurst, 2017). Competitive effects of temperate phages have been observed in many pathogens such as *Bordetella* (Joo *et al.*, 2006), *Escherichia coli* (Yu *et al.*, 2021) and *Pseudomonas aeruginosa* (Burns, James and Harrison, 2015; Bondy-denomy *et al.*, 2016; Davies, *et al.*, 2016). We hypothesised that phages could provide a similar competitive advantage in the colonization of legume hosts by their nitrogen-fixing symbionts, rhizobia.

In natural soil environments, rhizobia populations comprise many different strains which compete to colonise host plants and form root nodules (Wielbo *et al.*, 2011; Stasiak *et al.*, 2014). These different strains vary widely in their ability and effectiveness to colonise, occupy root nodules and fix nitrogen in host legumes (Wielbo *et al.*, 2011; Mendoza-Suárez *et al.*, 2021). Effective nodulating of hosts, however, does not always translate to effective nitrogen-fixing in host plants (Checcucci *et al.*, 2016; Burghardt and diCenzo, 2023). This variation means that many ‘cheater’ strains can occupy nodules and act as free riders while exploiting host-sanctioned resources in return for little to no benefit to the host plant (Denison and Kiers, 2004). Variation in strain competitiveness can be costly to the plant as strains range from effective nitrogen fixers to ineffective or no nitrogen fixers (Rahman *et al.*, 2023), causing a decrease in nitrogen fixation by effective strains in mixed nodules (Checcucci *et al.*, 2016; Rahman *et al.*, 2023). Competition for nodules is a particular problem for rhizobial inoculants which often show insignificant and/or inconsistent impacts on plant productivity (Martinez-

Romero, 2003; Pastor-Bueis *et al.*, 2019). Competition between introduced inoculants and naturalised or natural rhizobial populations is one of the major reasons for this variation in plant productivity (Denton *et al.*, 2003; Batista *et al.*, 2015). Since naturalised populations are well adapted to the local soil conditions, they are more competitive and can easily displace introduced rhizobial populations (Bashan, 1998; Catroux, Hartmann and Revellin, 2001; Bell *et al.*, 2019). This leads to low nodule occupancy by introduced rhizobial inoculants, lower plant productivity and inconsistent results (Babalola and Glick, 2012; Mendoza-Suárez *et al.*, 2021), which has a huge impact on the uptake of rhizobial inoculants by farmers. Increasing the competitiveness of rhizobial inoculants against naturalised rhizobial populations is thus key to increasing the consistency and performance of introduced rhizobia.

Previous studies suggest that temperate phages may be likely to increase the fitness of their host bacteria. Schwinghamer and Brockwell, 1978, found that lysogenic *Rhizobium leguminosarum* were competitively fitter than phage sensitive strains in liquid cultures (i.e. a well-mixed environment) as well as on peat (i.e. in a structured environment) (Schwinghamer and Brockwell, 1978). Similarly, another study on *R. leguminosarum* bv. *trifolii* strains RT9 and RT10 found that mixing of both the strains led to a reduction of phage sensitive RT10 strain both due to phage lysis and induction of another phage carried by RT10 (Takahashi and Quadling, 1961). In natural environments, rhizobia can benefit from antagonistic traits such as bacteriocin production (Burghardt and diCenzo, 2023). For instance, bacteriocins secreted by some *Rhizobium leguminosarum* strains have been found to have a positive effect on host competitive fitness in root nodules against susceptible strains, thus having higher occupancy in root nodules (Triplett and Barta, 1987; Goel, Sindhu and Dadarwal, 1999; Oresnik, Twelker and Hynes, 1999). However, another study in *Bradyrhizobium japonicum* found no effect of bacteriocins on competition (Gross and Vidaver, 1978). In addition, susceptibility to lytic or virulent phages can also affect competitive fitness in rhizobia. This has been shown in *Bradyrhizobium japonicum* and *Rhizobium leguminosarum* bv. *trifolii* where phage sensitive strains decreased in nodules when virulent phages were present, increasing nodule occupancy of phage resistant strains (Evans, Barnet and Vincent, 1979; Barnet, 1980; Hashem and Angle, 1990). The fitness effects of temperate phages on competition for nodules will be dependent on the balance between the costs of lysis within the host population and the benefits from lysing susceptible competitors (Touchon, Moura de Sousa and Rocha, 2017). Different susceptibilities of competitor populations to phage infection and killing will thus, play a huge role in determining whether phage mediated competition is of benefit or cost to the host

population (Brown *et al.*, 2006). In the presence of resistant competitors, there will be no benefit, as phages cannot infect the resistant population while in the presence of susceptible competitors, there will be a huge benefit as the phage will infect and kill susceptible populations through lysis. For temperate phages, the benefits may also be reduced over time as initially competitor populations are converted to lysogens themselves - resulting in resistance to infection. This resistance can, however, be reverted to susceptibility if highly unstable lysogens, which can lose the phage are formed.

Temperate phages may thus, be a good candidate to increase the competitiveness of rhizobial inoculants, but this has yet to be tested *in vivo*. Moreover, while most studies examine fitness effects on isogenic populations it is important to capture the phage mediated competition effects in more diverse environments. Including rhizobial strains with different phage susceptibilities as competitors would help to better understand the variety of competitive effects that phages can exert on the host rhizobia. To exactly investigate this, I measured the competitive fitness of rhizobial lysogens both *in vitro* and *in vivo,* in clover mesocosms.

3.2 Methodology

3.2.1 Fitness effect of vTRX32-1 on *Rlt* TRX19 in isogenic competitions

3.2.1.1 Strains and growth conditions

Experiments were conducted on temperate phage - vTRX32-1 to measure the competitive fitness of *Rhizobium leguminosarum* bv. *trifolii* (*Rlt*). The phage was isolated from *Rlt* strain TRX32 itself isolated from clover nodules in York, UK (Kumar *et al.*, 2015). The focal bacteria used in the experiments is *Rlt* strain TRX19 which was isolated in the same study from the same location (Kumar *et al.*, 2015). All experiments were conducted using marked strains: TRX19 with GFP (carries GFP and gentamycin resistant markers) and TRX19 with MC (carries mCherry and gentamycin resistant markers). In order to integrate phages into the bacteria, 20 μL of concentrated phage cultures were applied to the middle of the agar plates covered in a soft agar lawn of the desired recipient. The plates were incubated for 72 hours at 28ºC. Phage integrated strains, were then isolated from regrowth within the zone of clearing and confirmed by Polymerase Chain Reaction (PCR).

3.2.1.2 Creating a lysogeny resistant strain

To test for the role of competitor lysogenisation on the fitness effect of phage carriage, a knockout strain was produced which lacked the integration site in the bacterial genome. vTRX32-1 attaches at the following 12 bp motif – CAGTTGGCGGAATT, also known as the attB site located within a tRNA-Leu (Ford *et al.*, 2021). Integration disrupts the bacterially encoded tRNA-Leu, but is complimented for by the presence of a different tRNA-Leu, which lacks this motif, carried within the phage genome. To replicate this, we designed a knockout strategy to replace the attB containing tRNA-Leu with the phage encoded, attB free version. The phage encoded tRNA (amplified by primers tr4 and tr5, table 3.1) was cloned into a 7770 bp ds-DNA circular vector between flanking regions matching sequences either side of the bacterial tRNA (primers t6 and t2, table 3.1) using the NEB HiFi DNA assembly kit. The plasmid was constructed and kindly provided by Dr Ellie Harrison. The resulting plasmid was transformed into *Escherichia coli* DH5α and introduced to TRX19 GFP via conjugation. Potential knockout strains were selected on 50 mg/L kanamycin (selected for the plasmid) and 30 mg/L gentamicin (selected for recipient) and then re-streaked to ensure the loss of the plasmid. The knockout was confirmed using PCR (primers tr4, tr5, tr2 and tr6, table 3.1). The PCR program for the experiments was run at 95ºC followed by 25 cycles of 94ºC for 1 minute, 53ºC for 40 seconds and 72ºC for 1 minute 30 seconds with a final extension at 72ºC for 25 minutes. A 1% agarose gel was run at 130 V for 25 minutes for all the experiments. Lysogenization resistance was tested by applying concentrated spots of phages and isolating any colonies growing within the plaques after 72 hours. Resistance to lysogeny in colonies was confirmed using PCR (primers - maturase B and variable, table 3.1).

Competition experiments were done *in vitro* and *in plantae* with phage-free *Rlt* TRX19 Wild Type (WT), phage-containing *Rlt* TRX19 (P) and *Rlt* TRX19 Lysogenization Resistant (LR) strains.

3.2.1.3 Competition experiment in vitro

Phage containing *Rlt* TRX19 (P) and phage free control *Rlt* TRX19 (WT) strains were competed against isogenic phage free reference *Rlt* TRX19 WT and *Rlt* TRX19 LR strains. Each treatment had 6 biological replicates. To avoid marker effects the marker used to identify test and reference strains were switched: in the treatment replicates 1-3, test strains were labelled with GFP and the reference strains with MC. In replicates 4-6, test strains were labelled

with MC and reference strains were marked with GFP. The test and reference bacterial cultures were grown separately at 28ºC in 6 ml Tryptone Yeast (TY) microcosms and then mixed in a 50:50 ratio. The starting cultures were plated on the TY agar plates to estimate starting densities/ratios. 60 µL of this mix was inoculated in a 6 mL TY microcosm.

Table 3. 1 : Primer pairs used in the competition experiments.

Primer Name	**Sequence (5'- 3')**	**Target**
tr4	CGGTTTTGTGAACGCCCAGTTGGCGGAATTG	Exact phage tRNA
tr5	CTGCAAACAGCTTGGTGCCCAGTGAGGGAC	Exact phage tRNA
tr6	TCACTGGGCACCAAGCTGTTTGCAGGCGGGA	Bacterial tRNA flanking region_upstream_fwd
tr2	CGTTGTAAAACGACGGCCAGTGCCATCGATGCCGAGCTTGGAG	Bacterial tRNA flanking region_upstream_reverse
TRX-32-1 forward	GTCGAGTGCTTGACCTCCTC	maturase B - vTRX-32-1
TRX-32-1 reverse	ACCTCTTCTTGGTCGCTTCA	maturase B - vTRX-32-1
TRX-32-1 forward	CAGTCCTGCCACCTCAATGT	variable region - vTRX-32-1
TRX-32-1 reverse	ACGAAGAAATCCGTTGCCCT	variable region - vTRX-32-1

Microcosms were incubated at 28ºC with shaking at 180 rpm for 72 hours. Samples were plated at three time points - 24, 48 and 72 hours. Plates were visualised using GBOX-ChemiXX9 imager and images were analysed using the GeneTools software. Competitive fitness (w) of the treatments was calculated using formula 3.a (Lenski *et al.*, 1991; Wiser and Lenski, 2015). To calculate the proportion of phages integrated in the initially phage free bacteria, we performed PCR on 6 colonies from the reference (phage free) strains of all replicates at the three time points. Six replicates were used for PCR screening to account for easier handling of all replicates and it is a standard in some studies (Burns, James and Harrison, 2015). Since the

colonies formed are assumed to be clones, six to ten colonies here are enough to give a broad picture of the lysogenization rates.

$$w = \frac{ln\left(\frac{Test_{end}}{Test_{start}}\right)}{ln\left(\frac{Reference_{end}}{Reference_{start}}\right)} \quad \text{3.a}$$

In formula 3.a, *w* is relative competitive fitness, ln is natural logarithm, $Test_{start}$ refers to the population density of the test strain at the start of the experiment and $Test_{end}$ refers to the population density of the test strain after 24, 48 or 72 hours. $Reference_{start}$ refers to the population density at the start of the treatment and $Reference_{end}$ refers to the population density of the reference strain after 24, 48 or 72 hours.

3.2.1.4 Competition experiment in vivo

Plants were grown in sterile substrate media (4:1 sand and vermiculite) packed in 1L sterile pots. Clover seeds (variety Avoca, DLF) were treated with 3% household bleach for 30 minutes and rinsed thoroughly before being placed on a sterile filter paper for germination for 5 days. The 1L pots were autoclaved twice and placed in semi-sealed autoclaved sun bags to avoid any contamination. Single clover seedlings were planted in 850 g of the sterile substrate in the pots. The bacterial cultures were grown and mixed as described above and starting cultures plated. 200 µL of the mixed cultures were applied to seedlings. The pots were watered with 5 mL of sterile water and 5 mL of nitrogen free Jensen's media (Howieson and Dilworth, 2016). Treatment pots were then sealed and placed in a controlled environment chamber (16/8 hour day/night cycle at 22ºC/20ºC). Low chamber humidity (40%) was used to counter the impact of growth in isolation bags. The plants were watered with 10 mL of sterile water and Jensen's media mix every week, with Jensen increasing from 1 ml in week 1 to 2 ml in week 2 and so forth. After five weeks, the plants were harvested under sterile conditions. The shoots of the plants were separated from the roots and kept in a dry oven for 48 hours at 60ºC before weighing the dry biomass. Rhizosphere bacteria were quantified by plating soil wash onto TY agar. 5 g of the rhizosphere substrate (directly around the roots) was combined with 10 mL rhizobial wash solution (1.2 g MgSO4 and 0.1 mL Tween/polysorbate 40 per L) and vortexed intermittently for 30 minutes. The supernatant was diluted and plated on TY agar plates containing 0.03 µl/ml gentamycin and 1 µl/mL cycloheximide. Nodules were isolated, counted and surface sterilised in 3% household bleach for 30 minutes and rinsed with sterile water.

Sterilised nodules were mashed in TY media and supernatant was diluted and plated on TY agar added with gentamicin and cycloheximide. Competitive fitness (v) of the treatments was calculated using formula 3.b (Ross-Gillespie *et al.*, 2009). This formula is used due to the challenges of estimating population density in a complex mesocosm (Bird *et al.*, 2023).

$$v = \frac{\mathrm{x2}\,(1-x1)}{\mathrm{x1}(1-x2)} \qquad (3.b)$$

In formula 3.b, v is the competitive fitness of the test strain, x2 is the final proportion of the test strain and x1 is the initial or starting proportion of the test strain. Log10 of v was used for further analysis. The proportion of phages integrated in the initially phage free bacteria were counted from eight colonies using maturase B and variable region primers in colony PCR (Table 3.1).

3.2.2 Fitness effect of vTRX32-1 on *Rlt* TRX19 in competition with diverse strains

3.2.2.1 Strain selection

Twelve strains of *Rlt* - SM137, SM67, WS53, SM41, TRX22, WS11, SM158, WS59, WS51, WS119, WS276 and WS5 were used in this experiment. Strains - SM137, SM41, SM158 and SM67 were isolated from clover plants in Denmark (Cavassim *et al.*, 2020). TRX22 was isolated in York (Kumar *et al.*, 2015) while WS53, WS11, WS59, WS51, WS119, WS276 and WS5 were also isolated in York from clover nodules in 2017. The strains were selected to capture a range susceptibility to phage infection. This susceptibility was based on 1) the reduction in bacterial growth after addition of phage vTRX32-1 and 2) number of phage plaques.

3.2.2.1.1 Reduction in bacterial growth

Each of the 12 strains and the control - TRX19 were grown in the presence or absence of phages in paired cultures, with four replicates. Bacterial strains were grown to mid-log phase (i.e. for 48 hours) in microcosms at 180 rpm at 28ºC and diluted to a 1:10 ratio. 135 µl of the diluted cultures were transferred to 96 plate wells and 15 µl of phages from 10^7 PFU/ml phage culture was added to phage-infected treatments. Control wells were mock infected with TY media. The cultures were grown at 28ºC for 72 hours in a Tecan Spark microplate reader and OD600 was measured every 119 mins. Reduction in bacterial growth (RBG) for each strain was calculated using formula 3.c. The RBGs for each strain is shown in Appendix A (Figure A.1).

$$RBG = 1 - \left(\frac{OD600\ phage_{\ tn} - OD600\ phage_{\ t0}}{OD600\ phage{-}free_{\ tn} - OD600\ phage{-}free_{\ t0}}\right) \quad 3.c$$

In formula 3.c, RBG is Reduction in Bacterial Growth, OD600 phage is the absorbance of phage added bacterial cultures at 600nm, OD600 phage-free is the absorbance of phage-free bacterial cultures, t_0 is starting time point or time at 0 hours while t_n is time at n hour.

3.2.2.1.2 Efficiency of plating

Bacterial cultures for each 12 strains and control i.e. TRX19 were grown as previously described. 10 μL of different phage dilutions from 10^7 phage cultures were spotted on the plates with 4 replicates per strain. Plates were kept in the incubator at 28ºC and plaques counted after 24, 48 and 72 hours. The efficiency of plating was expressed by the density of plaque forming units (PFU) visible on the test strains compared to the TRX19 control.

The selected strains were put in three categories - strongly susceptible, weakly susceptible and resistant - based on reduction in bacterial growth at 60 hours and number of plaque forming units as shown in Table 3.2.

3.2.2.2 Competition experiment in vivo

Phage containing *Rlt* TRX19vTRX32-1 (P) and phage free control *Rlt* TRX19 (WT) strains were competed as previously but this time the competitor strain was one of the 12 competitor strains or TRX19 as a control. Competition treatments were prepared in three replicate blocks. Each strain had the phage-free and phage containing competition treatments in each block. All test (WT) or lysogen (P) strains were labelled with MC and all reference strains were labelled with GFP. Competition experiments were set-up in clover pots and placed in the Controlled Environment Facility as described above. However, the plants were inoculated only after a week following planting. This was done to ensure that the plants developed root hairs by the time of inoculation, allowing nodulation (Nazih and Weaver, 1994). This was to avoid extended period of competition in the substrate prior to nodulation. The plants were harvested after five weeks of inoculation.

Table 3. 2 : Categories based on susceptibility and number of plaques produced by the selected strains.

Category	**Strains**	**Reduction in Bacterial Growth at 60 hours**	**Number of plaques (PFU/ml)**
Strongly susceptible to lysogeny	SM137	0.29	2.8×10^8
Strongly susceptible to lysogeny	SM41	0.21	2.9×10^8
Strongly susceptible to lysogeny	SM158	0.23	9×10^8
Strongly susceptible to lysogeny	TRX22	0.3	1.4×10^8
Weakly susceptible to lysogeny	WS53	0.5	9×10^8
Weakly susceptible to lysogeny	WS11	0.54	3.9×10^5
Weakly susceptible to lysogeny	WS59	0.46	1×10^9
Weakly susceptible to lysogeny	SM67	0.4	3.3×10^7
Resistant to phage infection	WS51	0.69	None
Resistant to phage infection	WS119	0.68	None
Resistant to phage infection	SM276	0.2	None
Resistant to phage infection	WS5	0.66	None

3.2.3 Statistical Analyses

All statistical analyses was performed in R. The competitive fitness data was analysed using mixed model, lme() and groups were further compared using lsmeans(). Proportion of phages were analysed using linear model, lm(). Linear model was also used to analyse nodule number, nodule weight and plant weight data. lsmeans() was used for further comparisons.

