Biodiversity of rhizobia associated with sainfoin (*Onobrychis viciifolia*) in South Africa

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PREFACE

The research conducted and discussed in this dissertation was completed during the period of January 2020 to November 2022 in the Unit for Environmental Sciences and Management, North-West University, Potchefstroom, South Africa.

The research presented in this dissertation represents original work undertaken by the author and has not been submitted for degree purposes to any other university. Appropriate acknowledgements have been added in the text where use was made of the work of other researchers. The referencing style used in this dissertation is in accordance with the requirements of NWU Harvard.

Kelebogile Motlhamme

21/11/2022

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The first words that come to mind when I think of this MSc journey are: Ke sekilwe ke Jeso (I'm leaning on Jesus), Ke dutse ho yena (I am dwelling in Him), Fubeng sa hae ka jeno (On His chest today), Ke ya khatholoha (I find rest), Ke a pere Lerato (I am wearing Love), Ka matsatsi ohle (in all the days), and all I can say is, Thank you, Lord.

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ABSTRACT

Sainfoin (*Onobrychis viciifolia*) is a perennial crop with multiple agricultural and environmental benefits, as well as biotechnological applications. When used as forage, it can remedy the problem of bloating in livestock through condensed tannin production. Studies have shown that *O. viciifolia* extracts also display anti-inflammatory and antimicrobial activity, showing the potential of application in the pharmaceutical industry. Although resistant to drought, *O. viciifolia* is often prone to nitrogen deficiency. This problem can be mitigated by the symbiotic relationship that exists between the roots and rhizobia. These bacteria inhabit the root and facilitate nitrogen uptake by the plant through nitrogen fixation. Little information is known regarding the biodiversity of rhizobia nodulating the *O. viciifolia* root. This study was therefore conducted to isolate and characterise these bacterial species. Unbroken nodules were selected from the roots of three different *O. viciifolia* cultivars, one endemic (Esparsette) and two exotics (Perly and Taja). These nodules were then prepared aseptically and their bacteroids cultivated for molecular identification and phylogenetic analysis. DNA extraction and polymerase chain reaction (PCR) amplification methods were used prior to identification via the NCBI database using the BLASTn tools. The results revealed that in addition to rhizobial microorganisms that inhabit the *O. viciifolia* root nodules there were non-rhizobial microorganisms as well. The bacterial composition varied according to cultivar. The endemic cultivar, Esparsette, grown during this study in a greenhouse, comprised of rhizobial microorganisms from the genera *Rhizobium,* while the Esparsette obtained from a farm in Ficksburg comprised of *Mesorhizobium*. The exotic cultivars comprised of rhizobial microorganisms from the genera *Rhizobium*. The diversity of non-rhizobial microorganisms from the three cultivars included *Agrobacterium, Bacillus, Curtbobacterium, Enterobacter, Paenibacillus, Peribacillus, Pseudomonas*, *Pseudoxanthomonas, Spirosoma* and *Xanthomonas*. These identified isolates were screened *in vitro* for direct plant growth-promoting traits that included phosphate solubilisation, siderophore production, Indole acetic acid (IAA) production and nitrogen fixation. The rhizobial isolates from all the cultivars showed positive results for phosphate solubilisation and nitrogen fixation and varied results for the non-rhizobial isolates. Varied results for siderophore production and IAA production were observed for both the rhizobial and non-rhizobial isolates. It can be concluded that the bacterial population found within the nodules of different sainfoin cultivars comprises a diverse group of symbiotic as well as non-symbiotic microorganisms that are functionally diverse.

Keywords: Nodulating, *Onobrychis viciifolia*, Plant Growth Promoting, Rhizobacteria, Rhizobia, Sainfoin.

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CHAPTER 1: INTRODUCTION

1.1 Sainfoin and its untapped potential

Sainfoin (*Onobrychis viciifolia*) is a perennial legume used as forage or fodder that grows well in warm temperate regions. It is more frost and drought tolerant than alfalfa and clover species (Carbonero et al., 2011). Sainfoin can form symbiotic relationships with several genera of bacteria, *Mezorhizobium* and *Rhizobium,* found in the rhizosphere (Setu et al., 2019). It is due to this relationship that *O. viciifolia* retains the characteristic or ability to convert atmospheric nitrogen into a bioavailable form of nitrogen that can be used by the plant (Roychowdhury et al., 2015). The bacteria that assist the plant in accomplishing this are referred to by the collective name 'rhizobia'. These rhizobia colonise the root hairs and form plant structures called nodules (Setu et al., 2019). It is within these nodules that symbiotic nitrogen fixation occurs, and atmospheric nitrogen is converted to ammonium. This ammonium is then used by the plant to produce proteins (Asefa & Gidago, 2017).

Figure 1-1: Sainfoin (*Onobrychis viciifolia*) (https://www.i-flora.com; https://plantsam.com)

Onobrychis viciifolia can also have positive impacts on the environment. It can increase nitrogen use and minimise nitrogen transit from the soil, thus improving soil quality (Setu et al., 2019). This plantis an open pollinating plant which is pollinated by insects that feed on nectar; therefore, it enhances biodiversity within ecosystems on account of being a food source (Kölliker et al., 2017).

As a fodder, *O. viciifolia* can be mixed with grasses such as timothy hay (*Phleum pratense)* and wheat (*Triticum sp.*) or legumes such as alfalfa *(Medicago sativa)* and white clover (*Trifolium repens)* (Sheppard et al., 2019). When used in livestock nutrition, *O. viciifolia* becomes beneficial to the livestock due to the presence of condensed tannins and flavonoids (Kempf et al., 2015). This is because these compounds increase nitrogen utilisation in the digestive tract, reduce parasitism of the ruminant nematode *Haemonchus contortus* by reducing egg numbers in ruminants' digestive tracts, and decrease bloating which can be fatal (Sheppard et al., 2019). Condensed tannins also have many technological applications that include their use as a coagulant in water treatment; moreover, several researchers (Fraga-Corral et al., 2020; Pizzi, 2019) have reported their anthelmintic potential.

Onobrychis viciifolia generally does not show any severe pest and disease problems as compared to other legumes (Carbonero et al., 2011). For example, alfalfa (*Medicago sativa)* has several insect pests that do not affect *O. viciifolia,* such as weevil (*Hypera postica*) and pea aphids (*Acrythosyphon pisum*) (Issah et al., 2020). This further encourages the cultivation and sowing of *O. viciifolia*.

1.2 Importance of bacterial functional diversity

Due to their versatility and natural availability, microbial agents offer a sustainable and feasible alternative to the use of chemicals in agriculture in terms of soil fortification (Rashid et al., 2012). Singh et al. (2020) explains soil fortification as a sustainable way of improving soil nutrient status. Investigation of naturally occurring microorganisms that are found near the plant root in the rhizosphere aids in the implementation and use of compatible species when choosing inoculum and biofertilisers. The bacterial community composition found in the rhizosphere is influenced by different biotic and abiotic factors (Rashid et al., 2016). The plant itself is the most important determining factor when it comes to which bacterial strains interact with the roots in the rhizosphere due to the influence of the root exudates. The root exudates can facilitate plant–microbe interaction through a chemical response of the microorganism to exudates such as sugars, organic acids and amino acids that lead to root colonisation (Compant et al., 2010). Some of these bacteria positively influence the plant and are referred to as 'plant growth-promoting rhizobacteria' (PGPR) (Igiehon et al., 2017; Etesami et al., 2018). Rhizobia form part of total rhizosphere bacteria (rhizobacteria) that can colonise the root system in the presence of competing bacterial populations and soil microflora and aid in nitrogen fixation. Ultimately, these bacteria enhance or 'promote' the growth of the plant they are in close association with (Goswami et al., 2016). The plant growth enhancement is the function of the bacterial population. This function can only be performed by a particular bacterial population, or a diverse bacterial population with the same function. This 'functional diversity' determines the distribution and the

range of which microbial communities become active in ecosystems (Etesami et al., 2018; Taylor et al., 2020). Functional diversity can also be used to determine ecosystem productivity and vulnerability by various members' diversity and presence (Taylor et al., 2020). It also highlights the multi-dimensional aspect of species traits, for example, nitrogen-fixing rhizobacteria, wherein nitrogen fixation can be seen as a function of rhizospheric microorganisms to mediate the nutritional requirement or lack thereof in soil and aid in plant growth promotion. These groups of selective microorganisms are considered functionally diverse.

1.3 Molecular analysis of bacteria

Even though most soil microorganisms were previously regarded as difficult to cultivate, advances in molecular techniques for the isolation of bacterial strains have allowed for the investigation and discovery of information pertaining to rhizospheric bacterial communities (Koskey et al., 2018). The most used tools for studying the biodiversity of these microbes include polymerase chain reaction (PCR) based methods and sequencing techniques. Molecular methods reveal the diversity of rhizobacteria, including rhizobia, at the genetic, species and genus levels (Zafar et al., 2017; Shetta et al., 2011). Sequencing 16S rRNA is the most common tool used in current microbiology studies and has been used to identify and classify isolates from pure cultures and further determine bacterial diversity in the environment (Koskey et al., 2018). The 16S gene is regarded as a suitable phylogenetic marker, as it has both more variable and conserved areas that can be utilised to distinguish closely related species as well as conserved regions beneficial for revealing phylogenetic links between distant species. (Moura et al., 2020).

1.4 Problem statement

Nitrogen-fixing rhizobacteria and rhizobia are known as the leading bacterial groups that inhabit root nodules and convert inert atmospheric nitrogen to ammonia which can be used by plants (Carbonero et al., 2011). These bacterial groups can include *Rhizobium*, *Bacillus*, *Pseudomonas* and *Azospirillum* species (Rosier et al., 2018). The bacterial composition found in these nodules varies from plant to plant depending on the environment, cultivar, symbiotic genes present and the reciprocal molecular dialog that exists between the plant and bacteria (Burns et al., 2021).

As *O. viciifolia* is a crop that can become prone to nitrogen deficiency, knowing which bacterial members are capable of symbiotic nitrogen fixation and nodulation is important (Carbonero et al., 2011). This knowledge can be applied for plant growth-promotion purposes and implementation in the development of biofertiliser inoculums.

The present study determined the biodiversity of rhizobia associated with exotic Perly and Taja, and endemic Esparsette cultivars of *O. viciifolia*. The study was conducted using soil samples

collected from farmlands and this soil was used for planting the three cultivars, as well as sainfoin root nodules from sainfoin grown on a farm in the Free State. The study focused on the bacterial community found in nodules present on *O. viciifolia* roots.

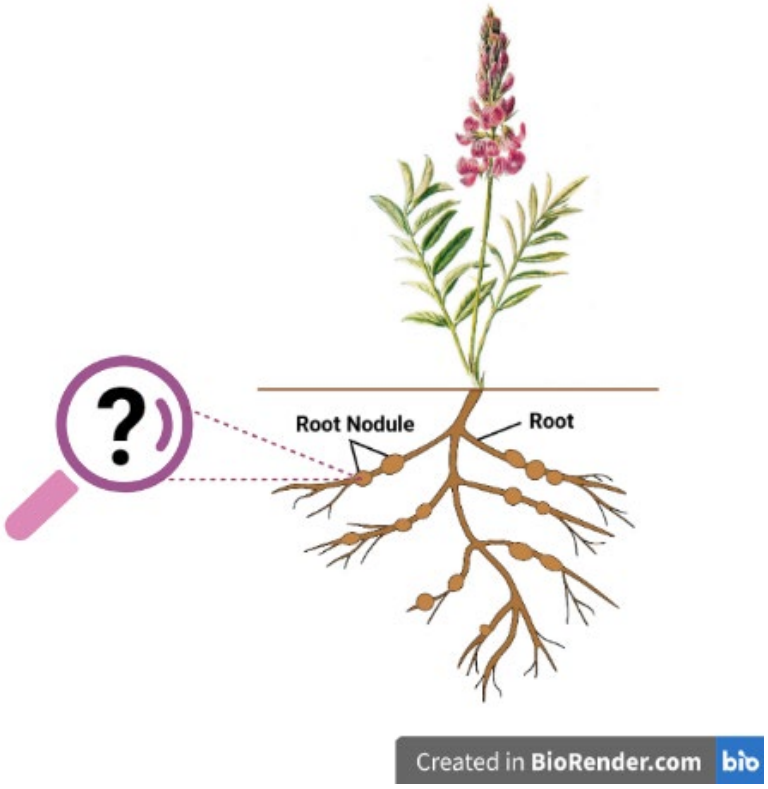

Figure 1-2: Sainfoin (*Onobrychis viciifolia*) taproot and root nodules

1.5 Research aim and objectives

This study aimed to determine the diversity of rhizobia associated with sainfoin (*Onobrychis viciifolia*) in South Africa and to screen these bacteria for their plant growth-promoting potential.