3.3 Results

3.3.1 Fitness effect of vTRX32-1 on *Rlt* TRX19 in isogenic competitions

3.3.1.1 Competition experiment in vitro

To compare the fitness effects of lysogeny, competition experiments were run in well-mixed environments in the lab. There was a significant difference in the fitness of the phage-carriage (Figure: 3.1, Treatment: DF = 1, STAT = 12.02, p = 0.0005). The extent of this fitness effect was also significantly affected by the propensity of the competitor strain to acquire the prophage. Time was not a significant factor (Figure: 3.1, Time: DF = 2, STAT = 0.99, p = 0.60) and there was no significant interaction between time and treatments in the model (Figure: 3.1, Phage x Treatment x Time: DF = 2, STAT = 1.63, p = 0.44). However, there is a clear increase in fitness benefit of the phage-containing strain overtime in competition with the lysogenization resistant (LR) competitor, rising from a 28% fitness benefit (Figure: 3.1, w = 1.32±0.26 for phage containing vs 1.04±0.02 for phage-free, t = -3.61, p = 0.003) to 72% at 72 hours (Figure 3.1: w = 1.72±0.11 vs 1.00±0.01, t = -5.32, p < .0001). In contrast, when competing against the wild type while the scale of the fitness impact of lysogeny was similar at 24 hours (Figure 3.1: 1.31±0.11 for phage containing vs 1.06±0.01 for phage-free) this was not significantly different in a direct comparison (p > 0.05) and if anything, declined by later time points.

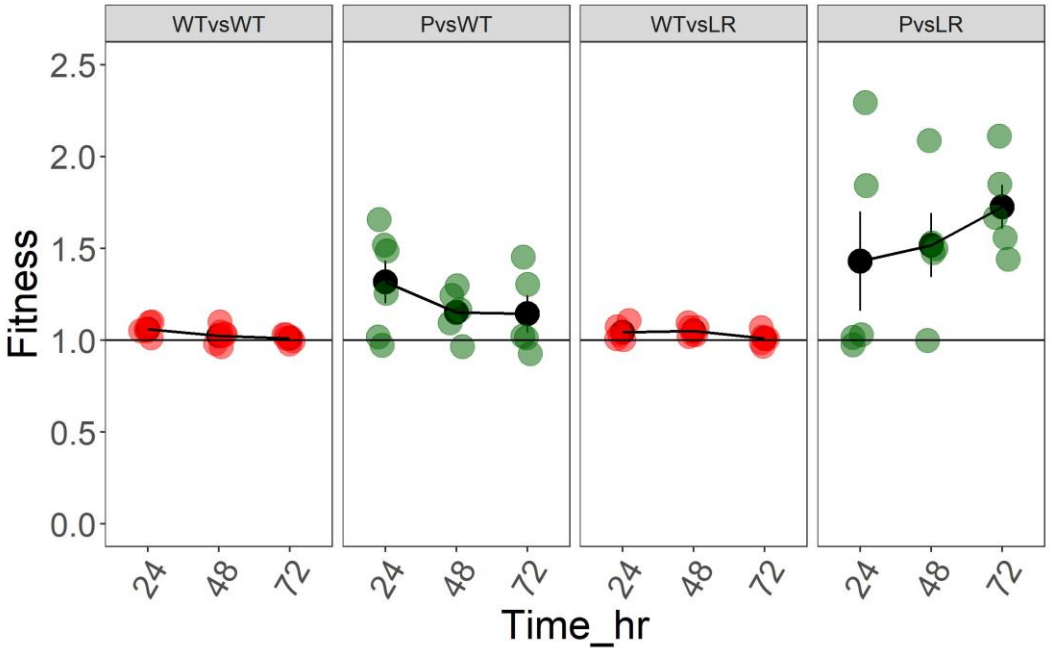


Figure 3. 1 : The competitive fitness effect of phage carriage in the presence of a wild type (WT) or lysogeny resistant (LR) competitor.

Phage free, wild type (WT - red) or phage containing (P - green) strains were tested in the presence of either the isogenic wild type strain (WT) or a lysogeny resistant competitor (LR) over 24, 48 and 72 hours. Coloured points represent different replicates and the black points represent the mean fitness of the treatments. The vertical bars show the standard errors of the mean fitness.

3.3.1.2 Proportion of phages in initially phage free competitors

The proportion of phages in initially phage-free competitor populations competed against the lysogenic strain i.e. WT from PvsWT treatment and LR from PvsLR treatment were compared using a mixed model. There was no significant difference in the proportion when modelled as an interaction between treatment and time (Figure: 3.2, Treatment x Time: DF =1, STAT = 4.12, p = 0.12). As expected, there was a significant effect in the treatments (Figure: 3.2, DF = 1, STAT =19.76, p = 8.75e-06), with the wild type strain gaining the phage at high rates by the end of the experiment. However, the lysogenization resistant strain also acquired the phage, but at lower rates. Interestingly, the proportion of lysogens changed overtime (Figure 3.2: DF = 2, STAT = 9.37, p = 0.009). There was no significant interaction between treatment and time (Figure: 3.2, Treatment x Time - DF = 2, STAT = 4.12, p = 0.12). However, when comparing between time points for each treatment, the proportion of lysogens in the WT population did not significantly diverge from the mean proportion of 0.88±0.08 at the three time points (Figure 3.2). In LR, however, there was a significant difference in the proportion of phages at 24 hours (mean proportion of lysogens - 0.66±0.22) and at 48 hours (mean proportion of lysogens -

0.03±0.03, t = 3.52, p = 0.007). In the pairwise comparison of WT and LR across different time points, there was no significant difference between WT and LR populations at 24 hours, but this was significant at 48 hours (Figure: 3.2, t = 4.11, p = 0.0007), where rates of lysogeny dropped to 0.03±0.03 in the LR population, and at 72 hours (t = 2.34, p = 0.03), where they recovered to 0.36±0.12.

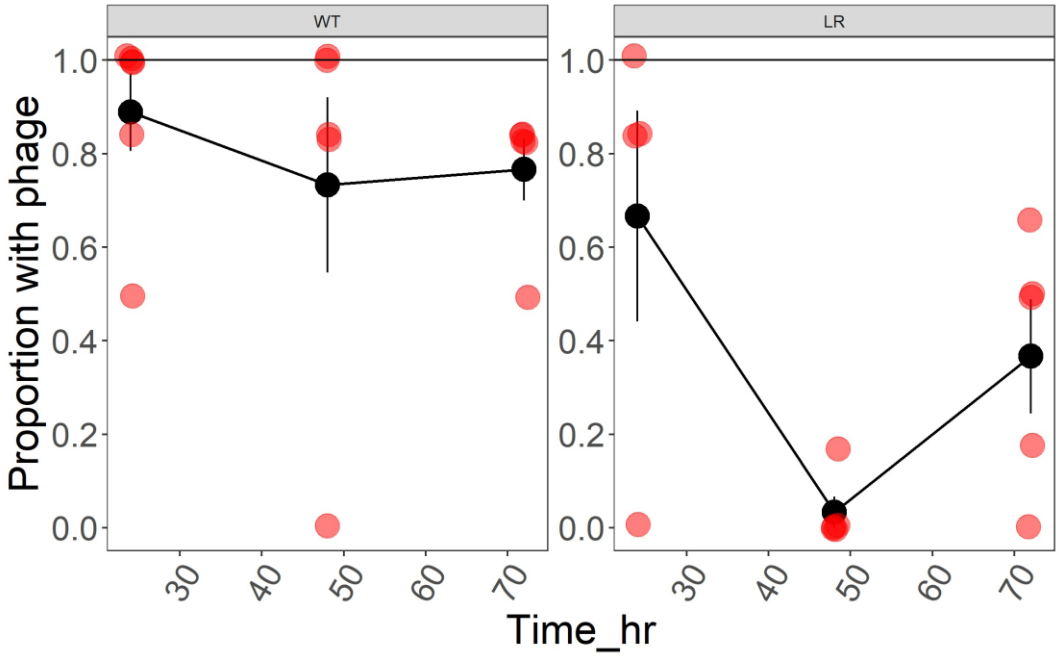
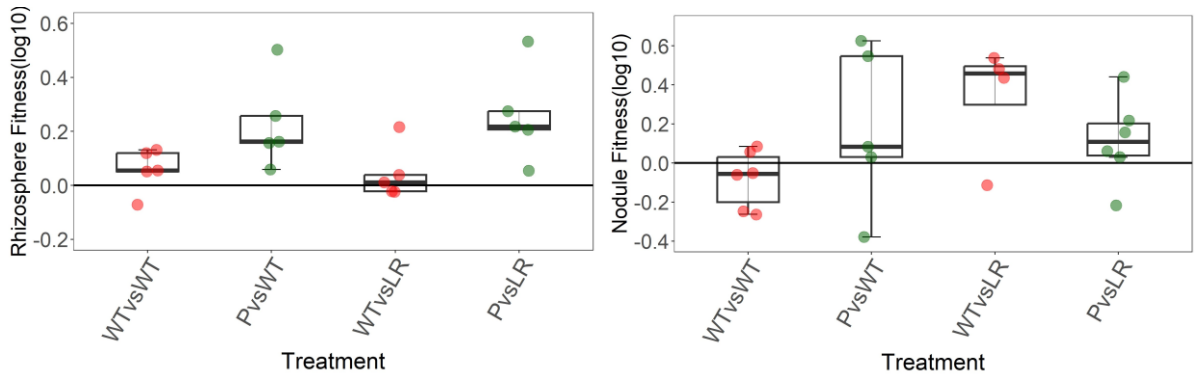


Figure 3. 2 : Proportion of phages picked from 6 colonies of each initially phage free wild type (WT) and lysogeny resistant (LR) colonies in PvsWT and PvsLR competitions.
WT is Wild type, P is Phage containing and LR is Lysogeny Resistant. The red points represent different replicates and the black points represent the mean fitness of the treatments. The vertical bars show the standard errors of the mean fitness.

3.3.1.3 Competition experiment in vivo

The fitness effects of lysogeny were then compared in both the rhizosphere and nodules of the symbiotic hosts, clover. In the rhizosphere, there was a significant difference due to phage presence (Figure: 3.3 (a), Phage: DF = 1, STAT = 9.76, p = 0.001). However, there was no difference in the treatments (Figure: 3.3 (a), Treatment: DF = 1, STAT = 0.017, p = 0.89) and interactions between phage carriage and treatments (Figure: 3.3 (a), Phage x Treatment: DF = 1, STAT = 0.12, p = 0.72). Pairwise comparisons showed a significant effect of phages in the LR strain which was a 21% fitness increase as compared to the phage free counterparts (Figure: 3.3 (a), w = 0.04±0.04 in phage free vs 0.25±0.07 in phage carrying, LR: t = -2.45, p = 0.04).

However, no significant effects were observed in the WT strain (Figure: 3.3 (a), WT: t = -1.9, p = 0.09).



(a) (b)

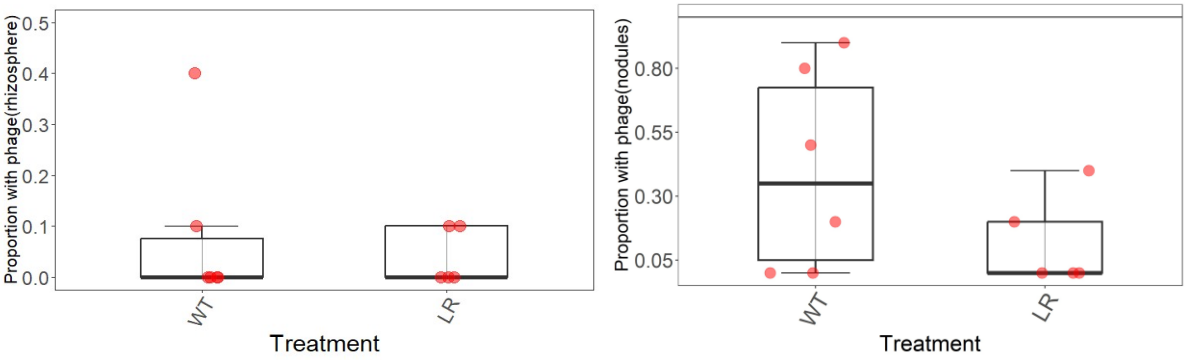
Figure 3. 3 : Competitive fitness effect of phage carriage in the presence of wild type (WT) and lysogeny resistant (LR) competitor in clover a) rhizosphere b) nodules.
Phage free, wild type (WT - red) or phage containing (P - green) strains were tested in the presence of either the isogenic wild type strain (WT) or a lysogeny resistant competitor (LR). Coloured points represent different replicates.

In the nodules, however, there was no significant difference in the treatments or phage presence (Figure: 3.3 (b), Phage: DF = 1, STAT = 0.0015, p = 0.96; Treatment: DF = 1, STAT = 3.18, p = 0.07). However, their interaction was significant (Figure: 3.3 (b), Phage x Treatment: DF = 1, STAT = 0.04, p = 4.17). In pairwise comparisons, no significant difference was found in WTvsWT (mean fitness = -0.07±0.06) and PvsWT (mean fitness = 1.18±0.18) (Figure: 3.3 (b), t = 1.36, p = 0.2). Similarly, in LR too, there was no significant difference in WTvsLR (mean fitness = 0.33±0.15) and PvsLR (mean fitness = 0.11±0.08) (Figure: 3.3 (b), t = -1.45, p = 0.18).

3.3.1.4 Proportion of phages in plant rhizosphere and nodules

The proportion of phages were tested in phage free WT and LR populations. In the rhizosphere, the rates of lysogeny were low with no significant difference between the phage free WT (mean proportion of lysogens = 0.08±0.06) and LR (mean proportion of lysogens = 0.03 ±0.02 ;) treatments (Figure: 3.4 (a), DF = 1, STAT = 0.32, p = 0.56).

In the nodules, however, the proportion of phages in the initially phage free WT was significantly greater than the LR populations (Figure: 3.4 (b), DF = 1, STAT = 3.78, p = 0.05). The mean proportion of phages in WT were 0.4±0.16 and in LR, 0.1±0.06. There were thus, 30% more phages in WT than LR strains.



(a) (b)

Figure 3. 4 : Proportion of phages picked from 10 colonies of each initially phage free WT and LR colonies in PvsWT and PvsLR competitions from a) rhizosphere b)nodules.
WT is Wild type, P is Phage-containing and LR is lysogeny resistant. The red points represent different replicates.

Nodule number (Appendix A, Figure A.2: DF = 1, STAT = 1.91, p = 0.16), nodule weight (Appendix A, Figure A.3, DF = 1, STAT = 0.47, p = 0.49) as well as plant dry weight (Appendix A, Figure A.4: DF = 1, STAT = 1.28, p = 0.25) were also not significantly affected by treatment.

3.3.2 Fitness effect of vTRX32-1 on *Rlt* TRX19 in competition with diverse strains

3.3.2.1 Competitive fitness in vivo

The fitness of test strains – WT (TRX19) and P (TRX19v32) was measured in competition against 12 non-isogenic strains – SM137, SM41, SM67, WS53, TRX22, WS11, WS59, SM158, WS119, WS276, WS5 AND WS51 – isolated from the same or similar communities, which represent a range of natural resistance profiles to the phage. 6 strains – SM137, SM67, TRX22, SM158, WS5 and WS51 were removed from the analysis due to low colony count. A mixed model was used to compare results from the remaining strains - SM41, WS53, WS11, WS59, WS119 and WS276 including the isogenic control - TRX19. The test strains showed different fitness effects against different competitors (Figure: 3.5, Treatment: DF = 6, STAT = 15.44 , p = 0.01) but there was no overall effect of phage (Figure: 3.5, Phage: DF = 1, STAT = 0.98, p = 0.32) or treatment and phage interactions (Figure: 3.5 , Phage x Treatment: DF = 6, STAT = 3.16, p = 0.78). No significant difference was observed in pairwise comparisons between phage-free and phage-containing treatments. In fact, for the 4 most susceptible strains, the mean fitness of the TRX19 lysogen was, if anything, lower than the fitness of its non-

lysogenic counterpart while in competitions against strains shown to be resistant to phage infection, the fitness of the lysogen was (non-significantly) higher (Figure: 3.5).

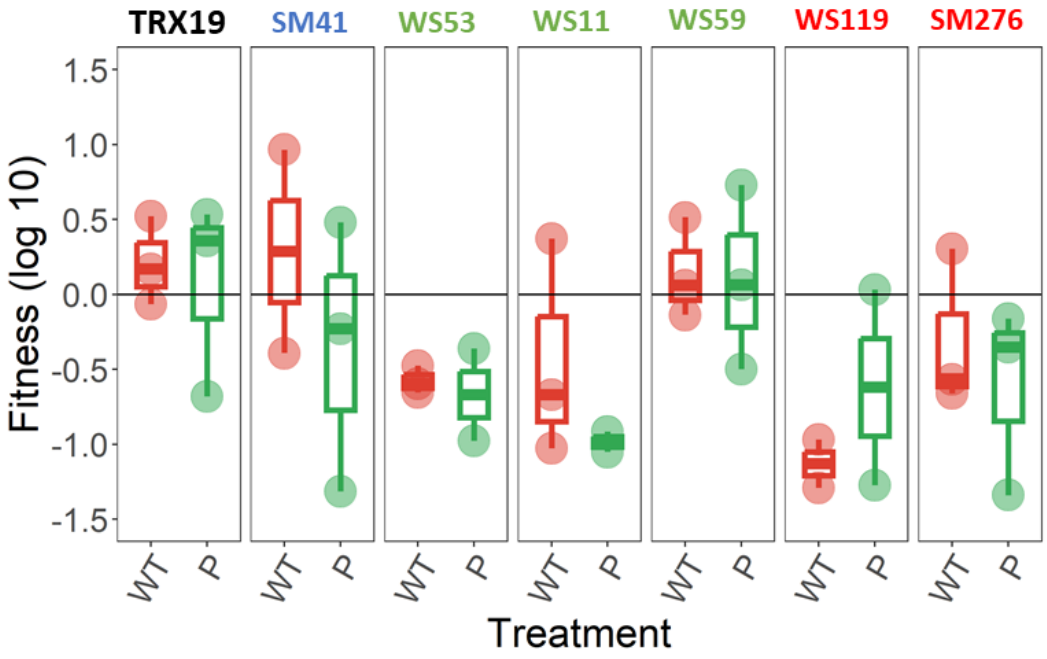


Figure 3. 5 : Competitive fitness of WT (Wild Type) and P (Phage-containing) against diverse non-isogens.
Control: TRX19, Susceptible and strongly lysogenic: SM41, Susceptible and weakly lysogenic: WS53, WS11, WS59, Resistant: WS119, WS276. Phage-free treatments are shown with red points and boxplots while phage treatments are shown with green points and boxplots.

3.3.2.2 Proportion of phages in nodules

12 colonies of phage free competitors from each treatment were tested for presence of phage using PCR primers (Table 3.1: maturase B and variable region). There was a significant difference in the mean proportion of lysogens between treatments (Figure: 3.6, DF = 6, STAT = 68.66, p = 7.69e-13). 4 strains had much higher rates of lysogeny at the end of the experiment than the control: TRX19 (Mean proportion of lysogens - 0.05±0.05): SM41 (mean proportion of lysogens - 0.69±0.16, t = 4.07, p = 0.002), WS53 (mean proportion of lysogens - 0.62±0.37, t = 3.19, p = 0.009), WS11 (mean proportion of lysogens - 1±0, t = 5.06, p = 0.0005) and WS59 (mean proportion of lysogens - 0.69±0.15, t = 4.07, p = 0.002). These strains were all susceptible to infection by the phage. In contrast, the 2 resistant strains showed no lysogens as might be expected.

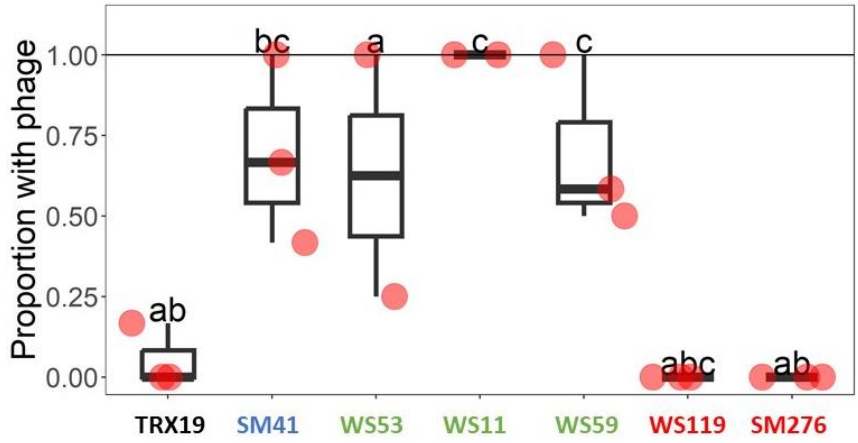


Figure 3. 6 : Proportion of phages picked from 12 colonies of each initially phage free diverse population treatments.
Control: TRX19, Susceptible and strongly lysogenic: SM41 and WS53, Susceptible and weakly lysogenic: WS11 and WS59, Resistant: WS119 and WS276 competitions from nodules. The red points represent different replicates.

The plant dry weight (Appendix A, Figure A.5, DF = 6, STAT = 10.10, p = 0.12), nodule number (Appendix A, Figure A.6, DF = 6, STAT = 0.59, p = 0.5) as well as nodule fresh weight (Appendix A, Figure A.7, DF = 6, STAT = 0.56, p = 0.5) were also not significantly different in the non-isogenic competitions.

3.4 Discussion

Improving competitive fitness is very important for the success of rhizobial inoculants (Batista *et al.*, 2015). Previous studies with bacteriocins show that they can increase the competitive fitness of their hosts. To investigate whether temperate phages could show similar results, I conducted competition experiments *in vitro* and *in vivo* (i.e. clover environment). I found that phages do increase the competitive benefits of their hosts *in vitro* and in the rhizosphere but not in the nodules. These results show that the phage-mediated benefits can be strongly dependent on the competitor population and the environment.

In well mixed *in vitro* environments, phages conferred an advantage to their hosts, but this advantage did not increase - and even declined over time - in competition with the isogenic competitor. Screening for lysogens in the initially phage free susceptible populations showed that phages integrated at a high rate by 24 hours, suggesting that rapid phage transfer might have negated any initial benefit of carrying the phage. In the lysogeny resistant (LR) strain, the phage gave a large fitness advantage at all the time points, increasing over time to 72%. This

pattern follows my predictions that the fitness effect of phages will be maintained at all the time points, as the LR strain is resistant to lysogeny but susceptible to lysis. However, even though the LR strain is resistant to lysogeny - as they lack the preferred phage integration site - a very high rate of phage carriage was observed in the LR population at 24 hours, which then declined. This maybe because the phage forms less stable lysogens in the absence of the attB site, resulting in higher rates of spontaneous induction or it integrates at an off-target site or a combination of both may lead to high fitness cost.

In the plant experiment with isogenic strains, significant fitness effects of phage carriage were observed in the rhizosphere - although the benefit was significantly less than that observed *in vitro*. The fitness in the rhizosphere was observed only in the LR population, where phage carriage gave a benefit of 21% over the phage-free competitors. This result suggests that because the rhizosphere is a structured environment, phage carriage might be giving a localized benefit to the lysogens (Brown *et al.*, 2006). This is because in a structured environment, free phages are more concentrated where their producers are present, killing the susceptible populations around (Brown *et al.*, 2006). The results observed in the rhizosphere are similar to those observed in *Rlt* strain carrying bacteriocins and temperate phages on peat (Schwinghamer and Brockwell, 1978; Triplett and Barta, 1987). Moreover, even though the phage-free competitors in both groups were not significantly different in their susceptibilities to phage integration in the rhizosphere, I observed the fitness effects of phage carriage only in the susceptible LR strain, which was expected. I expected the same fitness effect in the nodules, but even though nodules are also structured environments, I did not observe any fitness benefits in them. Similar results have been observed in *Bradyrhizobium japonicum* (Gross and Vidaver, 1978) where bacteriocin producing *Bradyrhizobium japonicum* had an inhibitory effect on sensitive strains *in vitro* but in the nodules, this effect did not give it a competitive advantage. This might be because the nodule represents a much more complicated structured environment than the rhizosphere where an unknown mechanism ensures that both phage-free and phage carrying rhizobia are selected for nodulating the plants. Yet, as observed in the *in vitro* experiment, both the competitor strains gained the phage over the course of the experiment in plants. Interestingly, this was only significantly lower than the WT strain in the nodule populations, where lysogenization rates in the WT population were much higher than observed in the rhizosphere.

In the non-isogenic competition experiments, interestingly, there were no differences in the fitness effects of the phage when competing against highly susceptible strains - which rapidly became lysogenised - and resistant strains - which remained uninfected. Moreover, phages were present in all the phage-free strains, except the resistant strains. The phage proportion varied widely in the other strains, with ~3% of initially phage-free TRX19, ~65% initially phage-free and strongly susceptible to lysogeny and ~65%-100% of initially phage-free and weakly susceptible to lysogeny. However, the non-isogenic results should be cautiously used to draw conclusions as we used only three replicates and in some cases, only two replicates in this experiment.

These results suggest that phages may not be the most reliable in efforts to improve inoculant competitive fitness. The high rates of integration appearing early on in the *in vitro* experiment shows the speed at which the phage can infect and transfer to new hosts. Similarly, the high lysogeny rate in nodule isolates, of either the isogenic strain or susceptible competitors shows that while its initial bacterial host may not gain an advantage in infecting the plant, the phage is highly efficient at infecting the plant, via its bacterial symbionts. Temperate phages might thus be useful as efficient genetic payloads to deliver beneficial genes to rhizobia that could enhance their performance as inoculants. Moreover, *in situ* microbiome engineering using Mobile Genetic Elements (MGEs) is increasingly used in agriculture to enhance desirable traits in different bacteria (Sheth *et al.*, 2016; Hu, He and Singh, 2017). Using natural temperate phages as *in situ* natural engineers for inoculant efficacy might thus be a potential path for future inoculant improvement when combined with essential participatory approaches (discussed in chapter 4).

Chapter 4 - Soil microbiome and soil inoculant perceptions in the UK farmer and urban grower community

4.1 Introduction

4.1.1 Background

Legumes are an important crop worldwide, as a source of protein for human diet as well as cover crops to maintain soil nitrogen. Rhizobia fix nitrogen in legumes and can thus be used as relatively low cost, sustainable 'bio-fertilizers'. But uptake by farmers and growers is low (Denton *et al.*, 2003; Bell *et al.*, 2019), majorly due to inconsistency in the effectiveness of rhizobial inoculants (Omotayo and Chukwuka, 2009; Bashan *et al.*, 2014; Mukhongo *et al.*, 2016). Thus, efforts of both industry and research have focused on improving the efficacy of rhizobial inoculants. However, there remains a huge gap in research on understanding the perspectives of the end users on inoculant use and uptake (Catroux, Hartmann and Revellin, 2001; Kaminsky *et al.*, 2019). Understanding how growers relate to their soil and its microbiome already, as well as their perspectives on microbial products, is needed. Vitally, such research must be done early as lack of early engagement with the users or public in technology design is one of the main reasons for the low social robustness of technologies.

Many studies show that engaging with end users of technologies early on can create feelings of trust and transparency as well as provide deeper insights on themes that experts sometimes perceive as insignificant (Tsouvalis and Waterton, 2012). An example of this is a study done in Austria to create a dialogue between genome researchers working on lipid metabolism associated disorders and citizens. The dialogues led to the emergence of new themes. Topics such as 1) the practice of using mice in research and 2) the future effects of the knowledge produced in society, that are usually considered less significant by experts (Felt and Fochler, 2008; Tsouvalis and Waterton, 2012). The engagement of end-users can also help in sustaining new technologies, thus, fostering social paradigm transitions and collective envisioning of the future. The co-production of content by the public and researchers upstream of public participatory projects such as the Solar Energy in Future Societies (SEFS) project in Stockbridge, England, is an example of collectively imagining the futures of technology (Krzywoszynska *et al.*, 2018). The SEFS project enabled the public participants to address their concerns such as economic and technical feasibility on different themes around renewable energy. In addition, there were many spill over effects such as social learning i.e. social

behaviours learned by observing and imitating the behaviour of others (Jensen, 2018), which helped experts to realize the motivations and desires that have the potential to make new technologies around local energy systems socially robust.

An understanding of end-user perceptions is an important pre-requisite for technology transfer as well as the implementation of practices and policies such as for sustainable agriculture (Liebig and Doran, 1999; Petrescu-Mag, Petrescu and Azadi, 2020). In soil research, there are only a few studies, which focus on understanding the perceptions of end users to improve soil-based technologies such as soil inoculants. Most of these studies aim to explore the socioeconomic or technological factors that influence the adoption of soil technologies (Doss, 2006; Launio, Luis and Angeles, 2018). While others do focus on including farmer perceptions (Liebig and Doran, 1999; Barbero-Sierra *et al.*, 2016; Roesch-Mcnally *et al.*, 2018; Clay *et al.*, 2020; Veisi *et al.*, 2022), it is either to understand differences in expert and farmer perceptions of the topic at hand or to further explore pre-set themes which use empirical evidence collected by combining models and surveys or interviews (Liebig and Doran, 1999; Thompson *et al.*, 2019). Fewer aim to purely understand the perceptions of farmers on topics such as soil fertility and soil management (Grossman, 2003; Dawoe *et al.*, 2012; Clay *et al.*, 2020). For instance, a study on the perceptions of farmers on soil quality found that farmer perceptions were important in designing and picking indicators that would be most helpful in on-farm soil quality evaluation (Liebig and Doran, 1999). Consequently, indicators that farmers use to assess soil quality were found to be accurate and on par with the expert-led indicators.

There is a lack of studies to understand farmers' and growers' perceptions of soil inoculants and the soil microbiome, specifically. Public participation is used in this study to fill the gap by reaching two main objectives - 1) Understand the context of soil inoculant technology by studying soil microbiome perceptions, and practices around the soil microbiome in the grower community and 2) Explore the existing interactions of the grower community with inoculant technologies.

4.1.2 Public participation to improve technology uptake

‘Participation’ broadly means anything which represents or involves people or different stakeholders (Cornwall, 2008). Public participation is a means to describe and integrate non-scientific forms of knowledge with scientific approaches (Miller and Wyborn, 2020). It can be used to ensure accountability and empower society (Fiorino, 1990; Holland, Jones and Kardan, 2015) through incorporation of the views and concerns of future users of technological and

scientific products (Tsouvalis and Waterton, 2012). This also helps to avoid the controversies or opposition from the public by involving them early on in the research. While these controversies can be seen as a way forward to correction and improvement in research as well as policies, they often emerge after the perceptions of the public have been shaped which leaves no room for collaboration between the experts and the public (Wilsdon and Willis, 2004). This increases transparency, robustness and success of the technology. It also helps to transform the goals or objectives of the research that are important for innovation in technology along with imagining the futures of these technologies collectively.

Participation gives rise to research that is interdisciplinary (collaboration is between researchers from different disciplines) and transdisciplinary (collaborators are researchers and stakeholders or members of the public) (Wickson, Carew and Russell, 2006; Holmes *et al.*, 2018). Transdisciplinary science thus blurs the lines between science and society as it helps to provide solutions and scientific knowledge transfer from bench to the real-life world and vice-versa. Moreover, participation has a different meaning or purpose in different projects or institutions such as to get a material incentive, empower or self-mobilise (Cornwall, 2008; Pallett and Chilvers, 2013; Holland, Jones and Kardan, 2015).

Fiorino classifies the rationales for public participation as substantive, normative and instrumental (Fiorino, 1990; Wesselink and Paavola, 2011). According to the normative rationale, the public participation process is a democratic right of citizens to be involved in taking decisions in the projects which are aimed at their interest (Fiorino, 1990). In the normative rationale, Fiorino stresses that the lay public can bring in a new perspective and in this process strengthen democratic rights. In the instrumental rationale, the participation of the public is important as it leads to better results and increased accountability of the project and decisions (Fiorino, 1990). The substantive argument states that the lay public will illuminate with their experience and understanding that which the experts may miss, leading to a wider understanding of the problem or project at hand (Fiorino, 1990). My research objectives fall primarily within the substantive rationale.

4.1.3 Using Participation in current study

The participation process in this study involves interviewing farmers and urban growers to understand inoculant use in the grower community. This involves exploring perceptions and practices around the soil microbiome to better understand the current trend of rhizobial and soil inoculant use in the farmer and urban food growing community, first hand. In addition, the aim

is to gain insights into practices that can be used for successful application of soil inoculant technology. This research is important to understand questions such as: Why are soil inoculants used by farmers and growers? Are there hidden barriers present to inoculant uptake? And if so, why?

Understanding current perceptions of and practices around the soil microbiome will be a foundation for further research into current trends in inoculant use and for developing practices, which can be used for successful application of soil inoculant technology. Beyond this project, participation may also help in the formation of public perception simultaneously with the conception and development of ideas around soil inoculants and soil microbiome technologies.

4.2 Methodology

4.2.1 Purposive sampling

Purposive sampling - selecting participants on the basis of their knowledge (Tongco, 2007) - was used to select and recruit participants. Legume growing farmers and urban growers were sought out as participants to gain information on soil microbiome, soil inoculants, and the practices surrounding the soil microbiome.

4.2.1.1 Pilot study

Legume growers were purposively selected in this project as most legume growers were assumed to interact much more with the soil microbiome as compared to non-legume growers. This is because they are aware of the presence of nitrogen-fixing rhizobia in legumes. In addition, legumes growers are the potential end users of rhizobial inoculant technology and thus, engaging them in the project for soil inoculant development is of utmost importance.

A pilot study was done before recruiting the participants and commencing the main interviews. Five participants were interviewed to test, further elaborate and identify any shortcomings of the interview schedule; and to fine tune the research questions and the interview schedule.

The legume growers were sought out using a survey that was sent to 25 soil and agriculture based organisations. These organisations were found using professional contacts known to my supervisors, friends and family; and at outreach events such as while presenting my research to the local library. I also used search terms on google to find participants. The terms used were

"Allotment organisations in England, Scotland, Wales and Northern Ireland", "Farming forums in UK", "Soil organisations in UK" and "Farming organisations in UK". I met some participants at the Real Oxford Farming Conference held in 2020 in Oxford. Many new participants were also recommended by existing participants leading to visibly concentrated locations of participants in Figure 4.1. This was not a concern in this study as the study is focused on individual narratives around the soil microbiome and soil inoculants rather than the representative ones.

4.2.1.2 Survey

A survey was designed and sent using the snowball sampling technique to various farmer, urban grower and allotment associations; unions and organizations as a first step to recruit participants. To ensure diversity of the sample size, 28 participants were recruited from England, Scotland, and Wales (Figure 4.1). 13 were urban growers and allotment holders, 14 were farmers and 1 was a farmer advisor. 53% participants identified as males and 46% as females. The age range of participants was 39 - 74. All recorded activities were conducted with the approval of the department ethics committee.

4.2.2 Semi-structured interviews

Semi-structured interviews are usually defined as open interviews, which focus on the perspectives or experiences and knowledge of the interviewees. The interviewer has an agenda to follow with questions on topics of interest. However, the interviewees perceptions and knowledge also influence the interview structure (Barbour, 2014). I used semi-structured interviews, which allowed flexibility to ask questions from pre-set topics but at the same time new sub-themes to emerge.

Figure 4. 1 : Location of the interviewees across the UK.

4.2.3 Interview schedule

The interview schedule was designed to explore perceptions and practices of participants on the topics of soil microbiome and soil inoculants. The schedule included questions aiming at exploring the following topics:

4.2.3.1 Perceptions and practices around soil microbiome

These questions sought to understand participant interactions with the soil microbiome and how these interactions affect participant decisions to use soil inoculants, providing the context before exploring inoculant use by participants. This helps us to understand why participants do what they do. For instance, the schedule explored 1) Do all participants care for the soil microbiome? 2) What actions are taken by participants around caring for the soil microbiome? 3) Is care a necessary form of relation for uptake of inoculants?