Objectives included:

1. Identifying the rhizobia and rhizobacteria associated with exotic Perly and Taja, and endemic Esparsette *O. viciifolia* cultivars by means of DNA-based molecular techniques.

2. Screening for potential plant growth-promoting properties of the rhizobia and rhizobacteria identified from the exotic Perly and Taja, and endemic Esparsette O. *viciifolia* cultivars.

3. Evaluation of nitrogen-fixing ability of the rhizobia and rhizobacteria identified from exotic Perly and Taja, and endemic Esparsette O. *viciifolia* cultivars.

1.6 Chapter layout

Chapter 1 provides an introductory overview of the study and describes the importance of rhizobial symbiosis with the legume sainfoin (*O. viciifolia*). The use of molecular identification and characterisation is also introduced. Additionally, the problem statement, aim and objectives are outlined in this chapter.

Chapter 2 reviews the available literature on *O. viciifolia* and its associated benefits relating to agriculture and pharmaceutics, together with its potential symbiotic relationship with different rhizobial and non-rhizobial microbes as a legume. This chapter also discusses the use of molecular methods in microbial identification and characterisation. The importance of microbial plant growth-promoting properties is highlighted.

Chapter 3 describes the methods used in this study. This includes the cultivation of bacterial species, molecular procedures such as DNA extraction and PCR amplification, as well as plant growth-promotion traits screening of the identified bacterial isolates.

Chapter 4 describes the results obtained from the molecular identification and characterisation of isolated bacteria. The results are then interpreted and discussed.

Chapter 5 describes the results obtained from the evaluation of plant growth-promoting effects of the identified bacterial isolates. The evaluation of nitrogen fixation of the identified rhizobia and rhizobacteria is included in this chapter as well as a brief literature-based comparison between the identified rhizobial genera pertaining to nitrogen fixation. The results are then interpreted and discussed.

Chapter 6 provides a general discussion and conclusion to the study as well as recommendations for future studies.

A complete list of references is provided at the end of this dissertation.

CHAPTER 2: LITERATURE REVIEW

2.1 Sainfoin

Sainfoin (*Onobrychis viciifolia*) is a perennial forage crop that is classified as a legume (Mora-Ortiz & Smith, 2018). This group of agriculturally important crops comprise lucerne or alfalfa (*Medicago sativa*), clover (*Trifolium spp.*) and birdsfoot trefoil (*Lotus corniculatus*). One outstanding feature about legumes is that they can convert atmospheric nitrogen into ammonium, which is a bioavailable form of nitrogen (Carbonero et al., 2011). This is due to a symbiotic association with bacteria found in the rhizosphere (Sheppard et al., 2019). These symbiont species of bacteria can stem from several genera (Mora-Ortiz & Smith, 2018). Sainfoin has gained attention from researchers due to its many beneficial agronomic, nutritional, and pharmaceutical attributes (Bhattarai et al., 2016).

Sainfoin belongs to the genus *Onobrychis*, which belongs to the *Fabaceae* family, previously called *Leguminosae* (Sheppard et al., 2019). Its English name 'sainfoin' stems from the French 'sain foin', which means 'healthy hay'. *Onobrychis viciifolia* is also known as holy grass, Esparsette and French grass (Amirahmadi et al., 2016; Kaveh et al., 2019).

Onobrychis viciifolia had been cultivated in many parts of the world, however, there has been a decline in recent years (Kaveh et al., 2019). Although *O. viciifolia* is native to South Central Asia, it was introduced to central European soils in the fifteenth century and has since been farmed for many years in different parts of the world including Asia, Africa, Europe, and North America (Mora-Ortiz & Smith, 2018).

2.1.1 Agricultural significance, and pharmaceutical and anthelmintic qualities

One of sainfoin's most prominent qualities is its drought tolerance in low rainfall areas due to its deep taproot system (Carbonero et al., 2011). Its tolerance to most pests and diseases makes it a valuable resource for pollinators, specifically in regions where honey production is a priority (Kells, 2001). *O. viciifolia* can be cultivated for use as both forage and fodder, which means it can be eaten fresh from the ground by grazing animals, as well as harvested and made into hay. It is regarded as one of the most palatable forage crops (Bhattarai et al., 2016). *Onobrychis* sp. produce several compounds in their plant structure, among which tannins are important. These plant tannins are a large group of natural phenolic compounds divided into three main subgroups: hydrolysable tannins, condensed tannins, and phlorotannins (Pizzi, 2019). The specific compound produced by *O. viciifolia* is condensed tannins and is produced in the leaves of this plant (Jones et al., 1994). This production of condensed tannins was viewed as an anti-nutritional

attribute in the past as it leads to decreased forage consumption; however, the voluntary intake of sainfoin by grazing animals was seen to be higher than that of alfalfa (Wang et al., 2006). It has a higher protein content compared to alfalfa due to its amino acid composition. Bloating is a digestive disorder that occurs in livestock such as cattle, sheep, and other domestic ruminants when animals are fed hay forages (Sheppard et al., 2019). It causes the formation of proteinaceous foam in the rumen, thus preventing gas from escaping. This can be fatal and is a serious problem for livestock breeders and farmers (Bhattarai et al., 2016). *O. viciifolia* consumption increases niterogen utilisation in the rumnen and remedies the bloating experienced by livestock (Sheppard et al., 2019; Kelln et al., 2020).

Condensed tannins produced in sainfoin reduce protein degradation in the rumen and this leads to lower ruminal ammonia concentrations (Jones et al., 1994). Therefore, condensed tannins prevent inefficient protein degradation in livestock (Bhattarai et al., 2016). Furthermore, *Medicago sativa* and *O. viciifolia* can be mixed and used as fodder to improve digestive health. Condensed tannins from *O. viciifolia* also reduce ruminal urine nitrogen losses and plasma concentration of essential amino acids (Kelln et al., 2020). The presence of condensed tannins and flavonol glycosides in *O. viciifolia* can also remedy nematode infections. *O. viciifolia* has an inhibitory effect on the agriculturally important sheep nematode, *Haemonchus contortus* (Bhattarai et al., 2016) and nematode egg excretion; it increases host resilience in goats to gastrointestinal nematodes, thereby providing an alternative to pharmaceutical treatments (Carbonero et al., 2011).

The condensed tannins produced by sainfoin can also be used in various industries such as leather, food, and pharmaceutics (Pizzi, 2019). When these tannins are present in edible plants and herbs they may prevent cancers, as they also contain epigallocatechin gallate which has inflammatory and antioxidant properties. Condensed tannins can also be used as potential medicinal agents and metal chelators (Phiwchai et al., 2017). Other significant properties include its use as antiseptic, antiviral, anti-carcinogenic and anti-inflammatory medications, which shows its potential applications in the pharmaceutical and nutraceutical industries (Pizzi, 2019; Fraga-Corral et al., 2020). Due to the complexation and antioxidant behaviour of sainfoin, its extracts can be used in the treatment of intestinal problems such as ulcers, gastritis, and irritable bowel syndrome (Fraga-Corral et al., 2020). Sainfoin is commonly grown with red clover (*Trifolium pratense*) and alfalfa (*Medicago sativa*) (Rufino-Moya et al., 2019). However, unlike other forage legume crops, sainfoin tends to develop nitrogen deficiency (Thies et al., 2001).

2.1.2 Diversity of rhizobia associated with *Onobrychis viciifolia*

Onobrychis viciifolia can form symbioses with rhizobia, which cause the plant to develop nodules, and with mycorrhizal fungi, which include a wide variety of fungal species. The plant benefits from

increased availability and mobilisation of nutrients such as nitrogen and phosphate (Carbonero et al., 2011). These symbiotic interactions occur between bacterial and fungal species found in the rhizosphere and legume plant roots. A specialised structure forms, called the nodule, and bacteria use the nitrogenase enzyme to reduce atmospheric nitrogen to ammonia (Gourion et al., 2015). The plant then uses this ammonia to produce plant proteins and amino acids. In exchange, the plant provides the rhizobia with photosynthetic by-products such carbohydrates (Schwember et al., 2019). Rhizobia infection typically occurs through the root hairs (Suzaki et al., 2015). A high level of specificity is present in the interaction between rhizobia and the host. Therefore, a mutual molecular dialogue between the host plant and the rhizobia is necessary for the successful infection of the roots by rhizobia (Schwember et al., 2019). Numerous bacterial genera and species may nodulate the same host species. Bacterial populations that can enter into symbiosis with the genus *Onobrychis* belong to the genera *Rhizobium*, *Mesorhizobium*, *Neorhizobium*, *Phyllobaterium*, *Bradyrhizobium* and *Ensifer* (*Sinorhizobium*) as well as members from the *Hyphomicrobiaceae* and *Methylocystaceae* families (Lafay & Burdon, 2007; Zafar et al., 2017). These bacteria are only a few of the members of microbial communities that exist in symbiosis with these plants. Although these microorganisms may form symbiotic relationships with the plants, not all of them are nodule-forming, nitrogen-fixing bacteria (Kawaka et al., 2018).

2.2 The symbiotic relationship driven by nitrogen fixation

The symbiotic relationships between legume plants and rhizobia are the most significant contributor to nitrogen fixation in most terrestrial ecosystems. In such a relationship, nitrogen fixation is carried out by root nodule bacteria in association with legumes (Luciński et al., 2002; Liu et al., 2018). Through this process, the plant receives readily usable ammonium, while the rhizobia receive a food source in the form of simple sugars from the plant (Reinprecht et al., 2020). An increase in rhizobia populations in the rhizosphere is a response to the release of nutrients by the legume hosts. One of the main reasons for insufficient nitrogen supplies is that it is present in the soil but in its organic forms and is thus unusable by the plant (Liu et al., 2018; Schwember et al., 2019). Leguminous plants can acquire a major portion of nitrogen directly from atmospheric nitrogen through bacterial nitrogen fixation (Reinprecht et al., 2020).

Chemical fertilisers can be replaced with biological nitrogen fixation (BNF), which typically takes place at moderate temperatures and involves nitrogen-fixing microorganisms (Lindström & Mousavi, 2019). Biological nitrogen fixation explains the process in which microorganisms convert atmospheric nitrogen into a form of nitrogen that can be taken up and used by the plant which that microorganism is in association with (Mus et al., 2018). Unlike chemical fertilisers, BNF does not have adverse effects on the environment such as eutrophication and the release of greenhouse gasses such as methane (Suhag, 2016) Nitrogen-fixing organisms are generally

categorised as symbiotic nitrogen-fixing bacteria, which are rhizobia found in the rhizosphere that form a symbiosis with leguminous plants and non-symbiotic nitrogen-fixing bacteria such as cyanobacteria, *Azospirillum* and *Azotobacter* (Rosier et al., 2018). Symbiotic nitrogen-fixing rhizobia within the class Alphaproteobacteria infect and establish a symbiotic relationship via the roots of leguminous plants (Koskey et al., 2018).

Two of the biggest groups of nitrogen-fixing bacteria associated with legumes are Proteobacteria and *Frankia* (*Actinobacteria*) (Suzaki et al., 2015). Rhizobia belonging to Proteobacteria are usually rod-shaped microorganisms that do not produce spores and are aerobic and motile. *Frankia* are slow-growing filamentous bacteria that form hyphae colonies without an aerial mycelium. They also feature vesicles and spores, which are distinctively differentiating developmental components that are essential to their survival (Suzaki et al., 2015; Koskey et al., 2018). Bacteria of the genus *Bradyrhizobium*, *Rhizobium* and *Sinorhizobium* can also induce the process of nitrogen fixation in legumes (Moura et al., 2020).

Only some prokaryotes can ‘fix’ atmospheric nitrogen by using nitrogenase, an enzyme that catalyses the conversion of N_2 gas to ammonia, and diazotrophic microorganisms from the bacterial or archaea domains can initiate BNF (Mus et al., 2018; Bloch et al., 2020). Despite the phylogenetic and ecological diversity among diazotrophic bacteria and their hosts, a synchronised interaction is always a prerequisite between the microbial entities and the host plant to achieve a successful nitrogen fixation system (Bloch et al., 2020). This procedure is crucial, as it lessens plants' need for nitrogen fertilisers, and by extension, agriculture (Zheng et al., 2019). Free-living diazotrophs belong to a small fraction of the plant rhizosphere ecosystem, namely, to Alphaproteobacteria (*Rhizobia*, *Bradyrhizobia*), Betaproteobacteria (*Burkholderia*, *Nitrosospira*), Gammaproteobacteria (*Pseudomonas*, *Xanthomonas*), Firmicutes, and cyanobacteria (Li et al., 2019). Rhizobia, a group of nitrogen-fixing soil bacteria that comprises the genera *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, and *Sinorhizobium*, coexist symbiotically with legume roots to produce nodules, a differentiated specific structure in which nitrogen fixation occurs (Mus et al., 2018). Biological nitrogen fixation is an important natural system for absorbing atmospheric nitrogen and converting it into a bio-available form of nitrogen through enzymatic reduction carried out by plants and their bacterial associations (Mus et al., 2018).