4.2.3.2 How growers assess microbiome changes

Investigating the assessment of the soil microbiome after the use of microbiome oriented practices or actions were important in the interview. Assessment shows that participants were curious to investigate and observe the effects of their practices.

4.2.3.3 Perceptions of soil inoculants

This section focussed on exploring existing perceptions of rhizobial and soil inoculants in the participants. Additionally, it aims to identify any challenges or barriers to using soil inoculants.

4.2.4 Thematic analysis using NVivo

Thematic analysis was used to analyse the data from the semi-structured interviews (Barbour, 2014; Castleberry and Nolen, 2018). The interviews were transcribed first hand by Otter (Otter.ai, 2021). Otter is an Artificial Intelligence speech to text converting application (Gaber, Pastor and Omer, 2020), which is commonly used for transcribing audio and video interviews in social sciences. NVivo (Lumivero (2020) *NVivo* (Version 13, 2020 R1), www.lumivero.com) was used to analyse the transcripts. NVivo is usually used to explore and find new sub-themes in the data according to pre-set themes. The data output from Otter was first categorised into pre-set themes (listed in the semi-structured schedule in Appendix C). This is also known as coding. Coding thus, is a process of arranging and re-arranging the previously transcribed data and assigning them to different themes (Wong, 2008; Silverman, 2013). Reassembling involves classifying the already coded data into appropriate themes (Silverman, 2013). Themes were pre-decided in the semi-structured interview schedule (after reviewing the literature review). Reassembling led to identification of sub-themes after manual interpretation. Some of these sub-themes are descriptions of the soil microbiome, uncertainty and scepticism around using soil inoculants, challenges and barriers to inoculant use; and knowledge needs around soil microbiomes. However, analysis and interpretation was not a single cycle but an iterative process where each cycle was repeated 4 times.

4.3 Results

4.3.1 Soil microbiome

Participants had different perspectives on the soil microbiome. These perspectives were identified and sub-themed into general perceptions, composition of the soil microbiome, appreciation of the soil microbiome, the gut microbiome narrative, uncertainty about the soil microbiome, caring for the soil microbiome, and gaps, future and knowledge needs (for detailed description see Supplementary 1, 3.1).

Some participants described the soil before describing the soil microbiome. The perspectives of participants are similar to soil classification in literature (Scoones, 2015) i.e. a natural resource that has a physical structure and chemical composition which can be measured as the amount of carbon, nitrogen or organic matter (Blum, 2005; Scoones, 2015). But soils also encompass a myriad of living things such as microorganisms, plants and small animals which make soils a living and thriving ecological system (Lines-Kelly, 2004; A. Krzywoszynska, 2019). Soils are seen as living systems by participants, a perspective that has only recently gained attention in the scientific community (Krzywoszynska, 2019; Delgado-Baquerizo *et al.*, 2021; Jing, Cong and Bezemer, 2022). Soil was seen by the participants as a complex system that is capable of sustaining itself without any external input (see quote by participant 6). In addition, soils were described beyond their ability to grow plants. They were an amalgam of hard labour and lived experiences of people who work intimately with soil and add valuable inputs to it (Engel-Di Mauro, 2014).

"There's a lot of bacteria and fungi, and all sorts of other things in the soil and…at the bottom of the chain, viruses…they all help to create soil because that's the difference between soil that you find in my field and sand that you find on the beach of the seaside. Because the sand on the beach is just nearly pure mineral, and nothing grows on that other, does it? And then it becomes a sand dune. And it gets colonized a little by things, and then it gets roots grown in it. And the roots, pump the root exudates with sugars out into the soil. And then little things, say bacteria initially are able to grow on these root exudates. And then when they get going a bit so the roots can grow some more…then there's something for the nematodes and protozoa…then all the other unmentionables…then it gets enough that the worms can start going through the soil and creating air spaces in it and then it all self-accelerates on it."
Participant 6

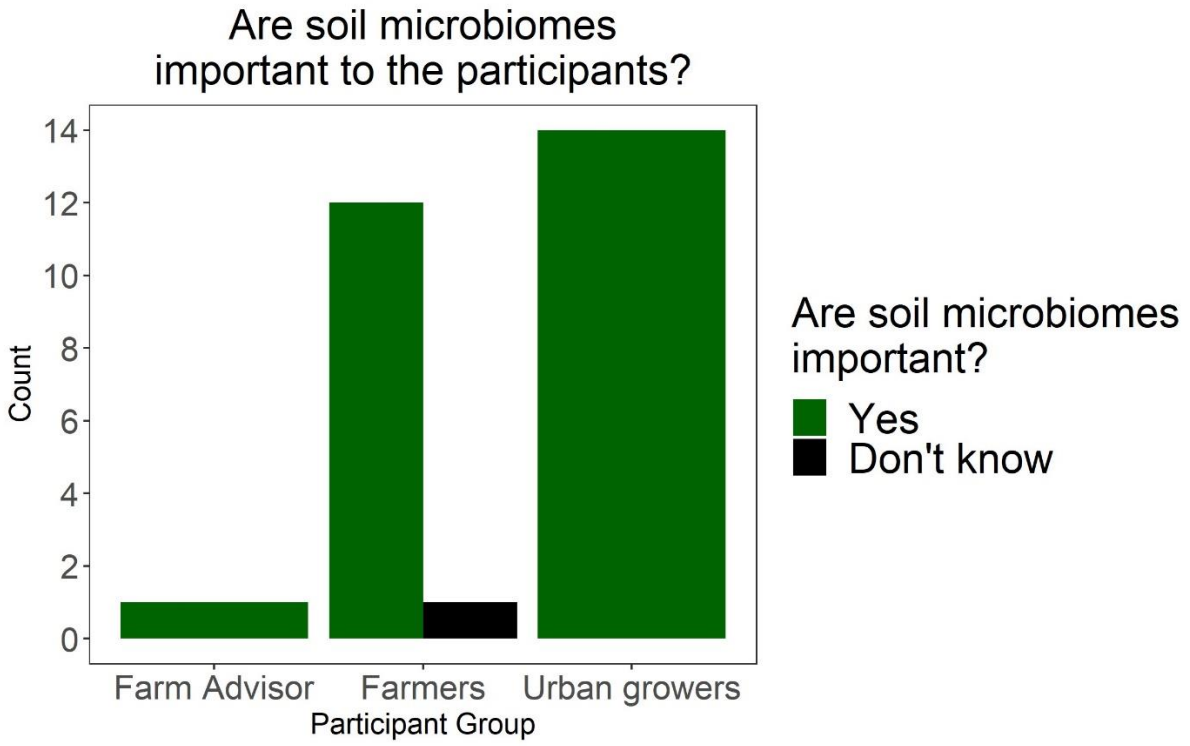


Figure 4. 2 : Are soil microbiomes important to the participants?
The bar graph shows if different participant groups consider soil microbiomes important in their farming/gardening. The farm advisor, 12 farmers and all the 14 urban growers consider the soil microbiomes as important for their farms/gardens/allotments while only 1 farmer said that they did not know if soil microbiomes were important for his farm.

The participants were able to speak about and reflect on the soil microbiome in a number of ways. This showed that soil microbiome is described and recognized by the many functions that it provides. This also shows the appreciation that participants have for the soil microbiome and the many processes that it carries out in the soil (Figure 4.2). Soil microbial interactions were described by participants as the foundation of soil that provide many benefits to plant growth and overall sustainability. The exchange of nutrients between microbes such as rhizobia, mycorrhizae and plants was mentioned. These symbiotic interactions are also recognized as pivotal for soil health, soil fertility and plant production by the scientific community (Denison and Kiers, 2011; Wangiyana and Farida, 2019). Interactions such as symbiosis have in fact been recognized since the beginning of agriculture (Fierer, 2017; Wang *et al.*, 2019). For instance - the benefits of rhizobial-legume symbiosis based crop rotation was mentioned in Chinese literature from at least 2000 years ago (206 B.C. to year 24 A.D.) (Zeng *et al.*, 2016; Wang *et al.*, 2019). Some participants also mentioned interactions such as competition and parasitism as important for the ecology and functioning of the soil microbiome. These interactions also contribute to increase or decrease in the populations of

their hosts leading to regulation of ecological processes. This keeps pathogens and diseases in check and as participant 16 states – "keeps the soil in the right balance". The right balance in the soil ecosystem was deemed important, but for the participants, current practices in agriculture meant that the right balance is lost. It was also mentioned that if we did not change our relationship with soils and the soil microbiome, it would be lost which will negatively affect agriculture. There was a general consensus that building a relationship with the soils by practices such as reducing inorganic fertiliser use as well as observing soils physically by touching or smelling them should be of utmost importance. This was seen as crucial for a personal connection or relationship with soils, which would steer off a more balanced soil ecosystem that supports better soil health and plant health.

"If you can get your biological life right, then I know that will mean a healthy soil…I guess it's important for helping us up resilience of the plants. I mean, I know it's perhaps slightly less important than vegetable growing, but things like mycorrhiza, how much that can extend the root system. And obviously, the things like the rhizobium in legumes as well, I suppose have the right balance…like the sort of antagonistic organisms against some of the soil pathogens as well." **Participant 16**

Some participants despite many problems on their fields were very optimistic about future of their soils and continued to work on the soil and the soil microbiome. This is only possible when growers see themselves as stewards of soil. The narratives on caring for the soil microbiome brought out that participants connect to the soil microbiome in their own ways. Some treat them as a living organism/pet residing in their own home while others see their importance as part of their economy, which also positively benefits their soils and crops. Caring for the soil microbiome for personal as well as environmental/sustainability reasons shows that stewardship was an important foundation for describing the soil microbiome. This narrative also points out that human-soil microbiome relationships can be altruistic and caring for the soil does not necessarily entail benefit by humans (Bellacasa, 2014).

Gaps and knowledge needs were identified by participants. A lack of standard tests for measuring the soil microbiome as well as a lack of expert help to maintain a healthy microbiome were mentioned as barriers to care for the soil. Participants pointed that apart from visual assessment, they cannot really tell of soil microbiome's functioning in the soil. There is also, a need to get help to maintain the soil microbiome and soil health. There is thus, an appetite for products, which actively sustain or support the soil microbiome and its functioning as well as a standard tool or test, which can also, measure this activity. Participant 3's narrative

shows that the term ‘soil microbiome’ may lead farmers to think that it is a scientific term that needs to be investigated by scientists more than farmers (Krzywoszynska, 2019). It is also evident that participants have a very wide range of perceptions around the term ‘soil microbiome’ as many participants did not differentiate between the soil microbiome, soil health and soil quality. Uncertainty was also seen around whether the soil microbiome was good or bad for the soil, whether it was a product that had to be applied to the soil or whether it could be cultivated using certain practices as stated by participant 3.

Interviewer: How important are the soil microbiomes to you personally in your farming?

“ I don’t know? Not directly? Because at the moment, I think that the microbiomes…can we add to them? How do we add them? Is it something by rotation. Is it something by different crops? I don't know. I mean, It’s still early days for me to learn. But obviously, you and scientists, need to look at the microbiomes and tell us what is right and what's wrong”.
Participant 3

There are thus, needs in the research community, which then need to be communicated to the farmer community. The onus of filling these knowledge gaps and needs was laid with scientists who are seen as the only knowledge bearers of the soil microbiome.

The above narratives on descriptions of the soil microbiome show that the perceptions about the soil microbiome vary widely between participants. This variation is due to educational background, experiences with soil as well as motivation. Regardless, all participants agreed that the soil microbiome is vital not just for crop health but for our own existence. Participants who thought that they did not know enough about the soil microbiome proved otherwise as through their anecdotes they did describe or sketch the soil microbiome according to their experiences and thoughts, which gave a peek into their perceptions. This shows that the growers are unable to connect their own experience with scientific understandings of the microbiome.

4.3.2 Practices around the soil microbiome

The participants followed practices to take care of the soil microbiome (Figure 4.3). There were many practices undertaken by participants aimed at changing and improving the soil microbiome. The practices were grouped into the following themes – minimum disturbance to soil, manures and composts, legumes and brassicas, using heritage varieties, alternatives to providing nutrients and new and innovative practice needs (for detailed description see Supplementary 1, 3.2). Table 4.1 shows the list of practices that were used or are considered

important by participants for soil and soil microbiome. Practices have been listed into different categories of themes identified by the interviewer.

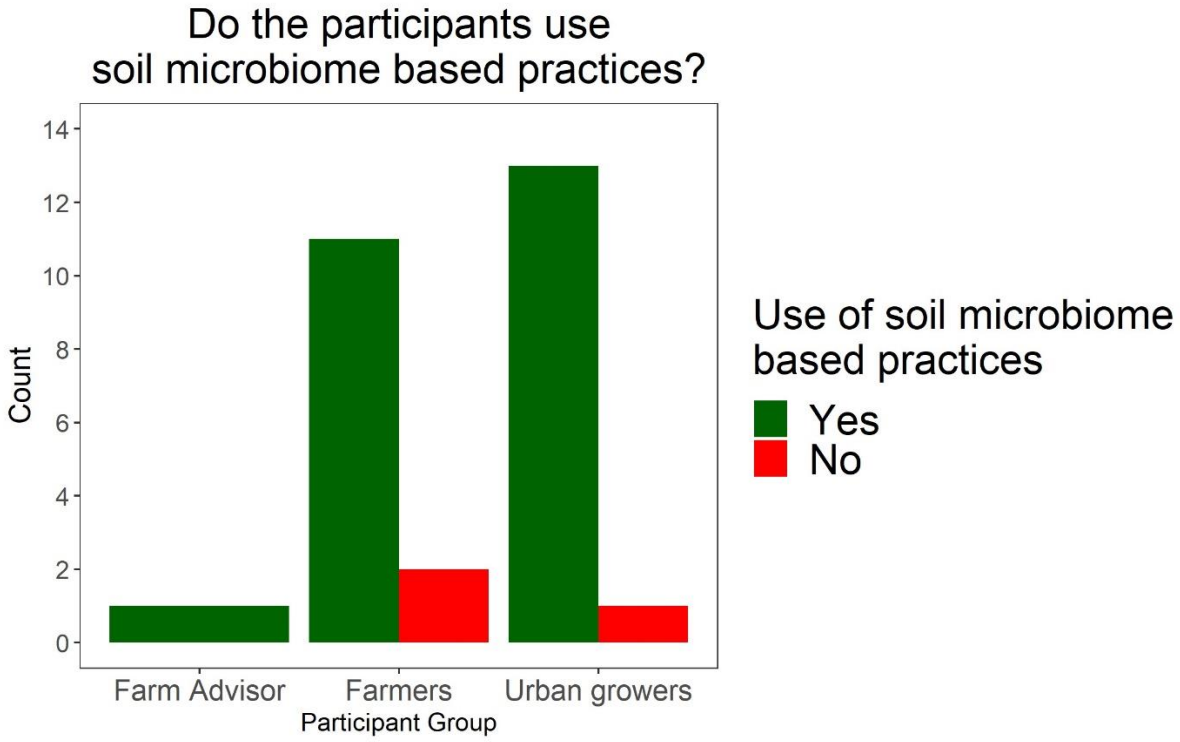


Figure 4. 3 : Do the participants use soil microbiome based practices?
The bar graph shows the use of soil microbiome based practices by different participant groups. The farm advisor, 11 farmers and 13 urban growers said that they follow practices to take care of the soil microbiome while 2 farmers and 1 urban grower claimed that they do not follow any soil microbiome based practices.

These themes show the breadth of actions taken by participants when caring for the soil microbiome or/and soil health. These practices also show the principles that participants follow, such as organic or regenerative when working with their soils.

A recent study in UK showed that farmers are generally aware of soil sustainability and use many sustainable practices to take care of their soils (Jaworski *et al.*, 2023). Yet, a lot is still unknown about the practices that farmers and growers undertake to take care of their soils. This section focused on discussing the practices that farmers use in detail to add information about practices around soil microbiome, soil health and soil sustainability to the existing literature. Farmers are motivated by their experiences, direct factors such as rising costs and indirect factors such as farmer identity and taking care of the environment to take on sustainable practices (Gosnell, Gill and Voyer, 2019). There was also a difference in practices according to farming/growing principles, primary motivation to grow plants - urban growers mostly grow

plants for their own use while commercial farmers grow crops for commercial food production and economic gains - as well as the plot size (Carlisle, 2016; Kirby *et al.*, 2021). Organic farmers mostly used practices such as using manures and composts to ensure organic produce while traditional farmers used a mix of practices such as composts, minimum and no-till, using tramlines to disturb the soil as minimally as possible. This was also to help prevent soil erosion. Urban growers use fewer cover crops as compared to conventional farmers due to limited land as well as differences in produce use. Urban growers did not follow a strict 6 or 7 year rotation pattern as opposed to commercial farmers who are much stricter in their rotation, which sometimes includes cover crops. Similarly, practices followed by urban growers were ensured to be on farm and local/regional and as sustainable as possible. Moreover, most urban growers are also, more likely to be early adopters while farmers are more likely to be mid or late adopters. This is because most new or innovative farming practices were tried by urban growers while farmers rarely wanted to try new practices, especially if there was no evidence of a benefit. This is understandable as urban growers can try new practices at small scale as they do not produce food commercially while farmers have to produce crops under constraints such as produce quality and quantity and thus, they do not want to risk trying new practices. In addition to the practices, participants also used indicators to measure the effects of their practices on the soil and the soil microbiome. Regardless, most practices were followed by both urban growers and farmers. Minimal disturbance of soil was followed by many participants, especially those who followed organic or regenerative practices such as minimum tilling and direct drilling. The main motivation being preservation of soil life to maintain the nutrients or fertility and water retention or infiltration capacity of soil. Second motivation to follow such practices is to increase carbon sequestration of the soil. These two motivations fall within the ecological sphere of traction to uptake sustainable practices as described by Gosnell, Gill and Voyer, 2019. There is also a general understanding within the participants that soil microbiome based practices do not yield results in the short-term but instead they are part of a long-term process which results in better soil fertility and plant production (Gosnell, Gill and Voyer, 2019; Beacham *et al.*, 2023).

Composts and manures are used in different parts of the world to enhance soil fertility (Grossman, 2003; Dawoe *et al.*, 2012; Petrescu-Mag, Petrescu and Azadi, 2020). Almost all participants used compost and manures. However, some also use chemical based fertilisers to provide nutrients to their plants (Figure 4.4). These composts vary in terms of production –

some are homemade while others are made commercially – usually by the local municipal councils in the UK. Homemade composts varied by the components used to make them. For

Table 4. 1 : Different practices used or considered important for the soil and/or the soil microbiome by the participants for the soil

Category of practices	**Practices**
New and innovative practices	Hügulculture[1], Johnson Su reactor[2], Bokashi[3], Moon-gardening[4], Kombucha[5]
Alternatives to fertilisers	Dried blood, Fish, Bones, Seaweed/Kelp, Lime, Volcanic rock, Sheep wool mat
Minimum disturbance to soil and life	No digging/tilling, Direct drilling, Minimum tilling, Keeping tram lines in same place, Biochar, Physical barriers such as net for pest management
Composts and manures	Mixed composts, Farmyard manure, Dairy slurry, Green manures, Mulching, Poultry manure, Mushroom and fungi compost, Earthworm compost, Comfrey/nettle tea, Wood chip compost, Sugarbeet factory waste, Bio-solids from sewage works
Cropping practices	Companion gardening, Rotation of crops, Leaving root residue in the soil, Strip drill, Indigenous/heritage varieties of grass, Legumes as cover crops, Crop diversity, Crop under sowing
Minimising chemical use	Organic farming

instance, some people used green waste from kitchen while others use green waste except weeds from their gardens/fields. Only a small minority used green waste that includes weeds in their composts. Mixtures of water and nettles, thistles or both, were quite common among

[1] Hügulculture is technique of raising soil bed, layering it with wood and then, covering it with soil and compost. Wood helps in water retention for longer time periods.

[2] Johnson Su reactor is a type of compost tank.

[3] Bokashi involves covering farmyard manure with a plastic sheet which is left for fermenting. The sheet doesn't let the carbon dioxide escape which preserves the organic matter and maintains carbon and other nutrients.

[4] Moon-gardening involves sowing seeds according to the moon cycle as it affects high and low tides and likely soil moisture.

[5] Kombucha – a fermented probiotic drink made by adding SCOBY, a cellulose cloth with symbiotic culture of bacteria and yeast to a mixture of tea with added sugar.

participants. Manures or animal droppings from cattle, sheep, horses, rabbits and poultry were also, commonly used as a nitrogen source for soil and the soil microbiome. Participants also, use different alternatives to provide nutrients to their soils. These alternatives include using dry blood, seaweeds, nails and horns. The primary goal of using these alternatives is to use nutrient sources that are local, cost-free, easily available, provide maximum nutrients and most importantly, are environment and ecosystem friendly (Durán-Lara, Valderrama and Marican, 2020). The alternatives such as sheep wool mats showed that participants used their creativity and knowledge to make the best use of the resources available to them. This also showed that most participants want to only use on-farm inputs for their allotments/fields. While some participants wanted to use on-farm inputs due to their organic or regenerative principles (Best, 2010), others did not trust the quality of off-farm inputs such as compost due to possible contamination, which leads them to use creative alternatives.

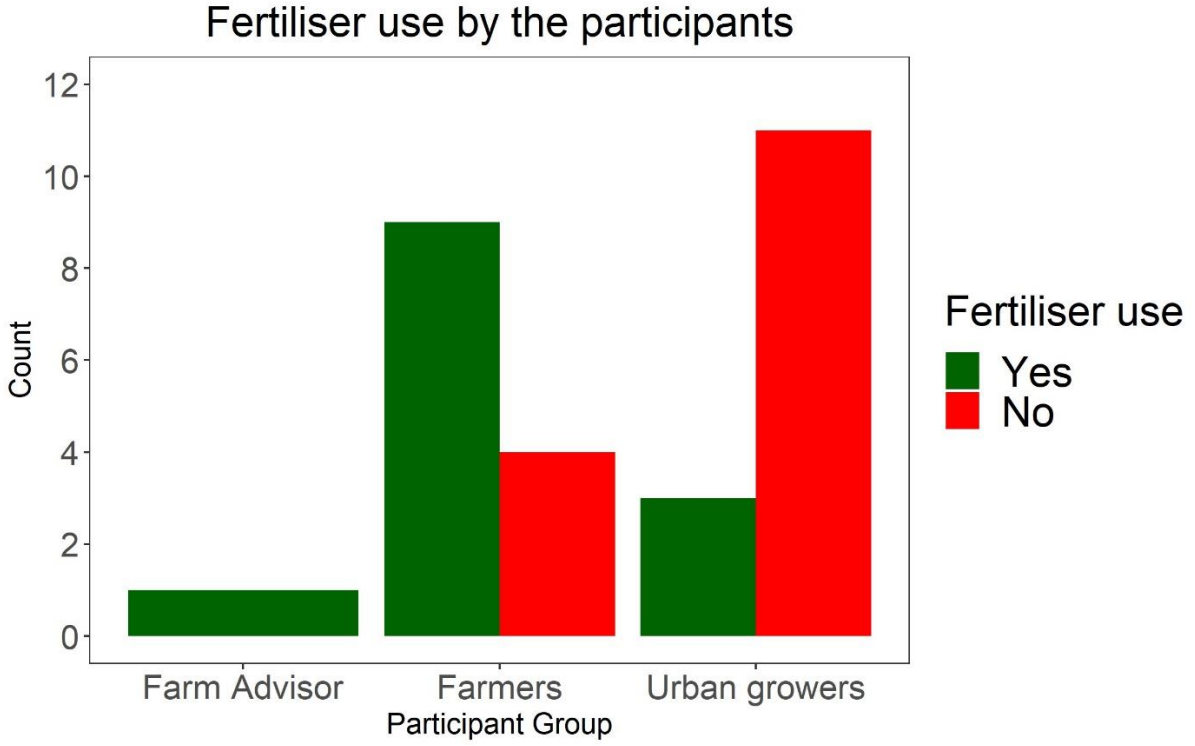


Figure 4. 4 : Fertiliser use by the participants.
The bar graph shows the use of chemical based fertilisers by different participant groups. The farm advisor, 9 farmers and 3 urban growers said that they use fertilisers while 4 farmers and 11 urban growers claimed that they do not use any fertilisers.

Legumes are gaining traction in the UK and Europe due to transition to plant-based diets as well as their importance in improving soil health (Durán-Lara, Valderrama and Marican, 2020; Cusworth, Garnett and Lorimer, 2021). Participants used legumes commonly as part of rotational cropping due to their ability to 1) fix nitrogen in plants and in the soil 2) break the

development of new diseases or prevalence of existing diseases in the soil. Legumes, thus, play an important role in the development of disease resistance of soil. Legume and brassica mixes are often used as under sown crop with cereals to prevent disease outbreak in the main crop as well as provision of nutrients to the soil. In addition, if a cereal crop has been affected by a disease, rotation with legumes and brassicas is a source of income for commercial growers in case the cereal crop yield is lost. An excerpt from Participant 6's interview sheds more light on the importance of legumes:

"So if we put…a cash crop in after the oil seed rape that's got some, let's say oats, rye, vetches and phacelia in it. Hopefully, we'll start building up the fungi in the soil before we put the next wheat crop in. It certainly helps build up worm numbers, because the fields we had were full of worm casts. It does help in to build up organic matter. And I think it probably is also reducing the amount of slug pressure on the next wheat crop. Because instead of drilling into what would be 100% brassica volunteers, it's a more diverse population. And if the slugs may be able to eat the decay in cash crops rather than the emerging wheat crop." **Participant 6**

Some participants showed the importance of keeping their fields and soils in synchronization with nature and the local ecosystem. Balance in nature was an important motivation for the practices that these participants followed. Some of the participants had learned from their previous experiences that working with nature was the best course for them to manage their soils and the soil microbiome. These participants used native plant species on their croplands as they were more productive and disease resistant as compared to the modern varieties introduced after the Green Revolution (Shelef, Weisberg and Provenza, 2017). Native plants are usually best adapted to the local soil conditions and the local soil microbiome plays an important role in imparting this adaptiveness, as they are part of the native ecology and form relationships with certain plant varieties (Cawley *et al.*, 2023; Touceda-Suárez *et al.*, 2023).

There were many practices that were new to the participants. Some participants had already used them on their soils while others planned on using them in the future. These participants had either read about the new practices in farming magazines or heard of them in the farmer community or in agricultural webinars. Some of these practices such as using kombucha (a fermented probiotic drink) by participant 4 were creatively modified for use on soils.

"Kombucha is…trendy as a drink. And it's like, there's a thing called a SCOBY. But I'm not quite sure what it is. It's not quite yeast or fungus. It's like a thing that grows...I just thought I'll put kombucha on the garden. So I've been making, like about…20-30 litres of it. And so then you pour it onto the soil and that's supposed to do all the kind of probiotics and whatever goes into the soil." **Participant 4**

The participants want to test the potential of these new practices to improve the soil and the soil microbiome. Innovation and creativity are part of the trial and error of grower experiences which leads to the addition of new knowledge and unique practices to their list of existing agricultural practices to sustain the soil and the soil microbiome (Vogl, Kummer and Christoph, 2016). These practices, if successful, diffuse in the farmer community and become part of local knowledge which is specific to farmer needs and their socio-economic and cultural conditions (Vogl, Kummer and Christoph, 2016). Farmer and grower innovation leads to the generation of diverse practices, which form the base of many innovations in agriculture. This is becoming increasingly recognised in research and innovation in agriculture which is essential for sustainability (MacMillan and Benton, 2014).

4.3.3 Assessing the soil microbiome

Assessing the soil microbiome and any changes to soil were described as a tough task by the participants. Participants assessed the changes in their soils in many different ways – some found visual qualitative assessments as most helpful while others used expert led quantitative tests (Figure 4.5). They used a variety of methods, including laboratory tools such pH and chemical composition (Nitrogen, Phosphorous, Potassium) based soil tests. Advice from peer groups (farmer or urban grower community) or farm advisors was also an important way to assess the soil microbiome's presence (for detailed description see Supplementary 1, 3.3). Participants use a range of indicators to determine whether the soil microbiome is functional.

Soil health assessment tests are undertaken by farmers to check the state of soils, especially before undertaking soil management practices and to observe the effectiveness of practices and progress of actions taken. Almost all participants use tests, either quantitative or qualitative, or a mix of both to get a sense of the health of their soils and assess the changes. These tests are done to take decisions - to maintain or improve plant yields while maintaining or improving soil health. The tests used by the participants were divided into qualitative and quantitative tests, broadly. Each of the testing categories were divided into physical, chemical and biological indicators, which have been listed in table 4.2. Physical indicators involved indicators related to the characteristics of soil such as soil structure and texture. Chemical indicators involve measuring characteristics such as macro and micronutrients in the soil while biological indicators depict the biological profile of the soil. The indicators mentioned and used by the participants were site-specific and linked to the functions that the soil microbiome provides.

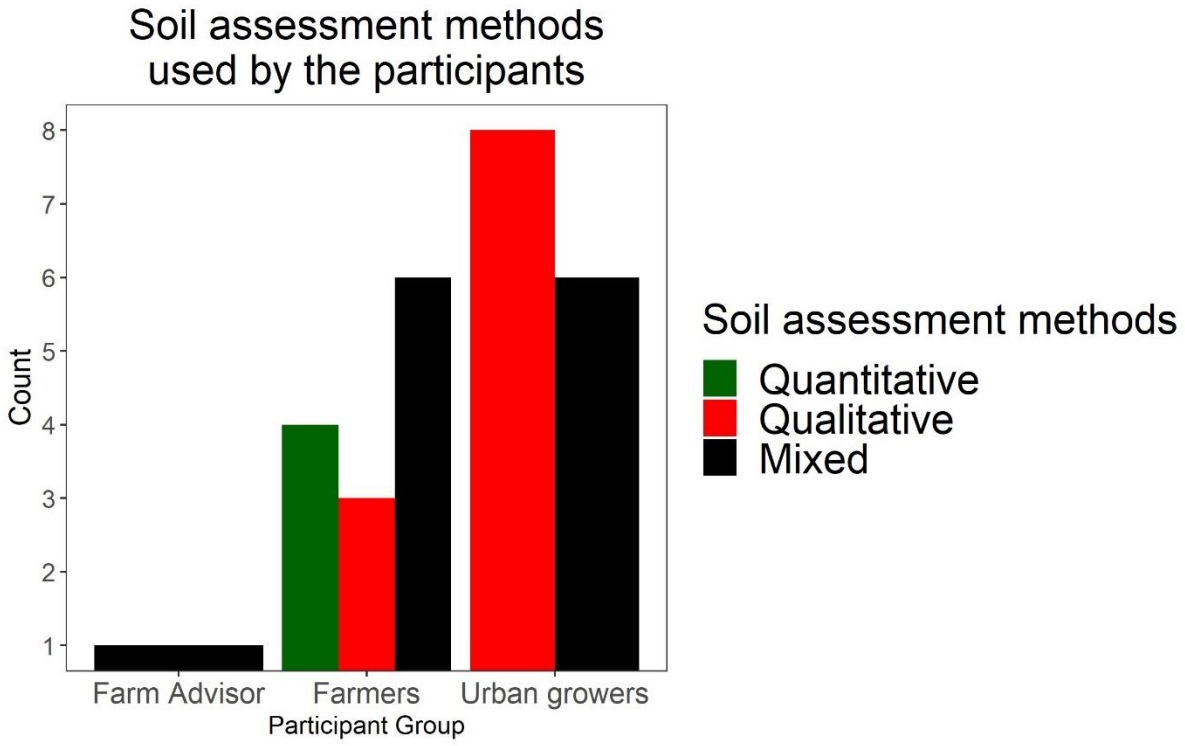


Figure 4. 5 : Soil assessment methods used by the participants.
The bar graph shows the different soil assessment methods used by different participant groups to assess the soil microbiome. Quantitative methods were used by 4 farmers while qualitative methods were used by 3 farmers and 8 urban growers. The farm advisor, 6 farmers and 6 urban growers said that they use mixed methods i.e. both the qualitative and quantitative methods to assess the soil microbiome.

Participants came in clearly with different perspectives on assessing the soil microbiome. Visual or qualitative assessments were commonly used by some participants to assess their soils and the soil microbiome. This includes looking at the colour, texture and smell of the soil. These participants also compare their soils to other farmers or growers in the community to ascertain if their practices are successful. Participants use their observation skills to notice indicators such as dung beetle population for soil microbiome assessment. Even though dung beetles may not be directly important to the participants here, they realise that it is important for the overall farm ecology and a direct indicator of the soil microbiome. Others use root nodules as an indicator for a healthy and functional soil as well as the soil microbiome. If they do not find the nodules, they try to find the issues that may be persistent in the soil and look for solutions. These solutions include using rhizobial or myccorhizal inoculants or rotating the crops. Many participants feel that plant health is a critical indicator of a healthy soil biology that often can be used to assess changes after practices. Plant productivity, quality and their palatability to cattle is another important indicator. In addition, crops colours other than green show deficiencies in soil and plants, which once identified, can be made available to plants.

"I do look to see if there are things crawling about and see how much activity is going on in the soil...I mean that is part of general sort of health, I would consider it so. Well, if I'm cultivating at all, or doing anything with the soil, I'm trying to take note of everything that's going on. What weeds are growing, how they're growing, if there's air in the soil, if it smells nice, trying to look at everything. If it starts to smell anaerobic then yeah, I'm in trouble. I will feel the soil and I'm trying to use as many senses as possible when looking at the soil. I've used the test kits in work to test the soil in the past…I'll test my soil occasionally, like once every seven years, not that often, just to see if there is any anomaly. " **Participant 23**

Quantitative soil tests such as pH, organic matter and nutrient analysis are used by many participants. Only one participant measured the bacterial and fungal levels in their soil. This helped her to assess if her farming practices affected the bacteria and fungi levels positively. The tests to measure the soil microbiome thus, exist but many factors play a major role in their use. Factors such as cost, accessibility and awareness about such tests are very important in determining who can use these tests. There is a keen interest in testing the soil microbiome but participants do not know of any tests that do comprehensive testing. Although there are tests that look at the chemical, physical and biological aspects of the soil, these tests do not provide a complete picture and are often available separately rather than a comprehensive kit. There is a heightened interest in looking at the soil microbiome closely and using it to enhance soil fertility, soil functioning and thus, plant production (O'Neill, Sprunger and Robertson, 2021). Some participants used both quantitative and qualitative methods to have a comprehensive understanding of their soil and soil microbiome and to make better estimates and conclusions about benefits or losses of undertaking particular practices.

Participants believed that healthy microbiomes are only present in healthy soils but they were uncertain whether the macro fauna is part of the soil microbiome. Yet, they saw the presence of macro fauna, such as earthworms, an indication of a healthy microbiome that support better food and vegetable production. They were also uncertain about whether they were looking for the right organisms or signs in the soil. Participant 10 for example stated that he knew what the signs of a good/healthy soil are according to his own experience but linking it to soil microbial presence was a challenge for him. This may be because terms such as 'soil microbiome' and 'assessment' are quite new in the farmer community and although they know that the soil microbiome is good for their soil, they link the indicators to a healthy soil or something that they can assess visually as opposed to the invisible 'soil microbiome'.

Table 4. 2 : Different physical, chemical and biological indicators used by participants in quantitative and qualitative tests to assess changes due to various practices

	Quantitative indicators	**Qualitative indicators**
Physical	Soil temperature	Touch - soil texture, soil friability, soil moisture, soil biting, soil shaping Visual - smooth fields, infiltration rate, soil structure, water drainage, soil colour, soil fertility Smell - earthy smell of soil
Chemical	Nutrient testing - nitrogen, phosphorus and potassium, forage mineral analysis, organic matter tests, carbon level tests and CO_2 burst, pH tests	Palatability of grass to sheep and cattle due to high potassium content
Biological	Total bacterial and fungal counts Plant yield	Micro fauna - mycorrhizal fungi's grey network Macro fauna - earthworms, dung beetles, scarab beetles, moles, insect presence and activity, hedge line diversity, above ground wildlife Plant - plant quality, plant leaf colour, plant material decomposition, root nodules, soil mushrooms, disease resilience in plants

"Earthworms are probably the easy example of something that…we want to see…we know that they're doing good in terms of aerating the soil, breaking up the soil, incorporating organic matter. So that's always positive. When we apply… organic manures, then, you know, it's great that you can see them there…I suppose in terms of…holding in your hand, like is this clay or whatever…I don't think I would come with anything that allowed me to make any judgement around soil microbes." **Participant 10**

Some participants state that it is very difficult to find indicators that show accurately the changes. While they do use visual and qualitative signs such as plant productivity to observe all the changes, these visual signs may not be only due to soil related practices but a mix of factors such as weather and rainfall. Even if the changes are due to the practices undertaken, it is not easy to distinguish which practice out of many, led to the changes assessed or seen. Others did not see any changes due to practices as they stated that it takes some time to appear.