2.2.1 Environmental factors affecting nitrogen fixation

A plant can attain the needed nitrogen supply through interactions with endo-symbiotic and endophytic symbionts through BNF, giving the plant the competitive advantage over plants that

do not fix nitrogen (Zheng et al., 2019). Free-living and symbiotic diazotrophs can engage in a variety of relationships and interactions with their host plants (Mus et al., 2018). Biological nitrogen fixation that occurs in the plant–rhizobia symbiotic association is mediated by endosymbiotic contact (Bloch et al., 2020). In rhizobia and legumes associations, the majority of microbial species that associate (i.e., have an endo-symbiotic relationship) with Fabaceae family legumes are Alphaproteobacteria (Lardi & Pessi, 2018). However, nitrogen fixation is a sensitive process influenced by nutrient and environmental conditions.

The nitrogenase enzyme, which is comprised of the nitrogenase protein and other related proteins and non-proteins, is sensitive to the presence of oxygen (Mus et al., 2018). Facultative anaerobes such as *Klebsiella* spp. and obligate anaerobes such as *Clostridium* spp. are also capable of fixing nitrogen, but only when oxygen is not found in the environment (Masson-Boivin & Sachs, 2018; Therien et al., 2017).

Biological nitrogen fixation is regulated by nitrogen supply and often responds to nitrogen demand or deficiency (Zheng et al., 2019). Biological nitrogen fixation is often active where and when nitrogen supply is low relative to other resources and nutrients. In addition, BNF is often suppressed following nitrogen additions through fertilisation (Li et al., 2019). Moreover, the addition of other nutrients such as water, light or phosphorous to an ecosystem can induce a demand for nitrogen (Li et al., 2019). Thus, additions of these limiting resources to the environment can stimulate enhanced nitrogen fixation.

2.2.2 Nitrogen fixation in legume establishment

Plants have a sophisticated microbiome that aids in the assimilation of nutrients, development, and defence. In legumes, nitrogen-fixing microbial partnerships are effective and well understood (Moura et al., 2020). Simple and complex sugars, amino acids and organic acids are all present in complex amounts in root exudates. The quantity and types of exudates will vary depending on the plant genotype and growth stage, soil texture, soil nutrients, and, most critically, the rhizosphere microbial populations (Lardi & Pessi, 2018). In turn, the rhizosphere's root exudate composition can affect the soil's microbial population and the availability of macro- and micronutrients, particularly nitrogen and phosphorus. Additionally, the root exudate can attract distinct bacterial and eukaryotic populations (Lardi & Pessi, 2018), for instance, pea plants emit homoserine to attract *Rhizobium* sp. (Calatrava-Morales et al., 2018). Additionally, plants can protect themselves by secreting phytochemicals that can stop the growth of specific microbial organisms (Rashid et al., 2016). The capacity to withstand these antagonistic compounds may be a key factor in a microbe's capacity to live inside the plant (Kurk, 2019). Legumes contribute a considerable quantity of nitrogen to both natural and developed environments, and the nitrogen

fixed by perennial forage legumes can be as high as the amounts of nitrogen fertilisers used in traditional agricultural methods (Andrews & Andrews, 2017).

The development and activity of nitrogen-fixing plants are restricted by several environmental constraints (Khalid et al., 2020). The process of nitrogen fixation symbiosis is closely tied to the physiological status of the host plant. Therefore, if limiting factors such as salinity, unfavourable soil pH, nutrient deficiency, mineral toxicity, temperature extremes, insufficient or excessive soil moisture, and insufficient photosynthesis impose restrictions on the vigour of the host legume, a competitive and persistent rhizobial strain is not expected to express its full capacity for nitrogen fixation (Khalid et al., 2020). These factors can also be viewed as environmental stresses experienced by legume nodules and their rhizobial symbiotic relationship, and a single stressor may have multiple effects. For instance, salinity may act as a water stress that affects the rate of photosynthetic respiration, or it may directly affect nodule metabolism (Etesami et al., 2018; Reinprecht et al., 2020). Lands with limited rainfall, temperature extremes, acidic soils with low nutritional status, and poor water-holding capacity pose the greatest challenges for rhizobia (Lardi & Pessi, 2018). Combining stress-tolerant cultivars with stress-tolerant rhizobia is important to reduce the impact of these pressures. Additionally, one method to lessen abiotic stressors on legumes is to co-inoculate rhizobia with other plant growth-promoting rhizobacteria (PGPR) species such *Pseudomonas*, *Azotobacter*, *Bacillus*, *Erwinia* and *Serratia* species (Benito et al., 2017).

2.3 Nitrogen-fixing bacteria

Numerous phyla contain nitrogen-fixing bacteria, and several of these phyla are known to associate with plants to fix nitrogen (Igiehon et al., 2018). Plants have created a variety of reciprocal strategies to ‘team up with’ and accommodate diazotrophs to absorb atmospheric nitrogen. The molecular dialogue, loose relationships with free-living nitrogen fixers, intercellular endophytic associations, and endosymbiosis are the main factors that impact how close a bacterial symbiont and plant host are to one another (Lindström & Mousavi, 2019).

The simplest type of nitrogen-fixing symbiosis involves interactions between plants and associative nitrogen-fixing bacteria, which are regarded as a subgroup of PGPR (Masson-Boivin & Sachs, 2018). These associative bacteria use chemotaxis to colonise the rhizosphere of many plants in response to root exudates. They can influence plant resource acquisition (nitrogen, phosphorus, and vital minerals), crop output, and growth due to their closeness to the root (Lindström & Mousavi, 2019).

Numerous diazotrophic bacterial species have developed beyond surface colonisation to grow and reproduce inside plant tissues without causing harm or evoking strong immune responses (Bloch et al., 2020). Due to their close associations to plant tissues, these bacteria are categorised as endophytes. Bacterial endophytes are common and have been isolated from tissue that has been surface-sterilised from practically every plant studied to date. They may be mutualistic or parasitic in their interactions with their hosts, and their affiliation may be obligatory or facultative (Gadhave et al., 2018). At the location of lateral root emergence, they often enter plant tissues through fractures or naturally occurring apertures. Research on bacterial endophytes has primarily concentrated on measuring the quantity of nitrogen fixed and identifying the diazotrophs (Gadhave et al., 2018), therefore, little is understood about the molecular mechanisms involved in establishing and maintaining the partnership. Bacterial endosymbionts are housed inside plant cells within the membranes of the plant after being ingested from the environment. Effective symbiosis development and maintenance depend on genetic factors in both the bacteria and the plant (Andrews & Andrews, 2017). Recognition, penetration, induction of host cell division, and differentiation of the endosymbiont lead to fully compatible symbiosis (Pankievicz et al., 2019).

Rhizobia are bacterial symbionts of legumes that use biological nitrogen fixation to fix atmospheric nitrogen (Schwember et al., 2019). Nitrogen molecules that the plant can absorb are created by rhizobia through the metabolism of atmospheric nitrogen, typically in the form of nodules, which are specialised structures on plant roots. Rhizobia benefit from carbon substrates derived from photosynthesis in exchange (Li et al., 2019). Nitrate fertilisers, which supply nitrogen to crops in the form of nitrogen, are expensive for both farmers and the environment. The manufacture of nitrogen fertilisers results in the release of greenhouse gases and consumes a significant quantity of non-renewable fossil fuel energy. The best researched examples of non-legume crops that benefit from rhizobia as endophytes are economically significant rice and wheat, although rhizobia can also function as non-symbiotic plant growth-promoting bacteria (PGPB) (Lindström & Mousavi, 2019; Calvo et al., 2019).

Rhizobia were formerly assumed to only comprise a part of the Alphaproteobacteria, namely the order Rhizobiales, which also contains many species that are not microsymbionts of legumes. However, some surprising results from the examination of the microsymbionts of wild legumes led to the identification of rhizobia from the class of Betaproteobacteria (Moura et al., 2020; Hassen et al., 2020).

Rhizobia and other PGPB, as well as those responsible for plant and animal diseases, are all part of the large groupings of gram-negative bacteria known as alpha- and betaproteobacteria (Dai et al., 2018; Moon et al., 2018). Legumes can be nodulated by a wide variety of bacteria, a fact

which has been underappreciated. Novel rhizobia species are still being described, including strains from unusual genera (Boakye et al., 2016; Hassen et al., 2020).

2.3.1 Rhizobial and non-rhizobial bacteria

Rhizobia occur in the nodules of both wild and domesticated legumes (Andrews & Andrews, 2017). Rhizobia, which include members of the genera *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Ensifer* and *Azorhizobium*, are principally responsible for root nodulation in addition to fixing atmospheric nitrogen, a significant component of the global nitrogen cycle (Gourion et al., 2015; Moura et al., 2020). Root nodules contain a variety of non-rhizobial bacteria in addition to strains that can be regarded as 'rhizobial' bacteria within its bacterial community population that have a significant impact on the survival, nodulation, and grain output of the crop (Sharma et al., 2020). *Methylobacterium* sp., *Agrobacterium* sp., *Phyllobacterium* sp. and *Burkholderia* sp. are among the non-rhizobial symbionts that have been identified and classified as belonging to the alpha- and beta-subclass of Proteobacteria. They can also form nodules and fix nitrogen in legume roots (Leite et al., 2017). Studies on root nodules have also identified Actinobacteria, *Microbacterium* sp., Firmicutes, *Bacillus* sp. and *Paenibacillus* sp. Through nitrogen fixation and phosphate solubilisation, these bacteria have positive effects on the host plants, promoting plant growth and increasing the availability of nutrients for the synthesis of vital biomolecules such as nucleic acids, amino acids, and proteins. This is reflected in improved plant growth (Benito et al., 2017; Sharma et al., 2020).

2.4 Nodule formation and host specificity

Legumes produce flavonoids that 'tell' rhizobia that the plant is looking for symbiotic bacteria when the soil nitrogen level is low (Suzaki et al., 2015). As can be seen in Figure 2-1, the plant is stimulated to produce 'deformed root hairs' because of the release of the Nod factors – these are lipochitooligosaccharides which are signalling molecules produced by rhizobia in response to flavonoids produced by the root, by rhizobial microorganisms (Liu et al., 2018). Rhizobia then create an infection thread that allows them to get past the root hairs and into the root cells. Specialised cell division takes place while the rhizobia are inside the root cells, resulting in the formation of the nodule (Suzaki et al., 2015). Rhizobia transform ambient nitrogen into ammonia, which the plant directly uses to synthesise amino acids and nucleotides, while the plant provides the bacteria with carbon sources, resulting in the establishment of symbiosis (Liu et al., 2018).

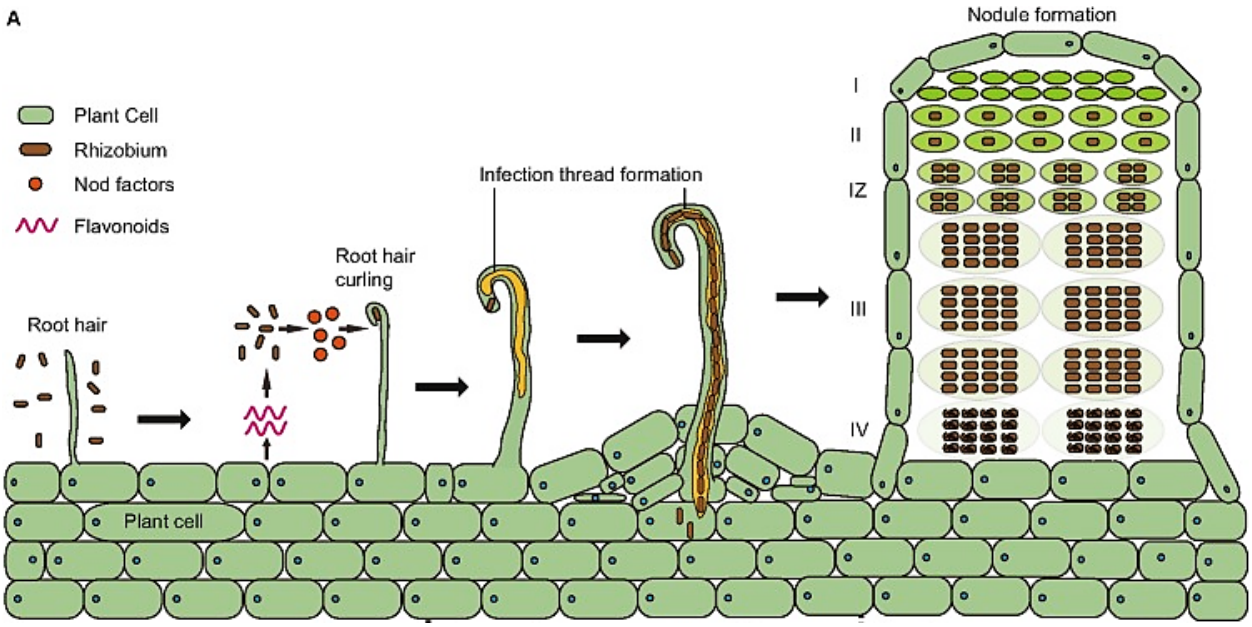

Figure 2-1: Figure showing root nodule formation (Wang et al., 2018)

The presence and sufficient colonisation of rhizobia in the soil is the first necessary step for nodule formation, as the bacteria and legume specification is highly selective (Benizri et al., 2001). In some cases, only a particular rhizobacterial species can form nodules on a particular legume species (Cooper, 2007).

The capacity of certain rhizobia species to develop nodules on particular legumes is known as host specificity (Andrews & Andrews, 2017). This results in a successful pairing of the legume and rhizobia, which promotes the production of nodules (which are deeply red within) and nitrogen fixation. Ineffective nodules (green or white interior) or an absence of nodules will result from an incompatible rhizobium–legume relationship, and nitrogen fixation will not take place (Gourion et al., 2015). The structure of the Nod factor and distinct root exudates (flavonoids) convey host specificity and symbiotic interaction (Andrews & Andrews, 2017). This is improved by bacterial recognition of the flavonoids produced by a host species, which gives the right flavonoid the chance to induce the expression of symbiotic genes in a specific rhizobial strain(s). Rhizobia produce a variety of Nod factors, and plant selection adds a second level of specificity to the relationship and offers the chance for plant–rhizobia choice (Rosier et al., 2018).

Phylogenetically, legumes and rhizobia are varied. No single rhizobial strain will form an association with a single legume. At both the species and genotypic levels, specificity exists (Wang et al., 2012). The same bacterial strains may infect and nodulate on one host plant but not on another. Additionally, incompatibility frequently occurs at later stages of nodule growth,

resulting in large differences in nitrogen-fixing efficiency across various plant–bacteria combinations (Wang et al., 2018; Martínez-Romero, 2009). Symbiotic specificity is the outcome of shifting signals from both the host and the bacterial sides; as a result, numerous recognition mechanisms have developed throughout the co-adaptation process (Wang et al., 2012). To create tools for genetically altering the host or bacteria to increase nitrogen fixation efficiency, it is imperative to understand the genetic and molecular foundation of symbiotic specialisation (Andrews & Andrews, 2017). Therefore, for the purpose of increasing the production of legumes, it is crucial to identify rhizobia strains in the fields of farmers and verify their ability to fix nitrogen, along with cross-inoculation (Kölliker et al., 2017).

2.4.1 Factors affecting nodule formation

If the environment and the plant are in poor condition, nitrogen-fixing rhizobia cannot reach their full potential in fixing nitrogen. The legume plant's functional status and the ideal environmental conditions that support the macro- and microsymbionts are important factors in the nitrogen fixation process (Etesami et al., 2018; Khalid et al., 2020). Rhizobia that fix nitrogen have varying levels of environmental sensitivity. The host plant and symbiotic rhizobia may be affected by environmental stressors (Martínez-Romero, 2009).

Plant root exudates have an impact on the rhizosphere's biological activities, as they include carbon compounds that draw in and promote microbial growth (Schwember et al., 2019). Closer to the root, microbial population sizes and activity levels rise. Because root-associated bacteria can change root exudation, it might be challenging to ascertain which provides what to the other (Lafay & Burdon, 2007). For instance, flavonoids may influence 'non-target' rhizobacteria and affect their behaviour, thus modifying the structure of the rhizosphere microbial population (Schwember et al., 2019). Although the overall quantity of the soil microbial population is considerable, the phylogenetic diversity is more constrained in the rhizosphere. Resources (exudates) in the rhizosphere support more active microbial communities (Rashid et al., 2016). At the plant–soil interface, population diversity is further reduced in the rhizoplane. Although less frequently discussed, exudation also significantly alters soil physical properties, which may further favour features that are rhizosphere-competent (Liu et al., 2018). There are intricate and reciprocal interactions between soil, plants and microbes that need to be considered. Some plant exudates serve to repel bacteria, while others serve as attractants to the microbes (Compant et al., 2010). The types of plants and microorganisms, as well as their physiological status, have an impact on the composition of these exudates. Additionally, these exudates encourage symbiotic relationships that are advantageous for plants and prevent the establishment of rival plant species (Martínez-Romero, 2009).

Actinobacteria, Bacteroidetes and Proteobacteria are members of these three major phyla, which co-occur in the bacterial root microbiota across a variety of soil types and diverse plant hosts (Benizri et al., 2001). This root-associated bacterial construct is built quickly a few days after seed germination and is largely drawn from the extremely diverse bacterial soil biome surrounding roots (Suzaki et al., 2015). With less variance discernible at the higher phylum rank, soil type is also one of the factors driving diversification of the bacterial root microbiota at low taxonomic ranks (at the genus level). However, root exudates are believed to be crucial in triggering substrate-driven competition between soil-dwelling microorganisms and differential proliferation of those bacteria for root colonisation (Benizri et al., 2001; Schwember et al., 2019).

The bacterial communities found in the rhizosphere are supported by the carbon substrates produced by the plant (Carbonero et al., 2011). A portion of the bacterial species found in the rhizosphere colonise roots as bacterial endophytes or as epiphytes on the root surface (rhizoplane) (Liu et al., 2018). Proteobacteria members in particular, are frequently found abundant in root and rhizosphere compartments, and diazotrophs from this phylum have evolved the capacity to create root nodule symbiosis, a type of mutualistic interaction with plant roots (Suzaki et al., 2015). In contrast to the taxonomically diverse root and rhizosphere associated bacterial communities that make up a network of microbe–microbe and plant–microbe associations, the root nodule symbiosis defines a highly specific plant–microbe interaction in which the host chooses the compatible nitrogen-fixing soil bacterium for intracellular infection, frequently via plant-derived infection threads, and then accommodates and configures this inside nodule cells (Schwember et al., 2019).

By creating a low-oxygen, carbon-rich environment inside nodules, the host enables bacteria to begin nitrogen fixation upon endocytosis (Lindström & Mousavi, 2019). Utilising signals from the shoot, the legume host regulates the frequency of infection episodes and nodulation. Due to symbiotic nitrogen fixation, legumes can flourish in environments with low nitrogen supply (Wang et al., 2018).

2.5 Microbial plant growth promotion in the rhizosphere

2.5.1 Significance of soil fortification using microorganisms

Soil quality and fertility are important factors that can influence the growth and health of vegetation (Rashid et al., 2016). Therefore, by increasing organic matter, soil aggregates will be increased, leading to the restoration and fortification of degraded soils. Soil structure degradation mainly occurs due to the loss of organic matter through land use changes, poor farming methods and

intensive soil management practices without rehabilitation (Rashid et al., 2016). This removal of organic matter leads to loss of soil fertility and soil structure. Crop production and crop yield depend on the nutrients available in the soil and good soil structure. Important nutrients such as phosphorus, potassium, iron and nitrogen play an important role in soil fertility and ultimately crop production (Kumar et al., 2018). As the demand for agriculture increases with an increasing population, chemical fertilisers are being used in ever greater amounts to remedy the nutrient deficiency in soils (Imran, 2021; van Dijk et al., 2021). However, the use of these fertilizers has led to air pollution and water eutrophication, and they contain toxic chemicals that can cause skin allergies and infections (Rashid et al., 2016). An alternative method to chemical fertilisers that farmers and agriculturalists have practiced is the use of biofertilisers. The use of biofertilisers is beneficial in soil nutrient balance as well as mitigating the release of greenhouse gasses (Suhag, 2016). Microorganisms that occur naturally in the soil and are introduced to the farming environment in the form of biofertilisers are a possible solution to increasing nutrients in the soil and restoring soil structure and fertility. Materials released from dead animal and plant matter increase soil organic matter and lead to improved soil structure and increased fertility. Fungi and bacteria can mineralise nutrients such as phosphorus, potassium, and nitrogen, leading to increased soil nutrient content (Rashid et al., 2016; Suhag, 2016).

2.5.2 Mechanisms employed by microorganisms to enhance the availability of nutrients

Bacteria play a role in increasing soil aggregates and nutrients, which is key to improving soil quality. They are present in the rhizosphere as single-celled, micro-colonies or biofilms in the moist areas of soil pores (Rashid et al., 2016). Some of these bacteria belong to the Rhizobiaceae family and are known to form nodules and fix nitrogen. Bacteria found in the rhizosphere are also responsible for the conversion of phosphorus from its organic to its inorganic state, along with the formation of siderophores to increase the phosphorus content in the soil. The production of siderophores, which according to Kumar et al. (2018) is an organic compound that chelates metals and increases their availability in the rhizosphere, can make iron soluble from organic matter available for plant use.

2.5.3 Using microorganisms to help crops withstand stress

According to Vimal et al. (2017), the main aim in agriculture is to initiate methods that will result in increased crop production as well as replenish nutrients, with the goal of agricultural sustainability. The effect of agricultural stress influences crop yield and production. The continuous effects of different agricultural stressors, be it biotic or abiotic, influence soil fertility (Kumar et al., 2018). Examples of abiotic stressors are heavy metal stress, drought, and salinity. These stressors can influence biotic stressors such as plant pathogens and pests, and reduce

crop productivity (Kumar et al., 2018). The effects of these stressors affect the plant structure, plant chemistry and the plant's gene expression. Ultimately, these effects can lead to soil infertility and the loss of beneficial soil microorganisms (Kumar et al., 2018). Critical supervision of the already available fertile land and striving towards abatement of the environmental stress can lead to optimum productivity in agriculture (Etesami et al., 2018; Vimal et al., 2017). A possible solution towards reaching optimum productivity in agriculture is by incorporating microorganisms in plant–root interactions to increase crop production and soil structure and fertility (Etesami et al., 2018). Microorganisms that occur in plant associations, including mycorrhizae and bacteria, increase the growth of plants and help the plants withstand various stressful agricultural conditions. Introducing these microorganisms can benefit and help improve crop yield and production.

2.5.3.1 Mechanisms used by microorganisms to help plants manage agricultural stress

Abiotic stress

Drought is an example of an abiotic stressor that affects South Africa and has negative effects on plant growth and crop production. During drought, the plant's water holding capacity and nutrient intake is affected, causing ineffective growth and protein synthesis within the plant (Etesami et al., 2018). Limited water content in crops caused by drought compromises the cell membrane structure and decreases cell division ability (Kumar et al., 2018). According to Vimal et al. (2017), drought can also change the amount of chlorophyll in plants, thus compromising the process of photosynthesis. Plant associations with microorganisms incorporate processes that help plants withstand the detrimental effects of drought and improve conditions for plant growth. Microorganisms that are beneficial to crops populate the rhizosphere and increase plant growth by using different processes such as producing phytohormones such as indole-3-acetic acid, which regulates plant growth by affecting the extent of stem elongation and produces abscisic acid that is responsible for the increase of drought resistance in plants (Ngumbi & Kloepper, 2016; Kumar et al., 2018). Plant growth-promoting rhizobacteria are responsible for the production of hormones in plants that increase growth along with cell division processes when experiencing environmental stress Etesami et al., 2018).

Salinity in soil suppresses plant growth by affecting germination and disturbs the physiological and chemical balance of plants (Vimal et al., 2017; Kumar et al., 2018). The increase of sodium and chlorine ions can be toxic to plants and disturb the nutrient balance in the soil, causing negative effects on the growth of plants. This becomes relevant for a country such as South Africa where chlorination is a significant step in water purification. If not handled properly, some of the chlorine may end up accumulating in the environment and irrigation systems. Studies have indicated that introducing plant growth-promotion microorganisms to plants decreases the

negative effects of salts on plant growth. These microorganisms can promote the growth of plants affected by salinity by using different processes (Kumar et al., 2018). These processes involve the production of phytohormones which help regulate the growth of plants. One of the most important processes employed by PGPR is decreasing the re-occurrence of microorganisms that cause plant diseases. Using PGPR along with bacterial strains such as *Pseudomonas* helps plants withstand the harmful conditions of environmental stress and increases the growth of plants experiencing saline stress (Kumar et al., 2014). Etesami et al. (2018) suggest that introducing PGPR to plants and soil can increase plant and root length, the total organic matter, water content, photosynthesis efficiency under salt stress conditions and enhance salt stress tolerance.