This may take a couple of years to a decade for participants to assess, especially through quantitative tests. Moreover, quantitative soil testing adds another task for the participants, which needs time, and in the case of commercial testing, money.

Another reason, which was essential for participants who decided not to use quantitative testing was that tests only gave a half picture of soils due to soil variability. These participants preferred qualitative techniques to observe the health of their soils, which linked to the importance of embodied expertise grounded in experience and observations. Some also mentioned the use of a microscope could help them to observe microscopic things such as mycelia in the soil. However, using these products and tests does not necessarily mean that they believe that any expert-led test was capable of giving a full comprehensive result. Participant 6 states the thoughts of many of these participants that measuring the changes in soil due to practices is not essential. The assessment here refers to both quantitative tests as well as visual assessments such as plant productivity and plant health, which are a part of almost all the participants' field routines. Moreover, the quantitative tests may show an enhancement in terms of a unit or two but since, the goal is to enhance the soil, participants believe that it is not important to use these tests or assessments.

"There's not a real point [of assessing], if you're trying to do everything you can…Well, you do everything you can to enhance it, you're not gonna say well, I've measured it is enhanced. I've got to stop doing that now. You actually will want to carry on enhancing it, even if you've enhanced it on here." **Participant 6**

The above narratives on different tests and indicators used show that there are diverse perceptions about quantitative and qualitative testing in the grower community. However, qualitative testing is mostly preferred. Additionally, there are many new and upcoming tests - some creatively invented by the participants and some commercially designed and trialled by companies - to measure the soil microbiome. Participants are interested in measuring at least some, if not all components of the soil microbiome, which helps them to assess the health of the soil microbiome and the soil. This also, helps them to take decisions such as whether to remedy the soil or provide certain nutrients for best outcomes.

4.3.4 Soil inoculants

The participants in this study have all heard about soil inoculants and have a general understanding about them (Figure 4.6), but some have not used them or do not feel that they

need to use them. The principal motivation for using or not using inoculants is the participants' assessment of the functioning of the microbiome in the soil.

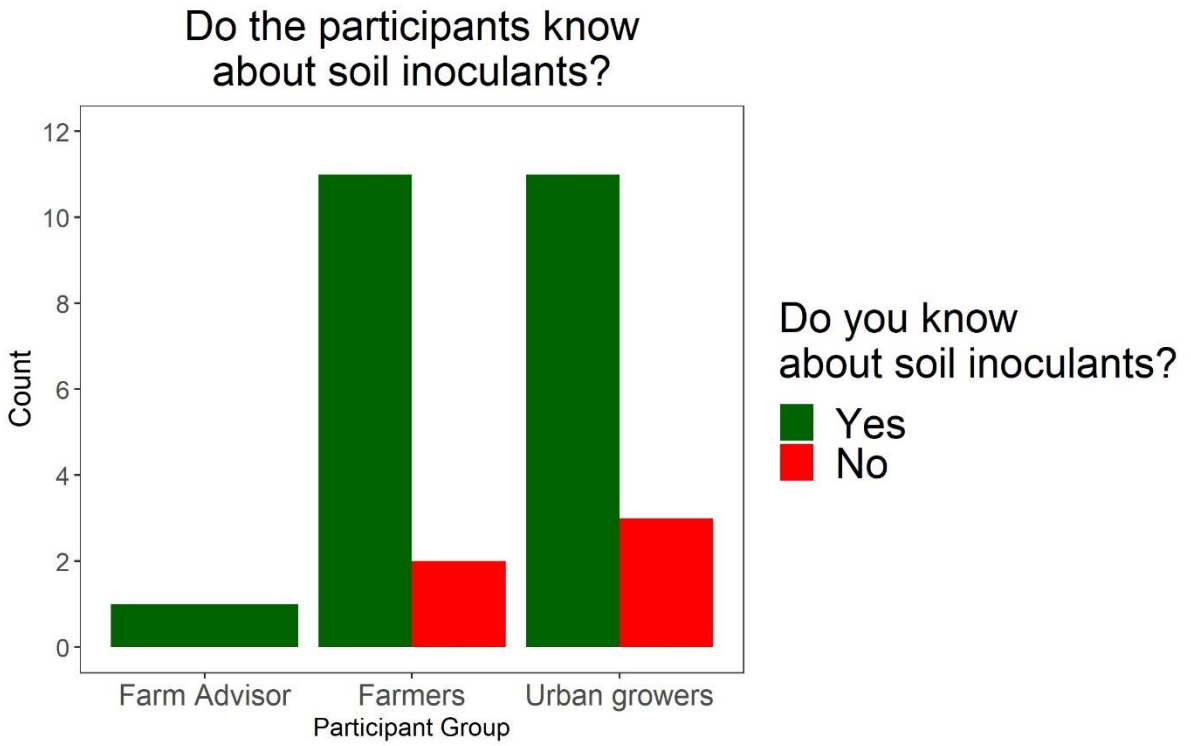


Figure 4. 6 : Do the participants know about soil inoculants?
The bar graph shows if different participant groups know about soil inoculants. The farm advisor, 11 farmers and 11 urban growers said that they know about soil inoculants while 2 farmers and 3 urban growers claimed that they have not heard about soil inoculants.

There was a high uncertainty around what counts as a soil inoculant, and the interviewees often turned to me as an interviewer and someone who represents the research community to confirm whether their perception was right. Some participants associate legumes with inoculants as through experience they know that legumes harbour rhizobia, which fix nitrogen that fits their definition of soil inoculants working as a plant nutrient provider. Similarly, other participants associate application of a product to soil inoculants. This parallel may be drawn through application of products such as fertilisers and pesticides. The term 'soil inoculants' also implies a product applied to soils. There was also, a component of guessing which describes the uncertainty involved. Participants either had not heard of soil inoculants or if they had, it was as a concept or as a product, they were not accustomed to terms such as 'soil or mycorrhizal or rhizobial inoculant'. There is thus, a lack of awareness around the terms but not the concepts of soil inoculants in the participants as most of them could describe their thoughts on soil inoculants but these often involved a tone of speculation or guesswork.

"*I'm not quite sure exactly what soil inoculants are. I think that I mean, is it right that the beans they're inoculating the soil when you're growing [them] in the soil? Is that the case? Are they inoculating the soil because they have those, those little white things on their roots, yeah, or is it about something else."* **Participant 1**

Participants had a varied experience of inoculant use. Some had experience mainly from using commercial products such as nematodes for pest/bio control (although there was some uncertainty whether these really count as inoculants). They drew parallels between microbial and macro-fauna based soil inoculants which could be used as plant growth stimulators and biocontrol agents in soils. Other participants were quite creative when describing soil inoculants. These descriptions were drawn from their experiences and principles, for instance, if they were organic or regenerative farmers, they used homemade compost teas, which they used as inoculants.

"*Inoculants, I'm imagining these things like even compost weed, or compost tea would be a soil inoculant, am I right? No, I haven't, but I think I would...perhaps…go to a nice wooded forest, have a bit of a dig find some mycelium, and maybe just get a cup full and make a tea from that, can be a natural form of…inoculant perhaps?"* **Participant 25**

Participants who had used inoculants mainly wished to improve their crop yields but the results varied for each participant. Some participants had higher yields while others struggled and had worse outcomes as compared to when they had not used inoculants. This generally translated into frustration and the decision to not use soil inoculants in future, as it is a futile addition that costs money and labour. However, others who used it for first time were hopeful and were looking forward to see positive results.

"It was quite good, actually…I did…for private clients and the roses [are] still growing quite well…So maybe there's something in it. Anyhow, to me, it seems more sort of natural than perhaps adding a chemical based fertiliser. But that's just, that's just my feeling as a sort of as a hobby gardener." **Participant 1**

The uncertainty surrounding inoculants, both in terms of their utility and the risks of their use, were a crucial theme in all interviews. Some participants did not want to add additives or soil inoculants from companies, as they did not believe the sources as well as the effectiveness of these products. These participants rather wanted to be creative and experimental with their own home brewed inoculants rather than getting a packet of commercial inoculants. This is an example of mistrust as well as uncertainty around using products from commercial companies. The idea that soils have a natural balance that could become upset with inoculants was very pronounced. Using non-native organisms was seen as potentially disturbing the natural balance

of their soils, and participants such as 21 expressed they did not require or want to use soil inoculants, as they were happy with the local soil microbiome as well as the plants yields. Allotment holders were generally much happier with the produce that they got and did not want to add another product, which was just an added cost to their allotment. They also mentioned that they took the necessary steps to ensure that the local soil and soil microbiome was actively functioning and thus, did not feel the need of using soil inoculants.

"I think the thing is, with the allotment sites…they've all been worked for quite a long time and they're worked by people. I think increasingly, they're much more likely to be organic, or organically worked, I feel like they're quite, lively. You feel like there's a lot of life in the allotment sites, if that makes sense." **Participant 21**

4.3.4.1 Soil inoculant importance

The usefulness and desirability of using inoculants was judged based on the participants' assessment of the strength of the microbiome in their soils. Some participants who have experience using soil inoculants consider soil inoculants are important, but only in areas where the local beneficial microbes such as rhizobia and mycorrhiza are absent or which do not have a legume legacy. Soil inoculants such as myccorrhizal preparations were seen as a promising source of plant nutrients in such areas. This was, however, influenced by positive results after using inoculants. Adding soil inoculants were considered unhelpful in areas that have a healthy soil microbiome. In such instances, soil inoculants are added labour and cost.

"I think because the soil in Western Australia, is so lacking in Rhizobium bacteria, we would think they [farmers] would actually inoculate the seed with Rhizobium bacteria before planting it…but I think soil in Britain has got enough Rhizobium bacteria. So we don't particularly need inoculants". **Participant 2**

Participants who have not used soil inoculants felt that inoculants are more apt for commercial purposes as small-scale allotments and gardens are managed much more efficiently and organically. This ensures that the soil microbiome within the soil is active and functioning, which means that there is no need of adding any inoculants to allotments or gardens. Soil inoculants can also be used to enhance the local soil microbiome that would be essential for food growing in future along with sustaining the soil.

"It must do to some extent, especially if there's very little there before you've got to start somewhere, haven't you? And, it is worthwhile trying to introduce these things to try and pull the soil back into some kind of life. But…you're talking more sort of large scale stuff, rather than, like for gardening, or, you know, some plots my size, it's more for sort of agriculture,

isn't it?...Because I think the thing is, with the allotment sites…they've all been worked for quite a long time and they're worked by people. I think increasingly, they're much more likely to be organic, or organically worked, I feel like they're quite, lively. You feel like there's a lot of life in the allotment sites, if that makes sense." **Participant 21**

There was a lot of scepticism around using soil inoculants. This stemmed from the difficulty of assessing the usefulness of inoculants. The small number of microbes introduced in comparison to the overall microbiome made it difficult for participants to be convinced of the effectiveness of inoculants. Inconsistent results in terms of crop yields also contributed to the scepticism, as inoculants were shown to work for some people while they do not work for others. However, soil inoculants were also seen in a positive light as a potential additive that can work well with other practices such as using compost and as a source of improving local soil biodiversity.

4.3.4.2 Scepticism and drawbacks to soil inoculant use

High cost of inoculants was commonly cited by participants as a reason for not using soil inoculants. In addition, if using soil inoculants did not yield positive results in the form of higher crop yields, it was wasted money. Participants were interested in spending money, only if there were proven positive results while soil inoculants do not have enough evidence of success and even if there is any evidence, it shows that soil inoculants are inconsistent in fields.

Participants found it very difficult to apply and visually confirm the application of soil inoculants. One participant came up with the feedback that inoculants should be in liquid form and there should be a dye on the inoculants for easier confirmation that soil inoculant application is complete.

"What I don't know, is how well they work. And whether they are economically justifiable. I also don't know…whether whatever it is that the seeds were inoculated with, has a kind of long-lasting effect beyond the current crop. So I think I don't know enough about them. It would seem to me that would be a good thing from a biological point of view. But again, from a sort of business point of view, I suspect though, that seed is expensive, and if part of the benefit is that it increases the biodiversity in the soil…that would seem to me a good reason for using it." **Participant 24**

A common drawback was inconsistent results. Few research trials in fields and farms, which show that soil inoculants are capable of omitting or reducing the use of fertilisers, was a major reason cited. Participants want to see more evidence from the farm trials, especially with no legacy of fertiliser use. Moreover, participants want to see consistent results that show higher

crop yields or at least on par with yields obtained after using fertilisers. Participants who had positive results also wanted to use soil inoculants that have a longer shelf/soil life so that the successive crops can also have the benefit (if any) of the soil inoculant presence.

Other participants while open to using soil inoculants were concerned about the lasting effects of soil inoculants. Soil inoculants may have the potential to disrupt the natural balance of the local microbiome. It is important and better that the local microbiome is provided with all the resources or nutrients to perform or function effectively instead of adding a non-native microbe mixture to the soils. Natural order or balance is essential to some participants, which they do not want to disturb. At the same time, when nutrients are added to soils, they might also affect this natural balance in some way such as if they are leaching instead of being absorbed by plants or microbes.

Lack of a standard or baseline to compare changes such as in yields and the soil microbiome after using inoculants was seen as another drawback to use soil inoculants. This highlights the issue of lack of comprehensive kits to measure the soil microbiome, as mentioned previously.

"I think we'd need to establish a baseline. Where are we now…with a soil microbiome of this amount of activity, biological activity, this is the output that we can get. So the microbiome at the moment enables us to produce say six times of dry matter a hectare, if our microbiome was to double, we could grow double the dry matter without buying in manufactured nutrients. Well, that would be very attractive, wouldn't it?...But we don't know where we're at at the moment." **Participant 12**

4.4 Discussion

This study sheds light on the different experiences with and perceptions of the soil microbiome by UK growers and farmers in order to better understand the potential for inoculant use. The experiences and perceptions are known to play an important role in technology transfer and adoption (Delgado and Stoorvogel, 2022). The thoughts and perceptions of participants varied widely. They ranged from appreciation to care to uncertainty and recognition of gaps around the soil microbiome. Symbiotic associations in the soil were an important component of appreciation of the soil microbiome. While many participants appreciate the functions that the soil microbiome carries out, there are also doubts and uncertainty amongst participants about the soil microbiome. The uncertainty is reflected in the questions asked about the soil microbiome by participant 3 - what is the soil microbiome? Is it naturally present in the soil or is it a product? Will it help in boosting plant production? This uncertainty stems from a

mismatch between terms that researchers and participants use to describe the soil microbiome. This is a reflection of the different contexts that researchers and farmers hold which are often related to the aims that researchers and farmers want to achieve (Ingram, Fry and Mathieu, 2010). Participants in this study want to learn more about the soil microbiome to improve their soils and productivity. However, the aim of the researcher is to understand the role that soil microbiome plays in daily interactions of participants with their soils and its potential for improving sustainable agricultural technologies for food production.

4.4.1 Soil microbiome understandings

All the participants clearly understand what the soil microbiome is in practice, but terms such as 'soil microbiome' make them less confident and more uncertain about their own perceptions and knowledge about the soil microbiome. The same is true for soil inoculants. Soil microbiome is often seen as a part of the soil and this was evident in the participant descriptions of soil health and soil fertility when asked about the soil microbiome. These different stances show that participants hold variable depths of knowledge (Dawoe *et al.*, 2012). Some participants view the soil microbiome from a broad perspective as they drew parallels between the gut microbiome and the soil microbiome that is similar to several scientific studies. For instance, the gut microbiome is similar to the plant root zone or rhizosphere as both environments provide a large surface area and diverse niches for diverse microbes to proliferate (Blum, Zechmeister-Boltenstern and Keiblinger, 2019). Others perceived the soil microbiome with a detailed perspective, which included describing the constituents and the functions that the soil microbiome performs in their soils and plant productivity. Taking care of the soil microbiome was important for participants as it was seen to promote good food, which can nourish participants' health. The nourishment and health of soil microbiome has been indeed found to influence food quality and thus, health of consumers (Blum, Zechmeister-Boltenstern and Keiblinger, 2019). This awareness of consequences on human health of how soil and soil microbiome is treated, was evidence that food growers care about the soil microbiome and the soil. But maybe consequences on their own health made them care more for the soil microbiome.

4.4.2 Practices and the soil microbiome

Participants described many practices dedicated to improving soil health by caring for the soil microbiome. The variety of practices such as using composts, manures and alternatives to nitrogen fertilisers that the participants use to take care of their soils and the soil microbiome

are again evidence of the participants' attentiveness and care for the environment. Participant practices' indicate that environmental concern is relevant when considering agricultural technology adoption (Best, 2010). In addition, the variety and innovativeness of the practices described showed innovative growers and farmers should be considered as innovators, and their experiences and experiments should be incorporated in thinking about the future of agricultural technologies.

4.4.3 Indicators for soil microbiome

Observing and assessing change are shown to be important to participants' understanding and appreciation of the soil microbiome as well as the efficacy of inoculants. Researchers and agronomists often use indicators to ascertain soil quality and to take decisions on the necessary efforts to conserve soil and agricultural productivity. Most of the indicators used by experts are quantitative. Quantitative indicators are value derived from physical (such as soil texture), chemical (such as nitrogen or phosphorous content) or biological (microbial mass) properties of soil which can be compared to a reference value (Bünemann *et al.,* 2018; Maurya *et al.*, 2020). In addition, quantitative indicators are usually based on analytical laboratory approaches and require measuring data systematically with standardised processes or procedures (Bünemann *et al.,* 2018). I found that the participants used a mix of expert-led quantitative indicators such as pH and nutrient tests, and intuition-based qualitative indicators such as presence of insects and earthworms to measure soil health as a proxy for microbiome's functioning in the soil. Most participants have a clear preference for qualitative indicators as they are embodied in their own experiences and align better with their own practices. While quantitative indicators and assessments were also used and interested many participants, they are an added cost and often hard to interpret (O'Neill, Sprunger and Robertson, 2021) which makes them less favourable over qualitative assessments. A need for comprehensive soil tests, which included measuring the soil microbiome, was also, often mentioned. This was because a thorough understanding of what is happening in their soils is important to some participants. The participants are curious to know if their practices actually have a positive impact on the soil microbiome (Maurya *et al.,* 2020). Overall, linking inoculants with appropriate indicators that are desirable by growers and farmers should be considered when planning for inoculant applications.