Increasing industrialisation has caused an increase in heavy metal contamination, which negatively impacts crop and human health (Kumar et al., 2018). Some of these metals are required, in small amounts, in the environment for chemical processes performed by plants, and if their levels elevate, they suppress the development of beneficial microorganisms in soils (Etesami et al., 2018). A large variety of microorganisms exist that can withstand high levels of heavy metal content in the soil and increase the growth of plants (Kumar et al., 2018). According to Etesami et al. (2018), heavy metal tolerant PGPR can increase the growth of plants experiencing heavy metal stress and have simultaneously reduced the harm and destruction caused in plants experiencing heavy metal stress. The production of phytohormones regulates different chemical processes that increase plant growth and remedy the effects of exposure to heavy metal stress. The use of PGPR to combat heavy metal toxicity has led to an increase in plant biomass by improving the uptake of nutrients such as nitrogen and phosphorus, helping plants tolerate heavy metals through the reduction of metal movements through the roots to the leaves and ameliorating metal toxicity through bio-absorption (Kumar et al., 2014; Etesami et al., 2018).

Biotic stress

The roots of plants are susceptible to increased populations of soil disease-causing microorganisms as well as beneficial microorganisms. Excretions of roots and different chemicals synthesised by plants draw a diversity of microorganisms. Disease-causing microorganisms such as viruses and bacteria trigger the destruction of soil fertility and crop production. Biotic stress elements involve the disturbance of nutrient balance and structural abnormalities (Pandey et al., 2017). Some plants can alter their gene expression to allow them to overcome stress by adapting to the environment around them (Kumar et al., 2018). This has led to a reliance on beneficial microorganisms for the prevention of pathogen prevalence. These beneficial microorganisms suppress the growth of pathogenic microorganisms and can thus be used to control the growth of pathogens, introducing a new alternative to the use of chemical fertilisers and pesticides. The

use of these microorganisms is more convenient for farmers, as it is affordable and an effective way to manage disease-causing microorganisms by providing defensive mechanisms that strengthen the cell wall and increase metabolites (Kumar et al., 2018). Co-inoculation of PGPR along with mycorrhizal fungi amend the detrimental effects of biotic stressors. The PGPR form symbiotic relationships with plants and increase their stress tolerance by inhibiting the growth of pathogens (Vimal et al., 2017). These microorganism associations increase the growth of plants while simultaneously safeguarding them from phytopathogens as well as deficiencies that result when undergoing biotic stress. They also increase nutrient mobilisation and reduce the success rate of diseases; thus, these relationships and associations are important in the environment when aiming at successful agricultural development (Kumar et al., 2018).

Applying inorganic fertiliser to the soil is a popular way to replace nitrogen and improve heavy-metal tolerance in soil environments. However, the cost of nitrogen and phosphoric fertilizers has soared, especially in developing nations (Etesami et al., 2018). Therefore, it can be difficult for farmers to add additional nitrogen and phosphorous fertilizers to the soil to prevent nutritional deficits. Utilising PGPR and rhizobia is useful for sustainable agricultural operations given the detrimental environmental effects of artificial fertilizers and rising expenses (Goswami et al., 2016). The utilisation of microorganisms with the capacity to solubilise mineral and organic phosphorus or fix nitrogen is thus one area of growing interest (Goswami et al., 2016).

Plants can be inoculated with specific PGPR to boost the native population, which will mobilise phosphorus from scarce sources and enhance plant nutrition (Hussain, 2017). Utilising mixed inoculants is another emerging technology or strategy that aims to stimulate plant development by fusing various microorganisms' unique mechanisms. Co-inoculation or mixed inoculants have a huge potential to increase crop health and productivity and can produce good yields (Benito et al., 2017; Hungria et al., 2020).

Rhizobium and phosphorus-solubilising bacteria co-inoculation has reportedly been shown to increase plant growth more than either of their individual inoculations (Paudyal & Gupta, 2018). *Bradyrhizobium* sp. and *Bacillus* sp. co-inoculation in soybean plants has been shown to significantly enhance the number of nodules generated, root weight, and total nitrogen (Bai et al., 2003). Additionally, it has been observed that co-inoculation with suitable rhizobia improves and stimulates the development of nodules and increases crop output in legumes (Benito et al., 2017). According to reports, nitrogen-fixing *Rhizobium* species and endophytic PGPB collaborate to increase the effectiveness of nitrogen fixation in crops (Li et al., 2019).

Additionally, co-inoculation has been effective in a number of limiting circumstances, such as low-phosphate soils (Rashid et al., 2016). However, co-inoculation with phosphate-solubilising

bacteria can make phosphate available for plant nutrition and, in the case of legumes, help to secure the benefits of biological nitrogen fixation. In these circumstances, biological nitrogen fixation is typically compromised (Masson-Boivin & Sachs, 2018).

Plant growth-promoting microorganisms play an important role in restoring and replenishing soil nutrient stores by influencing biochemical cycles. The utilisation of these microorganisms as bio-agents in the management and tolerance of agricultural stress through nutrient improvement has led to the protection of crops even in stressful conditions (Sundh et al., 2021). Plant growth-promoting microorganisms used in biofertilisers promise sustainable agricultural development while increasing soil fertility and crop yield (Sundh et al.,2021). However, the understanding of the mechanisms in which these microorganisms work is not fully understood.

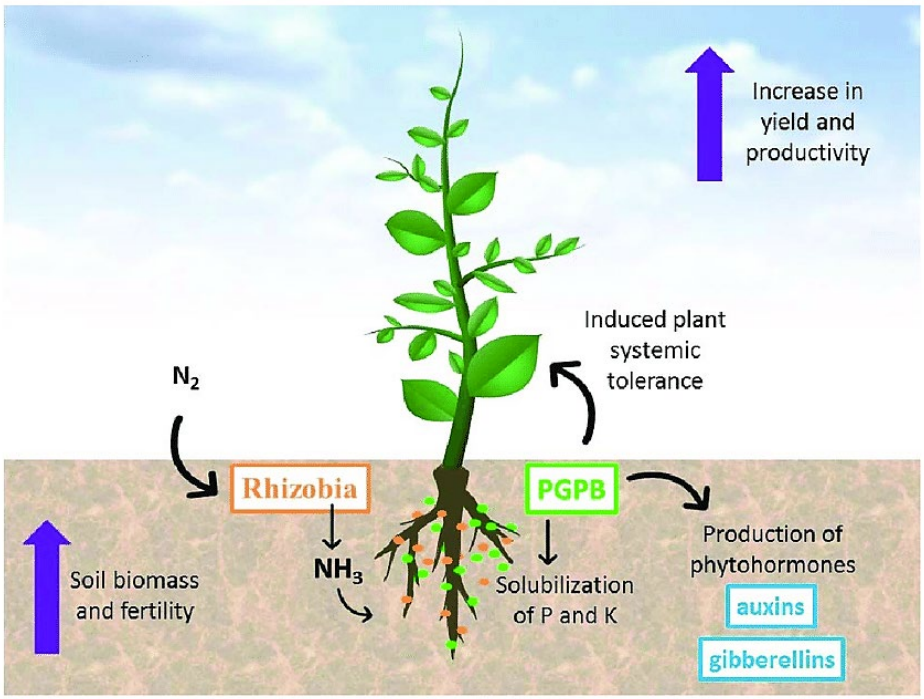

Figure 2-2: Benefits of the use of Rhizobia and other plant growth-promoting bacteria as biofertilisers or agricultural inoculum (Santos et al., 2021)

2.6 Methods for bacterial identification

Multiple methods are used to identify bacteria (Mishra et al., 2018). These different microbiological practices are used to properly identify isolated bacterial strains accurately. Molecular microbiology techniques have improved and revolutionised the bacterial identification process. Particularly, in microbial ecology, a wide range of methods for the detection and identification of specific microorganisms in environmental samples has been developed (Dickie et al., 2018). The most common practice comprises selective plating techniques used for culture purification and is useful

but limited, in that the bacteria cultured remains unidentified (Mishra et al., 2018). However, the importance of the culture dependent practices must not be ruled out completely, as the precise identification of microbes in bacterial cultures starts from the basis of morphology and cultural characteristics by performing Gram staining and observing the characteristics of a pure colony (Asefa & Gidago, 2017). Further classification is then carried out through biochemical tests, which categorise the organism by tracing its metabolic activity. Molecular techniques, which include phylogenetic probes or polymerase chain reaction (PCR)-based methods, allow for the detection of DNA sequences and can be used to justify and explain their occurrence in the environment (Dickie et al., 2018; Mishra et al., 2018). Molecular methods are used to carry out distinct classification and identification of bacteria by isolating genomic DNA and then sequencing the DNA fragment of interest. The processes involved in molecular bacterial identification include DNA extraction from a pure culture of bacteria, PCR amplification of the 16S rRNA gene, evaluating the PCR product using gel electrophoresis, PCR product clean-up, sequencing of PCR product, analysis of the base sequence online via the basic local alignment search tool (BLAST) and, finally, identifying the closest match in the database (Dickie et al., 2018).

A DNA polymerase commences DNA synthesis at a precise location on a specific DNA strand using the PCR molecular technique (Kuslich et al., 2019). A DNA template can be amplified quickly and precisely in a few hours using the PCR process. The target DNA template, a set of primers, polymerase enzyme, and nucleotides make up the PCR reaction mixture. The primers anneal to the corresponding sequence of the DNA strand as the template DNA is produced throughout each cycle of the reaction. New copies of the target region are produced in each cycle by the polymerase enzyme, which also catalyses the addition of a nucleotide to the end of each primer (Kuslich et al., 2019). The 30S subunit of prokaryotic ribosomes contains the 16S rRNA gene in addition to the 23S and 5S RNA molecules. The 16S gene has conserved sections that are helpful for determining phylogenetic relationships between distant species and variable regions that are utilised to distinguish closely related species, making it an excellent phylogenetic marker (Poretsky et al., 2014). Because it plays a crucial role in ribosome assembly, the gene that encodes the 16S rRNA is highly conserved among bacteria. The 16S rRNA gene has developed into an appropriate DNA segment for phylogenetic analyses and the categorisation of bacteria (Poretsky et al., 2014). The 16S rRNA gene sequences are used for a number of reasons, including the fact that almost all bacteria belong to a multigene family; the 16S rRNA gene's function has remained constant over time, indicating that the sequence is sufficiently conserved; and because its size (1500 bases) makes it simple to sequence while still being large enough to contain enough data to identify bacteria for informatics purposes (Poretsky et al., 2014). The 16S rRNA gene sequencing typically yields extremely good genus identification, however, it is less accurate when it comes to species identification (Khayalethu, 2013).

2.6.1 Impact of molecular methods in bacterial identification

Rhizobia and rhizobacteria have been widely characterised in numerous studies due to their high diversity and importance in agriculture (Koskey et al., 2018). These studies of diversity involve sampling of nodules from fields, rhizobia isolation from nodules, identification of the isolates at the genus and species levels, molecular characterisation through genotyping of isolates and description of new rhizobium species. Molecular methods have been used to successfully detect rhizobia and rhizobacterial diversity with precision at the genetic strain, species and genus levels (El-Zanaty et al., 2014). Sequencing the 16S rRNA gene has been used mainly to identify and classify isolates from pure cultures and estimate bacterial diversity in the environment even without the use of culture techniques. The use of PCR has proven valuable in the screening of rhizobia and rhizobacteria (Benito et al., 2017; Koskey et al., 2018). Gyogluu et al. (2018) described the detection and phylogenetic analysis of rhizobial housekeeping genes in environmental samples using PCR.

2.7 Conclusion

It is evident that the symbiotic relationship between sainfoin and rhizospheric bacteria is important in agriculture. Although there is a broad understanding of these bacteria, our knowledge about them is still limited. A better understanding of the specific microbial members and their functions will be beneficial for utilising symbioses for soil health.

CHAPTER 3: MATERIALS AND METHODS

3.1 Reagents and glassware

Reagents used during molecular analysis include a 1kb molecular weight GeneRuler and GelRed nucleic acid gel stain were obtained from Anatech Instruments (Pty) Ltd. (Olivedale, South Africa). The DNA extraction was done using the commercial DNA extraction kit, *Quick*-DNA Fungal/Bacterial Miniprep Kit, from Zymo Research. The primers used for the polymerase chain reaction (PCR) amplification, U8-27 and R1494-1514, were obtained from Integrated DNA Technologies, Inc. For bacteroid cultivation, the growth media used – yeast mannitol salt agar, consisted of yeast extract (1.00 g), mannitol (10.00 g), dipotassium phosphate (0.50 g), magnesium sulphate (0.20 g), sodium chloride (0.10 g), calcium carbonate (1.00 g) and bacteriological agar (15.00 g), and was obtained from Sigma-Aldrich (Pty) Ltd. (Johannesburg, South Africa). Required glassware was purchased from Lasec South Africa (Pty) Ltd. (Midrand, South Africa). Glassware used for all procedures were washed in tap water with soap, wiped with 70% ethanol and thereafter rinsed thoroughly with deionised water.