4.4.4 Soil inoculant perceptions, use and drawbacks

Descriptions of the soil microbiome, actions taken to conserve the soil and the soil microbiome along with the various tests to assess the soils and the soil microbiome in this study, show the human-soil microbiome interactions in the food growing community in parts of the UK. These interactions, perceptions and concepts influence participant decisions to use soil inoculants on their soils. The perceptions on soil inoculants were based on whether participants were farmers or urban growers/allotment holders and whether they followed some farming principles such as organic or regenerative. Urban growers and allotment holders who mostly followed organic or regenerative farming practices perceived composts, manures and teas as home-brewed soil inoculants, while most commercial farmers understood inoculants as commercial products which could be applied to the soil.

This research also found strong scepticism towards the use of inoculants. This scepticism is rooted, firstly, in the lack of knowledge about soil inoculants in the participants which also elicits that there is little to no knowledge transfer about soil inoculant research, design and development occurring. This lack thereof, affects the adoption of soil inoculants (Cawley *et al.*, 2023). Secondly, the assessment of soil inoculant desirability was linked to participants' experiences of its use. Most of the participants with positive views had used soil inoculants and had seen positive results while participants who had negative or no outcomes or had never used soil inoculants were often sceptical. Strong positive evidence of results, especially with regard to yield, was needed to encourage participants to invest in inoculants. The lack of a standard test to capture and compare changes after soil inoculant use, added to this scepticism. In addition, added cost, non-ease of application, unknown effects on the local microbiome along with inconsistent results were common barriers to using soil inoculants. Suspicion towards inoculant producers, was a further barrier.

The insights gathered in this chapter further strengthens the case for participatory approaches in designing and developing technologies before they are put on shelves into the market for end users to use. This can help in proper integration of technologies in the grower community based on the background contexts as well as the needs of the end users. This study also, helps to add to literature that participation is important not just for successful uptake of technologies but also, for development of newer contexts and successful knowledge transfer. This will bridge the gap, uncertainties and trust issues between researchers, companies and the end users.

Chapter 5 - Conclusion

This chapter aims to discuss the findings of this thesis in the wider context of rhizobial inoculant improvement and use in sustainable agriculture.

Research around the globe is focusing increasingly on augmenting soil microbiome based technologies in the drive to achieve net zero emissions and Sustainable Development Goals (Fagunwa and Olanbiwoninu, 2020; Northrup *et al.*, 2021; Kendzior, Raffa and Bogdanski, 2022; Verstraete *et al.*, 2022). This is becoming more urgent in the current climate crisis as the planetary boundaries are being pushed beyond their carrying capacities (Richardson *et al.*, 2023). This thesis specifically touches two SDG's i.e. SDG 2: Zero hunger and SDG 15: Life on land, which are directly linked to agriculture.

Technologies such as rhizobial inoculants with improved efficacies can be an important tool for reaching these sustainability goals in agriculture. A typical approach to improving rhizobia efficacy is to identify ideal rhizobia traits in highly controlled, axenic growth conditions. However, ineffectiveness associated with rhizobial inoculants continues to be a major challenge for their success in the natural environment, which is often due to displacement by native/naturalised rhizobia. Improving inoculant competitiveness is thus, key to both improving their effectiveness, and in doing so, increasing the uptake of rhizobial inoculants by farmers. MGEs are naturally occurring members of microbial communities that constantly transfer genes within bacteria, essentially carrying out engineering works at all times (Frost *et al.*, 2005; Heath *et al.*, 2022). Microbiome engineering - improving inoculants using Mobile Genetic Elements (MGEs) - could provide an effective way to improve elite strains (Haskett, Tkacz and Poole, 2021). This can be done by transferring genes that are functional or improving the functioning of existing genes in bacteria (Wang *et al.*, 2013) or, could be used to increase the competitiveness of the host. Moreover, if Mobile Genetic Elements that are naturally present in the bacterial genome are used, they might be much more acceptable as compared to Genetically Modified Organisms (GMOs) which raise many environmental and societal concerns (Zhang, Wohlhueter and Zhang, 2016).

In this thesis, I used the temperate phage vTRX32-1 to investigate its effects as a natural engineer in rhizobia. I found that there is no intrinsic cost of carrying the temperate phage to rhizobia despite the downregulation of many genes in one strain. Moreover, the nitrogen-fixing and symbiotic ability of rhizobia is not affected due to the phage. Rather, the phage provides

competitive benefit in well mixed conditions and some structured environments such as the rhizosphere - but not in the nodules. This could be due to higher rates of phage integration in the competitor strains, especially *Rlt* TRX19 wild type. This means that this phage might not be the best bacterial weapon in case of conflict. However, this does not mean that it cannot be used to enhance microbial inoculants using other approaches/techniques. Given that the phage has a high rate of integration without disrupting bacterial functioning, it might, for instance, be useful as a DNA or genetic payload that can be engineered to deliver beneficial genes to the rhizobial populations. Previous work has shown that using plasmids as 'microbial engineers' can indeed increase desirable traits in plant associated rhizobacteria (Setten *et al.*, 2013; Haskett *et al.*, 2022). However, using these cellular engineers is not easy in the real world as the benefits that they deliver might come at an intrinsic cost, which varies in different environments depending on the community and environmental interactions. It would thus be necessary to closely monitor the effects of engineered bacteria in different environments.

Insights from chapter 4 show that the food growing community is very caring and attentive towards the soil microbiome, and balance in the soil microbiome is of utmost importance to them. In order to ensure that the future of soil microbiome technologies is successful, ensuring that these human-soil microbiome interactions are involved must be considered early on. To facilitate this, bottom up research involving end users using participatory approaches such as interviews and/or focus groups for informed design, development and implementation would be essential. For instance, in this thesis, participant interviews could have led to a collaborative research process in which participants' questions and practices around soil microbiome could be brought in for experimental design, enriching it with user perspectives. Nonetheless, these interactions can be used for informing future researchers, especially, in interdisciplinary or transdisciplinary sciences.

To facilitate participation for discussions around soil microbiome and rhizobial inoculants in this thesis, I took a step back and interviewed members of the food grower community. The goal was to find current understandings and practices around rhizobial inoculants as well as the existing relationships that food growers have with the soil microbiome. These relationships, firstly, provided background information on the perceptions around rhizobial inoculant use. Secondly, they gave me evidence of the actions as a list of practices that are often used knowingly or unknowingly towards the soil microbiome. Overall, the interviews showed that there is lot of experiential knowledge around the soil microbiome that needs to be explored further as it can be used for improving inoculants.

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This study also highlights that there is a wide gap between research on microbial inoculants and its use in the grower community. This leads to many challenges and barriers, which may prevent the successful uptake, and application of current as well as future soil microbiomes based technologies. A lack of assessment, lack of shared language as well as the lack of trust were three major challenges that were identified in this study. For instance, soil microbiome and inoculant based tests were found missing in the real world. It would thus be helpful if future inoculant and soil microbiome research focuses on building comprehensive soil based assessments that have the characteristics of participant used qualitative assessments and expert led quantitative assessments that are standardised and recognised by regulatory bodies. Similarly, co-adapting, co-learning or even co-producing terms would be required for better understanding, communication and articulation of contexts and goals from end-users to researchers and vice-versa. In order to initiate this, participatory collaborations in the initial phases of technology innovation would be essential. In addition, participation can help in alleviation of mistrust due to much better understanding and co-creation of knowledge around these technologies together. These insights show that deeper and systematic collaboration is crucial, that users' questions and issues must be taken into consideration early in the research process and in this way, this study poses an important challenge to the microbiome research community to place its work more firmly in the real worlds of growers.

To conclude, this work demonstrates that although the temperate phage may not provide large competitive advantages, its integration does not interfere with rhizobia-plant symbiosis even though many important bacterial genes are downregulated in its presence. Going further, for future soil microbiome research, this phage might be useful as a natural engineer for improving the functionality of locally adapted soil microbiome. But for successful integration, use and application of such technologies, bottom-up research and participation of end users would be essential.

Appendices

Appendix A

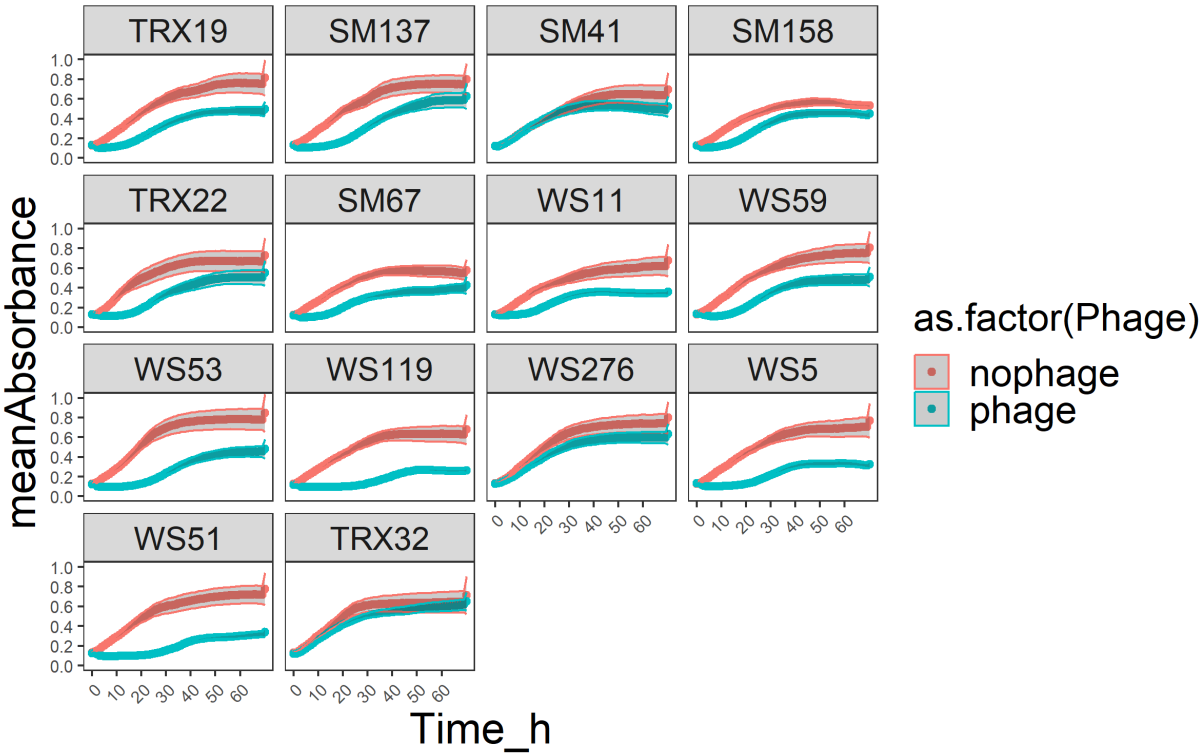

Figure A.1 : Mean absorbances of different strains selected for the community experiment in presence (green) and absence (red) of phage vTRX32-1.
The grey ribbons show the standard deviations across four replicates. TRX32 was the positive control. Control: TRX19, Susceptible and strongly lysogenic: SM137, SM41, SM158 and TRX22, Susceptible and weakly lysogenic: SM67, WS11, WS59 and WS53, Resistant: WS119, WS276, WS5 and WS51.

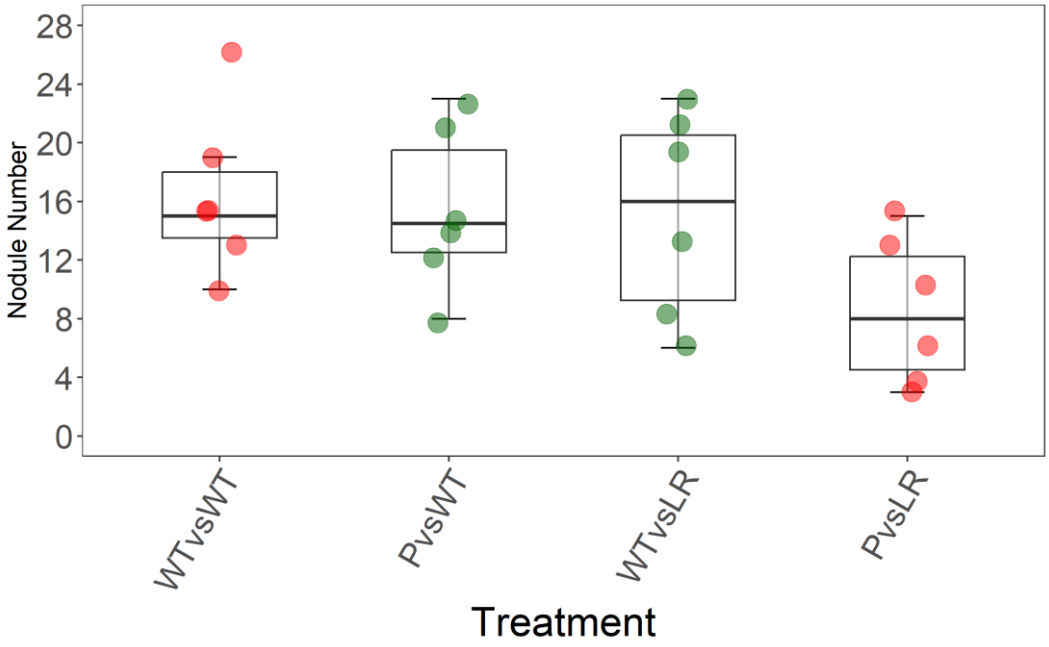

Figure A.2 : Number of nodules from WT and LR plant competitions.
Phage free, wild type (WT - red) or phage containing (P - green) strains were tested in the presence of either the isogenic wild type strain (WT) or a lysogeny resistant competitor (LR). Coloured points represent different replicates.

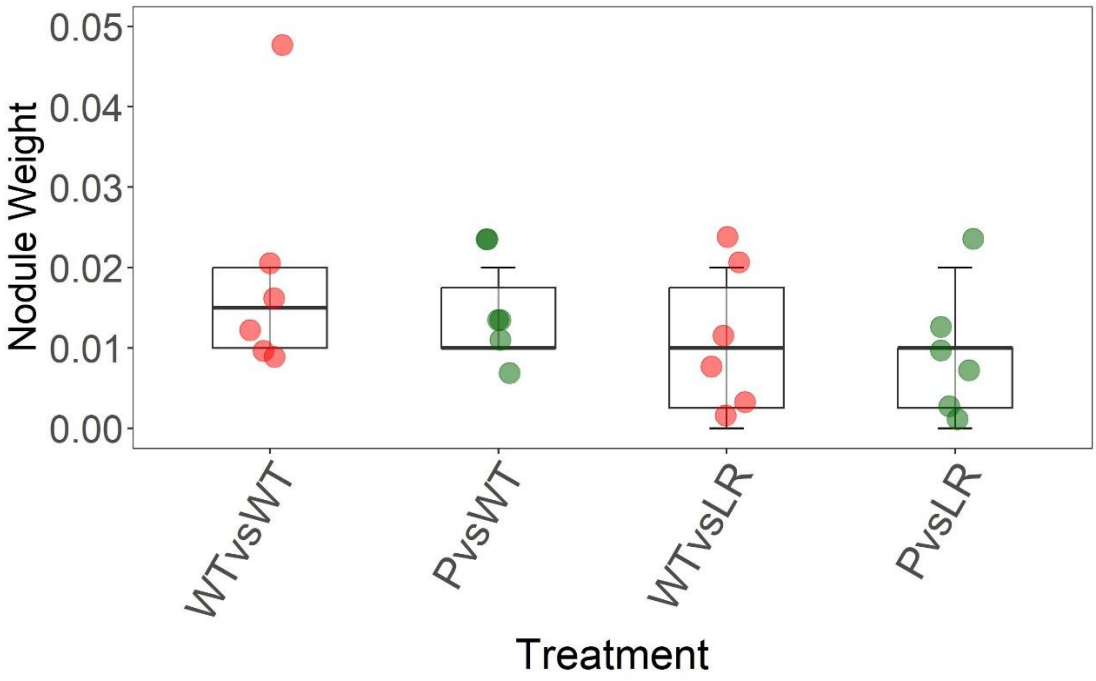

Figure A.3 : Fresh weight of nodules from WT and LR plant competitions.
Phage free, wild type (WT - red) or phage containing (P - green) strains were tested in the presence of either the isogenic wild type strain (WT) or a lysogeny resistant competitor (LR). Coloured points represent different replicates.

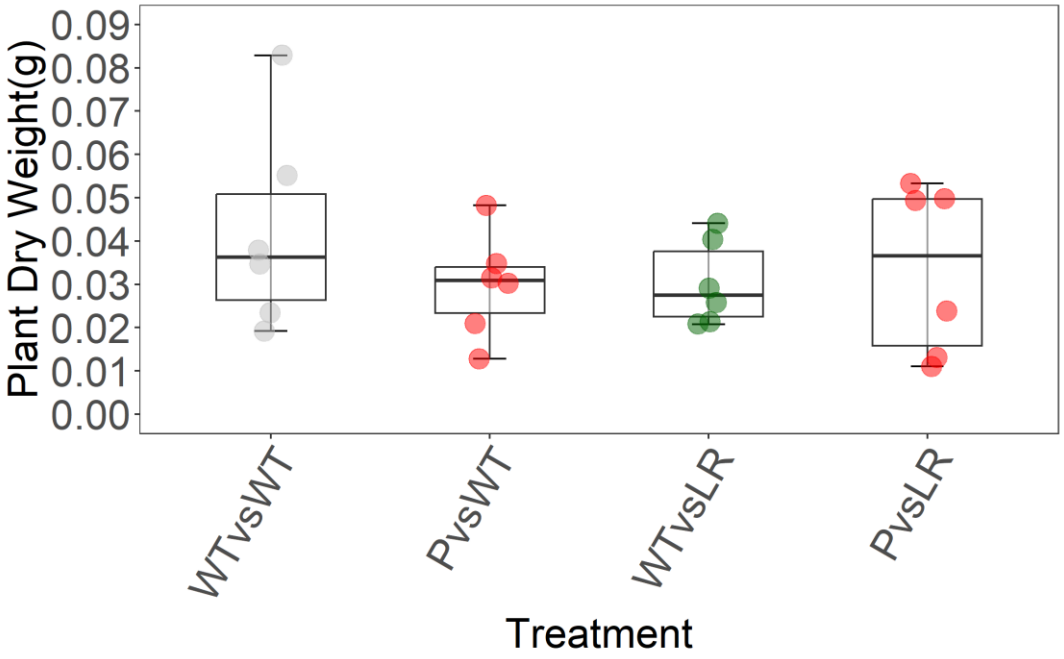

Figure A.4 : Plant dry weight of isogenic competition treatments.
Phage free, wild type (WT - red) or phage containing (P - green) strains were tested in the presence of either the isogenic wild type strain (WT) or a lysogeny resistant competitor (LR). Coloured points represent different replicates.

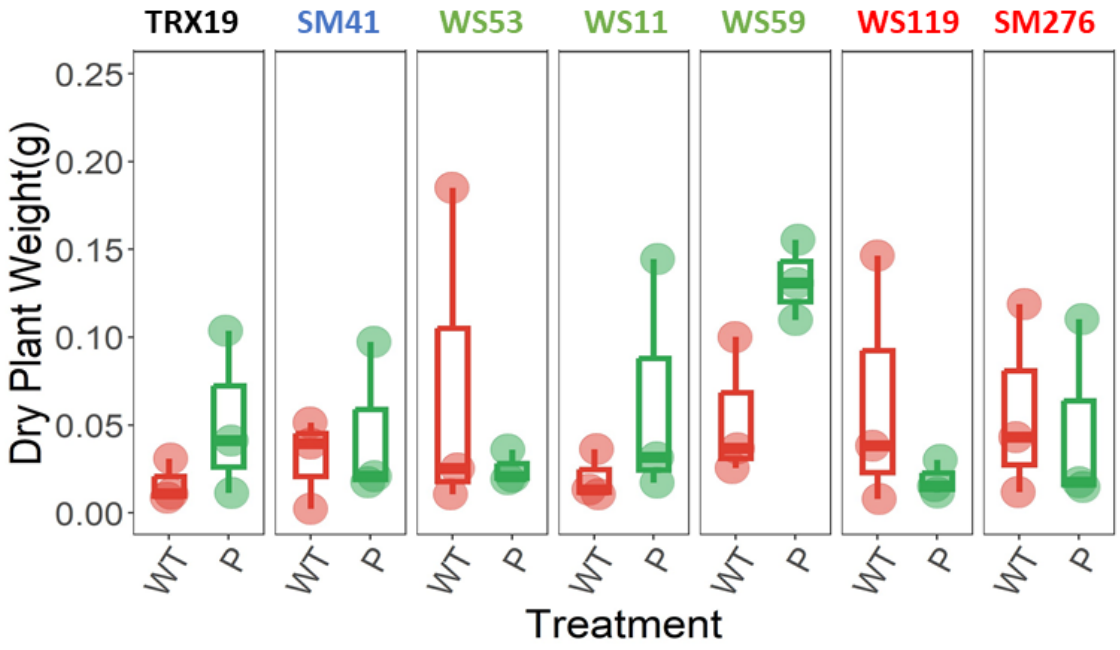

Figure A.5 : Plant dry weight of non-isogenic competition treatments.
WT is Wild type, P is Phage-containing. The red and green points represent different replicates.

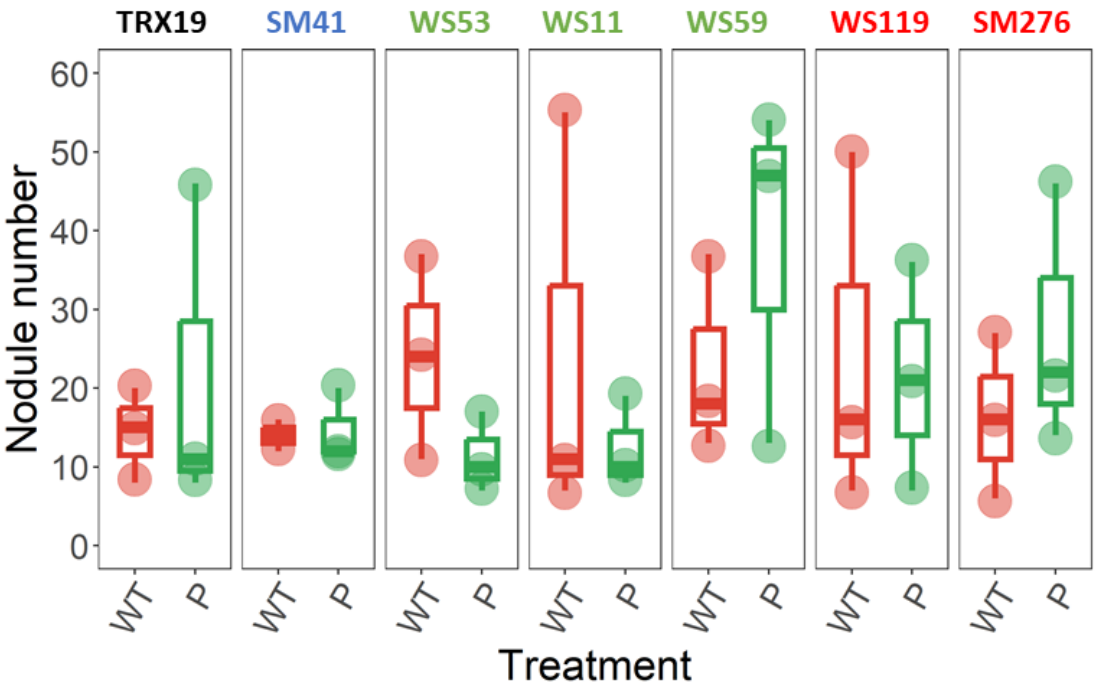

Figure A.6 : Number of nodules in non-isogenic plant competitions.
WT is Wild type, P is Phage-containing. The red and green points represent different replicates.

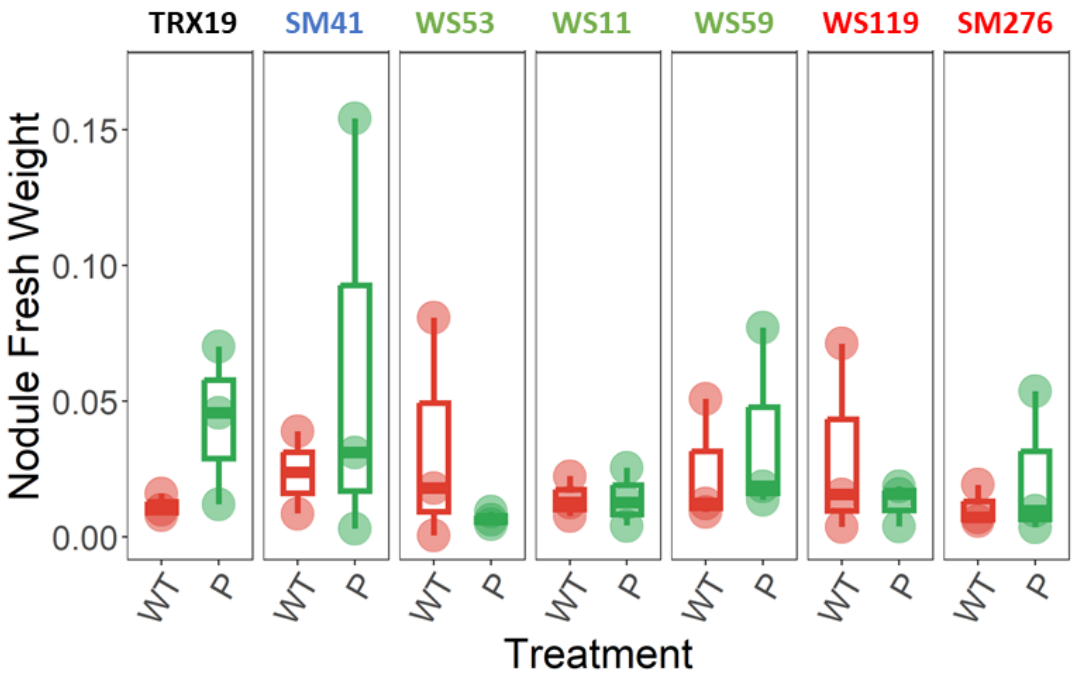

Figure A.7 : Fresh weight of nodules from non-isogenic plant competitions.
WT is Wild type, P is Phage-containing. The red points and green points represent different replicates.

Appendix B

Participant info sheet

1. PhD Research Project Title:

Utilizing the soil microbiome for sustainable agriculture in the UK: drawing from rhizobial inoculants and farmer knowledge

2. Invitation paragraph

You are being invited to take part in a PhD research project. Before you decide whether or not to participate, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. Thank you for reading this.

3. What is the project's purpose?

My PhD project aims to understand the farmer and soil microbial interactions. This would involve talking to legume farmers and urban growers through survey, interviews and group discussions. Farmer and urban grower participation would will help me to:

1. Identify soil microbiome related practices
2. Explore ways to make inoculant technologies more useful/successful in the real-world context through focus discussions
3. Co-produce an agenda of user-relevant future research questions and trajectories in soil microbiome research

4. Why have I been chosen?

You have been chosen for this survey because you are involved in farming and related activities with knowledge and insight in the soil microbiome related practices.

5. Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep (and be asked to sign a consent form) and you can still withdraw at any time without any negative consequences. You do not have to give a reason. If you wish to withdraw from the research, please contact Mary Eliza using the details provided later in this information sheet.

6. What will happen to me if I take part? What do I have to do?

If you decide to take part, I will conduct a semi-structured interview with you. Semi-structured interviews are interviews which are more like a normal conversation, in which a number of topics are explored. The interviews will be virtual/on call or in person depending on the Covid-19 situation. The audio will be recorded, and may be transcribed. The first interview will last for around an hour. I may also ask to visit your place of work, and conduct

an interview with you there (only if Covid-19 permits and after completing the risk assessment from the University).

I will also recruit a 'Farmer Advisory Board' for my research which will consist of interested interviewees. The aim of the board being to discuss and explore ways in which the soil microbiome (and rhizobial inoculants) performance can be enhanced. I will ask you to be part of this board in the interview and if you decide to be a member of the 'Farmer Advisory Board', I may ask you to participate in multiple focus group discussions with other interested participants and/or soil researchers as part of this board. Focus groups are small groups (6-10) of people who are interviewed together. The focus groups will be conducted at least 6 times. They will also be recorded and transcribed virtually.

7. What are the possible disadvantages and risks of taking part?

There are no expected discomforts or disadvantages arising from your participation in this study. In line with University of Sheffield Animal and Plant Science Department's risk assessment procedures a full risk assessment has been undertaken to ensure the safety of all participants and staff involved in the research.

8. What are the possible benefits of taking part?

Whilst there are no immediate benefits for those people participating in the project, it is hoped that this work will lead to improved understanding of the soil microbiome and rhizobial inoculants for both participants and researchers alike.

9. Will my taking part in this project be kept confidential?

All the information that we collect about you during the course of the research will be kept strictly confidential and will only be accessible to members of the research team. You will not be able to be identified in any reports or publications unless you have given your explicit consent for this. If you agree to us sharing the information you provide with other researchers (e.g. by making it available in a data archive) then your personal details will not be included unless you explicitly request this.

10. What is the legal basis for processing my personal data?

According to data protection legislation, we are required to inform you that the legal basis we are applying in order to process your personal data is that 'processing is necessary for the performance of a task carried out in the public interest' (Article 6(1)(e)). Further information can be found in the University's Privacy Notice https://www.sheffield.ac.uk/govern/data-protection/privacy/general.

11. What will happen to the data collected, and the results of the research project?

The audio recordings from the interviews will be written up into anonymised transcripts by a University of Sheffield approved transcription service. These transcripts will then be analysed by the research team to achieve the research objectives.

The results of this research may be shared with farming and soil research organisations (such as Legume Technology, Soil Association, PGRO and AHDB). The results may also be shared with national policymakers. They will further be published in academic journals and included in my PhD thesis. They may also be published in local or national media, as well as on the

internet. However, any information you provide is confidential, and no information that could lead to the identification of any individual will be disclosed in any reports on the project, or to any other party. No identifiable personal data will be published.

Any identifiable personal data that is obtained through the research process will be destroyed as soon as possible once it is clear that this will not affect the research purpose.

Due to the nature of this research it is very likely that other researchers may find the data collected to be useful in answering future research questions. For that reason we would like to archive the anonymised transcripts in a data repository provided by the UK Data Service. This process will follow the UKDS guidelines on preparing data for deposition and no identifiable personal data will be stored.

Will I be recorded, and how will the recorded media be used?

The audio recordings of your activities made during this research will be used only for analysis and for illustration in conference presentations and lectures. No other use will be made of them without your written permission, and no one outside the project will be allowed access to the original recordings.

12. Who is organising and funding the research?

The project is funded by the Institute of Sustainable Food, University of Sheffield.

13. Who is the Data Controller?

The University of Sheffield will act as the Data Controller for this study. This means that the University is responsible for looking after your information and using it properly.

14. Who has ethically reviewed the project?

This project has been reviewed and approved by the Sheffield University's Animal and Plant Sciences' department ethics review procedure. The University's Research Ethics Committee monitors the application and delivery of the University's Ethics Review Procedure across the University.

15. What if something goes wrong and I wish to complain about the research?

In the first instance please contact Ellie Harrison or Anna Krzywoszynska who are my PhD supervisors if you wish to raise a complaint about the research using the contact details provided below.

If the complaint relates to how your personal data has been handled, information about how to raise a complaint can be found in the University's Privacy Notice: https://www.sheffield.ac.uk/govern/data-protection/privacy/general.

16. Contact for further information

Contact details for the research team:

- Mary Eliza, PhD student, Department of Animal and Plant Sciences, mary.eliza1@sheffield.ac.uk, 07704749519

- Dr Ellie Harrison, NERC Independent Research Fellow, Department of Animal and Plant Sciences, ellie.harrison@sheffield.ac.uk, 0114 222 4621
- Dr Anna Krzywoszynska, Faculty Research Fellow, Department of Geography, a.krzywoszynska@sheffield.ac.uk, 0114 222 7969

Finally …

Thank you for reading this sheet and taking part in the project. Please keep a copy of this information sheet and the consent form for your records.

Appendix C

Semi-structured interview Questions

Ethics-
Consent from, project details

Demography
Name
Age
Occupation in farming
Years in farming sector

Farming
Farming kind/type
Area of farm
Farm Ownership (Own/tenant)

The introduction
Can you describe your soil and farm? How long have you been farming? What kind of farming do you do? What machines do you use on your farm?

Soil microbiome

Research question- What are the farmers' perceptions about concepts surrounding the soil microbiome?

- How do the farmers assess the soil microbiome physically?

1. Are you familiar with the concepts soil ecology/soil microbiome/ soil life/soil microbes?
2. How important are these concepts to you in your farming?
3. If yes, why do you think the soil microbial life is important?
4. What do you think are signs of soil microbes presence in soil?
5. Do you use any methods for assessing soil quality/ functioning (for instance- Visual Soil assessment of plant roots (legumes) or assessment using Company services-Biolab)?
    - Why do you use this method?
    - How are the results recorded and displayed?

Farming practices

Research question- Do farmers use certain practices to improve the soil microbiome? If so, what are these practices?

- Is it because they think there is a relationship between these practices and the soil microbiome? Do they think this relationship always as positive?

1. Do you follow any practices which you think are better for the soil microbial life (have to be clear about microbial life than their effects on soil quality such as better soil quality)?
   Note to myself- If they answer no to the above question, follow up with next question which includes a list of practices which are thought to have a positive impact on the soil microbiome.
2. Do you do composting, vermicomposting, sillage? (list to be updated)
3. Why do you do these practices, why do you think these practices are important at all?
4. Do you notice any changes to the soil quality after them?
5. How do you assess these changes?

(legumes)

Research question – what are the perceptions of farmers or urban growers about legumes?

- Are there any difficulties associated with growing legumes regularly which they recognize or experience (such as high incidence of diseases – high pathogenic microbe diversity which ultimately leads to less abundance of good bacteria)?

1. Why are legumes important as a crop for you/ why do you grow legumes?
2. Have you ever noticed the root nodules of legumes? Do you look for the red/pink nodules?
3. How often do you grow legumes? Why/ why not grow them often?
4. Do you grow cover crops or leys (if not mentioned earlier)? If yes, why?
5. Do you use cover crops/leys for anything specifically- to sell it (as pasture crops) or use it as a green manure?
6. How often do you grow them? Why/Why not?

Soil inoculants

Research question- Do farmers think that soil inoculants are important (as they are an invention coming from science) and eventually form a part of the soil microbiome?

1. Have you ever heard about soil inoculants (explain what they are-Soil inoculants are microbial, such as bacteria and fungi, preparations that are used as a nutrient source or fertiliser or growth stimulant)?
2. Have you ever used them? Why/why not?
3. How often do you use them? Why/Why not?
4. (If answer yes to question 3) Are there any drawbacks of using them?
5. Do you think they form a part of the local soil microbiome/ local soil microbial life/soil ecology/ soil life?

   Thank you for your time, do you think we missed anything which you would like to discuss?

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Abbreviations and Glossary

CFU – Colony Forming Unit

DF – Degrees of Freedom

DNA – Deoxyribonucleic Acid

g – gram

GFP – Green Fluorescent Protein

GMOs – Genetically Modified Organisms

H-NS – Histone-like Nucleoid Structuring Protein

ICEs – Integrative and Conjugative Elements

IRMS – Isotope Ratio Mass Spectrometry

IS – Insertion Elements

kb – kilobase

L – Litre

LFC – Log Fold Change

ln – Natural log

LR – Lysogenization Resistant

MC – Monomeric Cherry, red fluorescent protein

mg – milligram

MGEs – Mobile Genetic Elements

$MgSO_4$ – Magnesium Sulphate

Mins – Minutes

mL – millilitre

mm – millimetre

mM – millimolar

NFs – Nodulating Factors

ng – nanogram

ORF – Open Reading Frame

P – Phage carrying

PCA – Principal Component Analysis

PCR – Polymerase Chain Reaction

PFU – Plaque Forming Unit

PPFD – Photosynthetic Photon Flux DensityRAST – Rapid Annotation using Subsystem Technology

RBG – Reduction In Bacterial Growth

Rlt – *Rhizobium leguminosarum* bv. *trifolii*

RNA – Ribonucleic Acid

Rpm – Rotations per minute

rRNA – ribosomal Ribonucleic Acid

SOS – Save Our Souls

tRNA-Leu – transfer Ribonucleic Acid

TY – Tryptone Yeast

UV – UltraViolet light

v – Relative competitive fitness

w – Relative competitive fitness

WT – Wild Type

µg – microgram

µL – microlitre