3.2 Study sites and sample collection

Two different sets of samples were collected and used in this study – first, soil was collected for growing sainfoin in a greenhouse, and second, sainfoin plants were collected from a farm.

Figure 3-1 shows the sampling design followed in soil acquisition and planting of the sainfoin cultivars: Esparsette, Perly and Taja. The soil samples were obtained from two farming regions: Reitz in Free State, South Africa (27.8010° S, 28.4318° E) and Vrede in Free State, South Africa (27.4524° S, 29.1507° E). Ten fields, five in Reitz and five in Vrede, were selected for soil sampling in each region. The soil sample collection was done at random points in each field in each region. The collected soil samples were used to grow three cultivars of sainfoin, namely, Esparsette – indigenous to South Africa, and Perly and Taja – which are exotic cultivars. The exotic cultivars were selected to observe the bacterial variation that can occur within the *O. viciifolia* plant. Each cultivar was planted in each of the soil samples collected from the 10 fields, therefore each cultivar was planted in 10 different soils. Overall, there were 30 pots per cultivar (3 replications per cultivar). Three months (90 days) after planting, sainfoin plants that grew, and established nodules were uprooted and transferred in a cool and dry container to the laboratory for further analysis within 48 hours. The nodules present on the roots were collected and analysed separately according to the different cultivars.

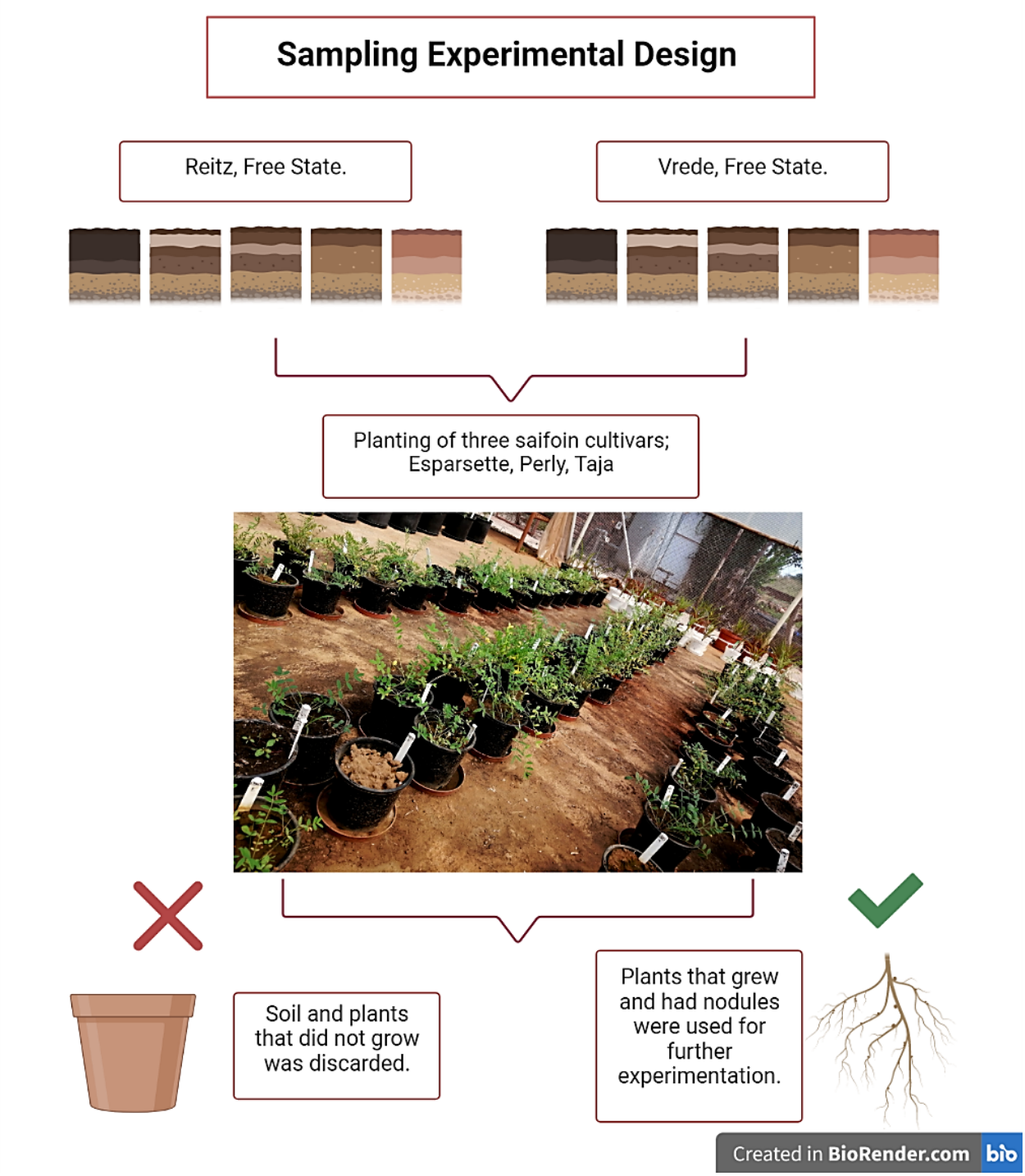

Figure 3-1: Schematic representation of soil sampling and planting

Sixty sainfoin plants were collected from a sainfoin farm in Ficksburg in the Free State, South Africa (28.8778° S, 27.8663° E). The original sainfoin seeds were treated with an inoculum that the farmer obtained from Stimuplant (https://stimuplant.co.za). There were two fields on the farm (field 1 and field 2) and 30 plants were collected at random in each field. The plants were uprooted and placed into marked plastic bags and stored in a cool and dry container and transported to the

North-West University Microbiology laboratory for root nodule examination and further analysis within 48 hours.

3.3 Sample preparation

3.3.1 Root nodule preparation

The plant roots were washed under tap water to remove excess soil. Nodules were identified and carefully excised from the roots and were surface sterilised with 70% ethanol for two minutes followed by 2.5% sodium hypochlorite for five minutes, then with 70% ethanol for 30 seconds and finally rinsed in distilled water thrice, for one minute each (Asefa & Gidago, 2017; Roychowdhury et al., 2015). Sterility tests were performed by spreading 100 µl of water from the last rinse with the distilled water onto nutrient agar plates that were supplemented with MSA (50% yeast mannitol agar) and incubated at 28°C for three days (Asefa & Gidago, 2017). The prepared agar growth plates were observed and screened for bacterial growth. Only the plates that did not contain bacterial growth were used as indicators for adequate nodule sterility. The prepared agar growth plates that exhibited bacterial growth were used to indicate which nodules required a repeat of the sterilisation procedure. The importance of the sterility tests was to ensure that there were no viable microorganisms on the exterior of the nodules that could contaminate the bacteroid solution.

The sterilised nodules were then transferred into sterilised Eppendorf tubes with 100µl of double deionised water and crushed using sterilised pestles and vortexed for three to five minutes. This was followed by adding 900µl of water to the tubes to make the stock solution. From the stock solution, serial dilutions with a dilution factor up to 10^{-9} or 1:1 000 000 000 were prepared and cultured onto nutrient agar plates that were supplemented with 50% yeast mannitol agar and marked accordingly based on the degree of dilution and cultivar. For example, a sample preparation from the Esparsette cultivar with the dilution series of 10^{-2} was marked ‘E10^{-2}’. Plates were then incubated at 28°C and inspected daily (growth is expected within one to three days). The significance of the use of the serial dilution technique was to reduce the bacterial concentrations to obtain single colonies. Single colonies were mostly observed on the plated spread with the 10^{-9} dilution factor.

3.3.2 Bacteroid cultivation and purification

Single colonies that appeared on the prepared spread plates were then sub-cultured separately on nutrient agar plates that were supplemented with 50% yeast mannitol agar until growth was observed (Asefa & Gidago, 2017). This procedure was repeated until uniform colonies were

observed. Two separate plates were prepared for every single colony observed, one for the gram staining procedure and another for molecular analysis.

Figure 3-2 shows the scematice representation of the workflow followed in the preparation of root nodules from the sainfoin cultivars: Esparsette, Perly and Taja, for molecular analysis.

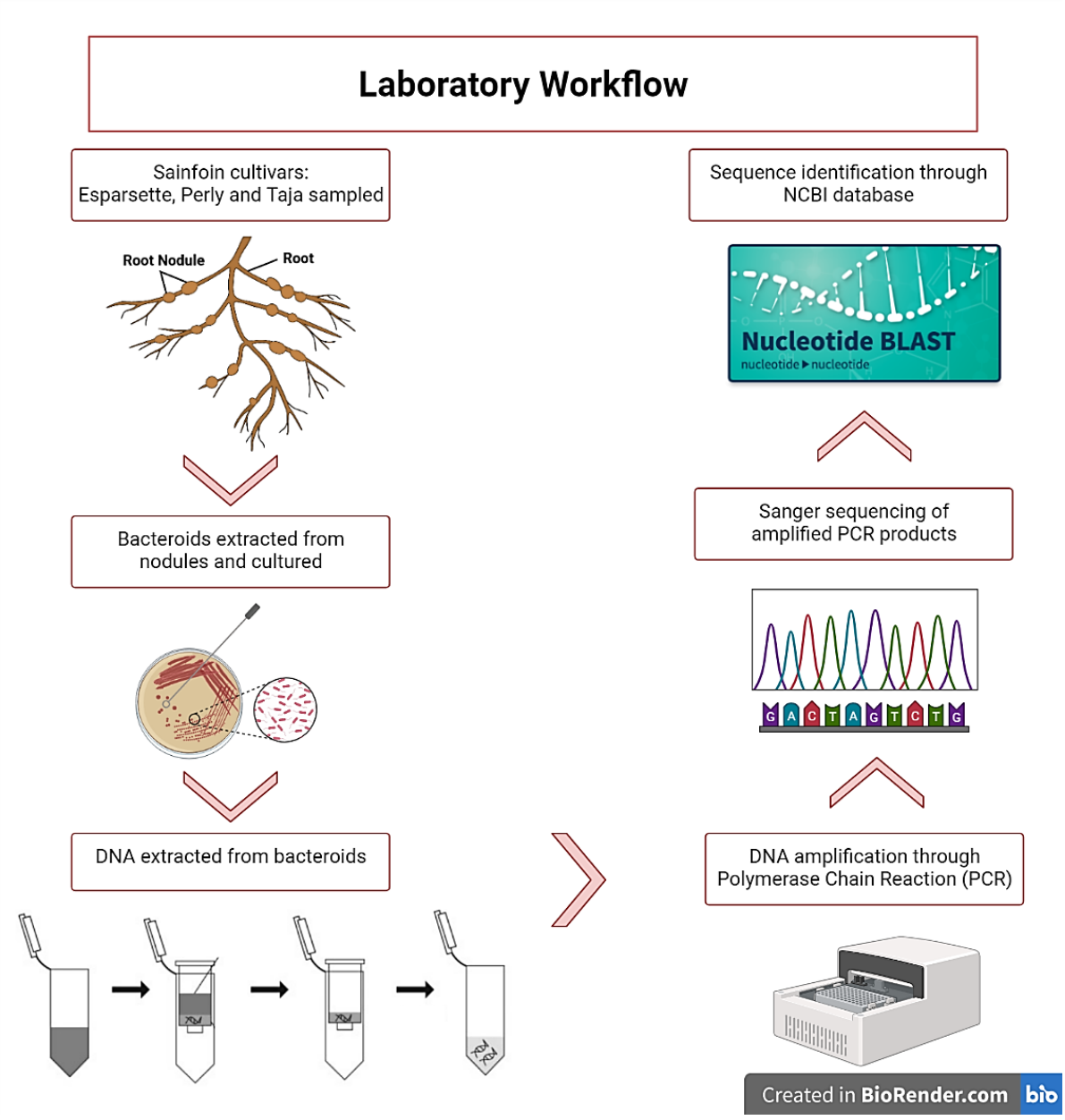

Figure 3-2: Schematic representation of laboratory workflow

3.3.3 Gram staining procedure

The Gram stain technique was performed to differentiate between gram-positive and gram-negative bacterial colonies. Rhizobia are gram-negative microorganisms, therefore only the colonies that tested Gram-negative were colonies of interest as rhizobial strains. A bacterial smear was prepared and fixated on a sterilised microscope slide. The heat-fixed bacterial smear was placed on a staining rack and stained with crystal violet for one minute, then Lugol's iodine was added and left for three minutes. This was followed by decolourising with 95% alcohol for about 30 seconds. Finally, the entire film was covered with safranin for three minutes and rinsed off with distilled water. The slide was left to air dry completely before being examined under the microscope (Asefa & Gidago, 2017). The importance of Gram staining is to classify bacteria into two groups, gram-positive or gram-negative, based on their cell wall constituents.

3.4 Statistical analysis

The Shannon-Weiner index was used to calculate species diversity between the three sainfoin cultivars (Favero et al., 2021).

3.5 Molecular analysis

3.5.1 DNA extraction

The DNA was extracted using the Zymo Research Quick-DNA Fungal/Bacterial Miniprep Kit ® from the purified isolates cultured on nutrient agar plates that were supplemented with 50% yeast mannitol salt agar based on the protocol provided by the manufacturer (Zymo Research: https://www.zymoresearch.com). The DNA quality and concentration of the samples were measured using the Nanodrop spectrophotometer (ThermoFisher Scientific). The accepted parameters for the Nanodrop results were a measured DNA concentration which is above 10 ng/µl and a 260/280 ratio of absorbance value of 1.7 – 1.9. The reason for the accepted measurements was because a value ranging from 1.7 to 1.9 is indicative of a pure DNA sample (Lucena-Aguilar et al., 2016).

3.5.2 PCR amplification

For PCR amplification, the 16S rDNA gene was amplified using the following primers: U8-27 (5' – AGA GTT TGA TCM TGG CTC AG – 3'), which was the forward primer, and R1494-1514 (5'-CTA CGG YTA CTT TGT TAC GAC – 3'), which was the reverse primer (Heuer et al., 2001). The PCR was performed in 25 µl reactions consisting of 12.5 µl Master Mix (GoTaq G2 Colorless Master Mix), 1 µl of each primer, 8 µl of dH_2O, and 2 µl of DNA. The PCR conditions were as follows: initial denaturation at 95°C for two minutes, 30 cycles at 95°C for 30 seconds, annealing

was done for 30 cycles at 56°C for 30 seconds, and 72°C for one minute, and a final elongation (extension) step at 72°C for 10 minutes and stored at 4°C in the C1000 Touch™ thermal cycler (BioRad Hercules, California USA). Ultimately, the PCR product was loaded on a 1.5% agarose gel stained with GelRed and electrophoresis conditions of 80 V for 45 minutes. The agarose gel was loaded into a TAE buffer solution which comprised 20 Mm Acetic acid, 40 mM Tris and 1 mM EDTA at a pH of 8.0. The gel was then visualised under the ChemiDoc MP Imaging System (Bio-Rad, US) using Image Lab software (version 4.0.1) to check the quality of DNA bands.

3.5.3 Microbial identification and phylogenetic analysis

The PCR products were sequenced by Inqaba Biotec™ Laboratories (Johannesburg, South Africa). The forward and reverse sequences obtained from Inqaba Biotec™ Laboratories were checked and corrected manually using FinchTV (Version 1.4.0, Geospiza Inc.) and then a consensus sequence was generated for each isolate. The open access Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to accurately characterise the bacterial isolates according to the percentage similarities. The result from the database was attained, and the closest match for either the genus or species name was allocated to each isolate.

Flavivirga algicola and *Algibacter aestuarii* were used as an outgroup. Reference sequences were obtained from NCBI (GenBank). The available sequences (including the reference and outgroup sequences) of the 16S dataset were aligned using MAFF tools (Katoh & Standley, 2013) implemented in Geneious Prime® 2023.0.1, and both ends were manually trimmed. The Hasegawa–Kishono–Yano (HKY) model was selected as the most appropriate substitution model according to jModelTest 2.1.10 (Darriba et al., 2012). Bayesian analysis was conducted using MrBayes v3.2.6 (Ronquist & Huelsenbeck, 2003) in Geneious Prime® 2023.0.1 to construct phylogenetic relationships among the bacterial isolates. A random starting tree was used for each locus and the chains ran for 3×10^6 generations. After discarding the 25% burn-in samples, the remaining samples were retained for further analysis. The posterior probabilities (PP) were estimated based on the Markov chain Monte Carlo (MCMC) algorithm, using the 50% majority rule (Larget & Simon, 1999).

3.6 Screening for plant growth-promoting attributes

The identified isolates were subject to screening for plant growth-promoting traits. The plant growth-promotion traits that were screened, which included phosphate solubilisation, siderophore production, indole acetic acid production and nitrogen fixation, are considered direct plant growth-promoting effects. These traits are mechanisms of plant growth promotion by rhizosphere

microorganisms that can be applied to soils through biofertilisers (Backer et al., 2018). These traits result in the direct promotion of plant growth through providing the plant with a compound that is synthesised by the microorganism or through facilitating the solubilisation, availability, and uptake of certain nutrients from the environment (Olanrewaju et al., 2017).

3.6.1 Phosphate solubilisation

The in vitro evaluation of phosphate solubilisation was performed by growing the isolates on glucose yeast media that comprised 10 g glucose, 2 g yeast extract and 15 g bacteriological agar per litre. Two separate solutions were prepared: (i) 5 g K_2HPO_4 in 50ml of distilled water and (ii) 10 g $CaCl_2$ in 100 ml of distilled water. These were added into the glucose yeast media prior to pouring into Petri dishes. The prepared bacterial media growth plates were then spot inoculated with the bacterial cultures and incubated at 28℃ for seven days. The formation of a clear halo around the inoculated area was indicative of positive phosphate solubilisation (Ambrosini et al., 2012).

3.6.2 Siderophore production

The in vitro analysis of siderophore production was evaluated by inoculation of the isolates into Kings B media and then spot inoculated onto Chrome Azurol S (CAS) agar and incubated at 28˚C for a maximum of 72 hours. Colour development of an orange or a clear halo around the inoculated area was indicative of a positive result for siderophore production (Ambrosini et al., 2012).

3.6.3 Indole acetic acid production

In vitro analysis of indolic compound production was assessed by bacterial inoculation in Kings B media at 28˚C for 72 hours and aggravated at 200 rpm. One millilitre of the bacterial culture was then transferred into Eppendorf tubes and centrifuged at 12 000 g for six minutes and the supernatant was mixed with equal parts of Salkowski reagent in a sterilised test tube. The test tube was then kept in the dark for approximately 30 minutes. A colour varying between pink and red was used as an indicator for positive indolic compound production. The samples were then measured using a spectrophotometer at 550 nm, and a standardised calibration curve was constructed (Ambrosini et al., 2012). From the standardised curve equation, the concentration of the Indole acetic acid (IAA) was calculated.

3.6.4 Test for nitrogen fixation

A nitrogen-free media was used to test the target isolates' ability to fix nitrogen. The biochemical authentication was done by aseptically inoculating the target isolates onto nitrogen-free Burk's media agar plates which were incubated at 28°C for one to three days. The plates were observed every 24 hours and the isolates that showed growth proved to be nitrogen fixers, and those that did not show growth were considered non-nitrogen fixers and were discarded (Trncik et al., 2022).

CHAPTER 4: MICROBIAL IDENTIFICATION

4.1 Results

In total, 54 bacterial isolates were isolated from the roots of three sainfoin cultivars, one endemic – Esparsette, and two exotic – Perly and Taja. The isolates were subject to Gram staining and analysis by molecular methods including DNA extraction, polymerase chain reaction (PCR) and phylogenetic analysis. After DNA extraction and sequencing, the bacterial isolates were screened, and repetitions were removed based on the 16S rDNA sequence after identification via the NCBI database.

4.1.1 Morphological characteristics of isolates

The morphological characteristics of 29 out of 54 initial isolates from root nodules of three sainfoin cultivars – one endemic, Esparsette and two exotics, Perly and Taja, were determined based on colonial morphologies on yeast mannitol agar (MSA) and Gram staining. Data on the morphological characteristics of individual isolates appear in Table 4-1. A large proportion (55.2%) of the isolates were white and rod shaped. Of this 55.2%, 31.2% were gram-positive and 68.8% were gram-negative. The second largest portion (13.8%) of isolates were yellow, rod shaped and were all gram-negative. The third largest portions were translucent-white (10.3%), of which all the isolates were gram-positive, and orange (10.3%), of which 33.3% were gram-positive and 66.7% were gram-negative. The smallest portions were milky-white (3.4%), of which the isolate was gram-negative, white-slimy (3.4%), of which the isolate was gram-negative, and orange-yellow (3.4%), of which the isolate was gram-negative.

Table 4-1: Isolate morphological characteristics as observed on yeast mannitol agar as well as microscopically and gram stain results

Isolate:	Colony morphology		Gram stain:
	Isolate colour:	Cell shape:	
E1	White	Rod	negative
E2	White	Rod	negative
E3	Yellow	Rod	negative
E4	Translucent-white	Rod	positive
E5	White	Rod	negative
E6	White	Rod	negative
E7	White	Rod	positive
E10	White	Rod	positive
E11	White	Rod	positive
P15	White	Rod	negative
P17	White	Rod	negative
P18	Orange	Rod	negative
P21	White	Rod	negative
P22	Yellow	Rod	negative
P23	Orange	Rod	negative
P24	Translucent-white	Rod	positive
P25	White	Rod	positive
P27	Milky-white	Rod	negative
P28	White	Rod	negative
P31	Translucent-white	Rod	positive
T33	White	Rod	positive
T34	White	Rod	negative
T36	Orange-yellow	Rod	negative
T37	White	Rod	negative
T38	Yellow	Rod	negative
T39	White	Rod	negative
Ef40	Yellow	Rod	negative
Ef44	White-slimy	Rod	negative
Ef46	Orange	Rod	positive

4.1.2 Bacterial 16S rDNA gene amplification

A total of 54 bacterial strains isolated from root nodules were screened for bacterial 16S rDNA gene fragments. The 16S rDNA gene fragment was successfully amplified in all the isolates. Figures 4-1 and 4-2 show bacterial isolate DNA that was amplified via PCR. The PCR products of the 16S rDNA gene were loaded into wells 2 – 20 and 22 – 40 for Figure 4-1 and wells 2 – 57 for Figure 4-2, with 1kb molecular weight marker loaded into wells 1 and 21 for Figure 4-1 and well 41 for Figure 4-2. The wells with the PCR products showed positive amplification. The agarose gel images were viewed via a UV transilluminator.

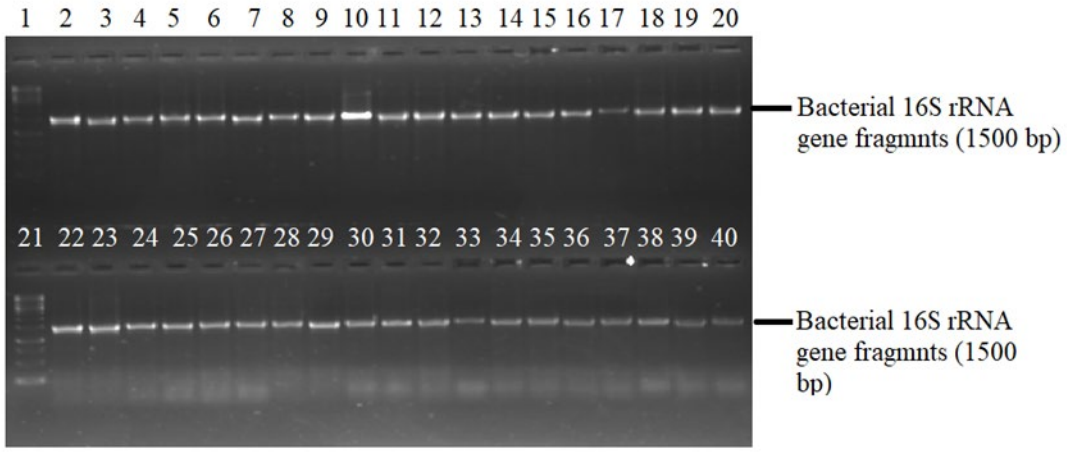

Figure 4-1: Agarose gel image showing 16S rDNA PCR for bacterial isolates (2 – 20 and 22 – 40) extracted and purified from the roots of sainfoin cultivars

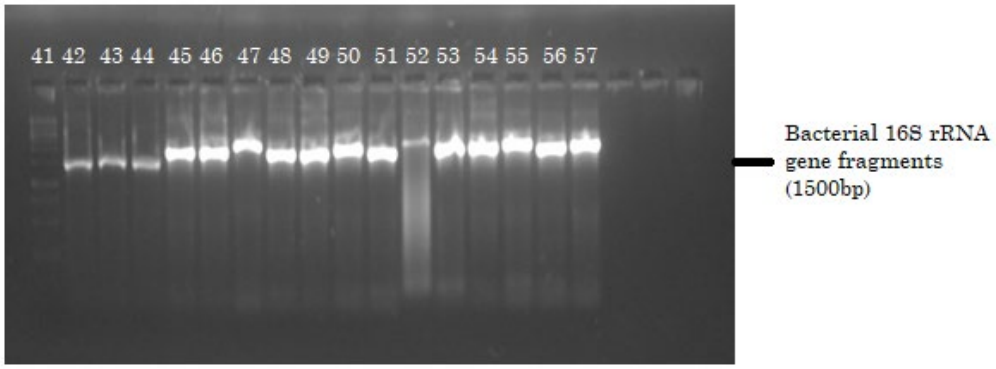

Figure 4-2: Agarose gel image showing 16S rDNA PCR for bacterial isolates (42 – 57) extracted and purified from the roots of sainfoin cultivars

4.1.3 16S rDNA sequencing analysis for identification of bacterial isolates

Bacterial 16S rDNA genes from the isolates were sequenced by Inqaba Biotec (South Africa). After editing and removal of duplicate sequences, 29 effective sequences with an average length of 900 bp were obtained. The percentage similarities of isolates ranged between 95% and 100%.

Analysis of the bacterial isolates revealed that a large proportion of isolates were from Esparsette, including “Esparsette” and “Esparsette Ficksburg”. The exotic cultivars, Perly and Taja, had lower proportions of isolates. Table 4-2 outlines identification results of the individual bacterial isolates from the different sainfoin cultivars as well as their most similar identities based on molecular

identification using 16S rDNA sequence of these isolates. Bacterial classes identified include Alphaproteobacteria, Gammaprotebacteria, Bacilli, Cytophagia and Actinomycetia. Isolate identification was based on similarity percentages >95% from the NCBI database.

Table 4-2: The bacterial strains isolated from different sainfoin cultivars characterised using 16S rDNA sequences

Cultivar:	Isolate:	Best matching identification on NCBI database:	Similarity percentage:	Query cover:	Bacterial Class:
Esparsette	E1	*Rhizobium leucaenae* (NR_118993)	99.89	100%	Alphaproteobacteria
	E2	*Sphingomonas leidyi* (NR_025324)	100	100%	Alphaproteobacteria
	E3	*Pseudoxanthomonas mexicana* (NR_113973)	100	100%	Gammaproteobacteria
	E4	*Paenibacillus tritici* (NR_157638)	98.89	100%	Bacilli
	E5	*Enterobacter mori* (NR_146667)	99.39	100%	Gammaprteobacteria
	E6	*Rhizobium glycinendophyticum* (NR_165782)	98.89	100%	Alphaproteobacteria
	E7	*Bacillus inaquosorum* (NR_104873)	100	100%	Bacilli
	E10	*Peribacillus frigoritolerans* (NR_117474)	100	100%	Bacilli
	E11	*Peribacillus simplex* (NR_112726)	100	100%	Bacilli
Perly	P15	*Sphingomonas leidyi* (NR_025324)	98	100%	Alphaproteobacteria
	P17	*Enterobacter asburiae* (NR_024640)	99.34	100%	Gammaproteobacteria
	P18	*Pseudomonas brassicacearum subsp. neoaurantiaca* (NR_116299)	99.78	100%	Gammaproteobacteria
	P21	*Rhizobium nepotum* (NR_117203)	100	100%	Alphaproteobacteria
	P22	*Xanthomonas arboricola pv. juglandis* (NR_113167)	100	100%	Gammaproteobacteria
	P23	*Spirosoma sordidisoli* (NR_164977)	99.56	100%	Cytophagia
	P24	*Paenibacillus borealis* (NR_025299)	99.89	100%	Bacilli
	P25	*Peribacillus frigoritolerans* (NR_117474)	100	100%	Bacilli
	P27	*Agrobacterium fabrum* (NR_074266)	98	100%	Alphaproteobacteria
	P28	*Enterobacter mori* (NR_146667)	99.44	100%	Gammaproteobacteria
	P31	*Paenibacillus peoriae* (NR_042092)	100	100%	Bacilli
Taja	T33	*Peribacillus frigoritolerans* (NR_117474)	99.89	100%	Bacilli
	T34	*Rhizobium mayense* (NR_109703)	100	100%	Alphaproteobacteria

	T36	*Pseudomonas brassicacearum subsp. neoaurantiaca* (NR_116299)	100	100%	Gammaproteobacteria
	T37	*Rhizobium nepotum* (NR_117203)	100	100%	Alphaproteobacteria
	T38	*Xanthomonas maliensis* (NR_136457)	100	100%	Gammaproteobacteria
	T39	*Enterobacter ludwigii* (NR_042349)	99.78	100%	Gammaproteobacteria
Esparsette (Ficksburg)	Ef40	*Pseudomonas vancouverensis* (NR_041953)	100	100%	Gammaproteobacteria
	Ef44	*Mesorhizobium helmanticense* (NR_157661)	100	100%	Alphaproteobacteria
	Ef46	*[Curtobacterium] plantarum* (NR_104943)	99.78	100%	Actinomycetia

Figure 4-3 shows bacterial diversity for each cultivar either as rhizobial or non-rhizobial isolates. Of the three cultivars, Esparsette (with inclusion of the Esparsette (Ficksburg) isolates) had the highest bacterial population for both rhizobial and non-rhizobial members, followed by Perly and Taja, respectively. The rhizobial isolates found to associate with the Esparsette cultivar were three in number, of which one was from the Esparsette (Ficksburg) group and two were from the Esparsette cultivar, and nine non-rhizobial isolates were found, of which two were from the Esparsette (Ficksburg) group, and seven were from the Esparsette group. Only one rhizobial isolate was found in association with Perly and the non-rhizobial isolates amounted to ten. Two rhizobial isolates were found in association with Taja and the non-rhizobial isolates amounted to four.

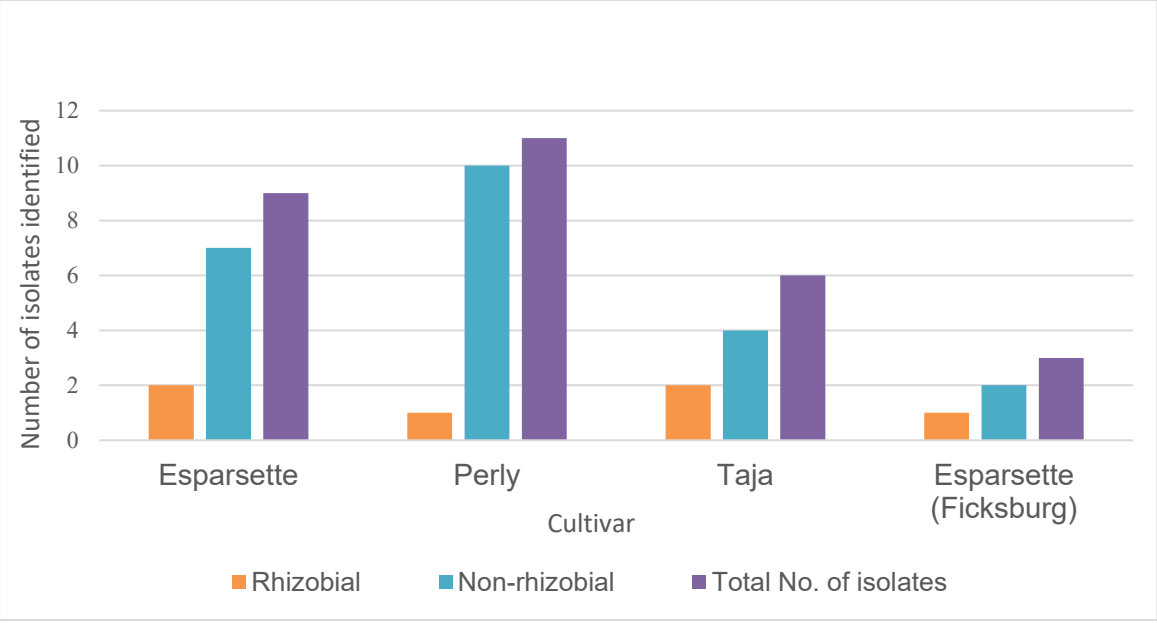

Figure 4-3: Graph showing bacterial diversity between the different sainfoin cultivars

Figure 4-4 shows the different bacterial classes identified in the three sainfoin cultivars, one of which was endemic – Esparsette, and two exotic – Perly and Taja. For Esparsette, there were three bacterial classes identified of which 45% were Bacilli, 33% were Alphaproteobactria and 22% were Gammaproteobacteria. For Esparsette (Ficksburg), there were three bacterial classes identified of which 34% were Alphaproteobacteria, 33% were Gammaproteobacteria d 33% were Actinomycetia. For Perly, there were four bacterial classes identified of which 37% were Gammaproteobacteria, 27% were Alphaproteobacteria, 27% were Bacilli and 9% were Cytophagia. For Taja, there were three bacterial classes identified of which 50% were Gammaproteobacteria, 33% were Alphaproteobacteria and 17% were Bacilli. Perly exhibited the highest diversity, comprising of four bacterial classes. The bacterial class unique to Esparsette was Actinomycetia and the bacterial class unique to Perly was Cytophagia. The three bacterial classes that were common among the three cultivars were Alphaproteobacteria, Gammaproteobacteria and Bacilli.

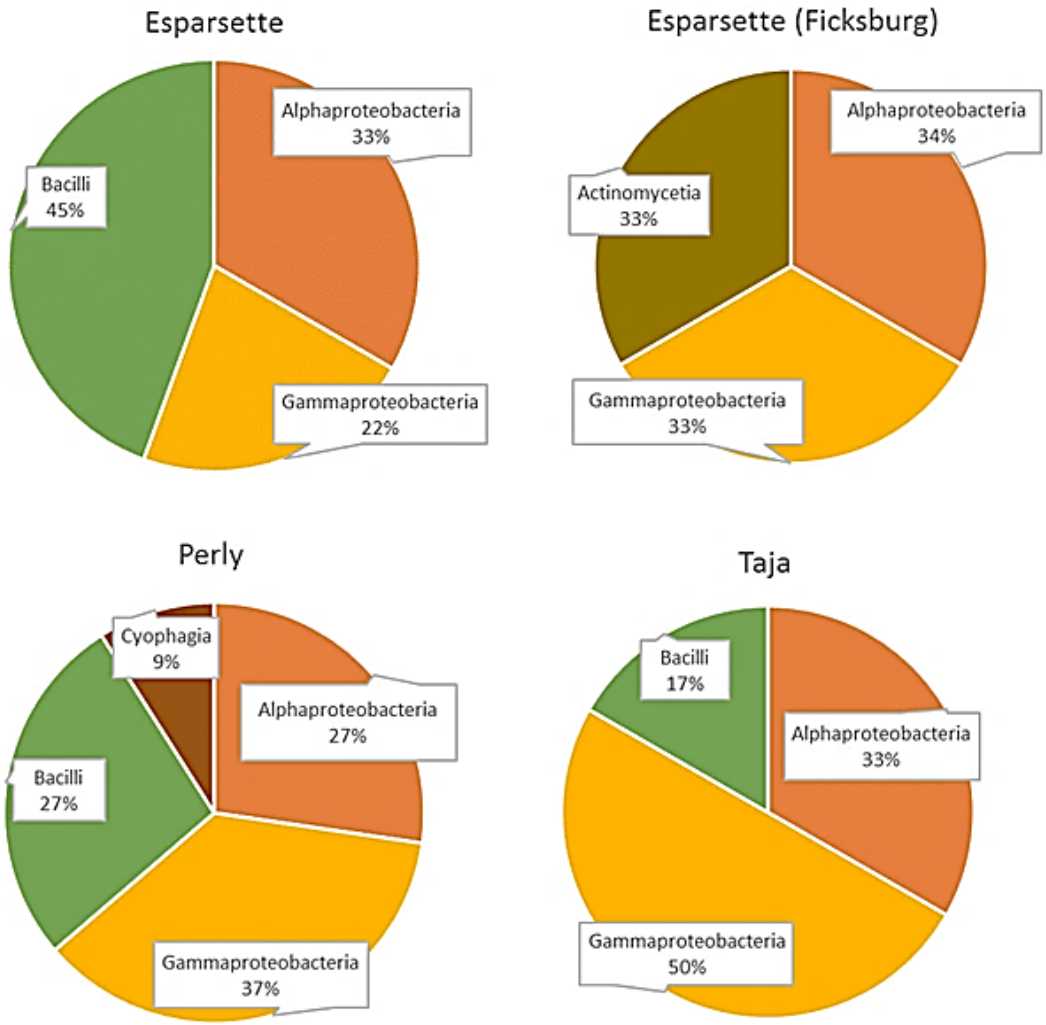

Figure 4-4: Bacterial diversity of isolates based on bacterial class between the different sainfoin cultivars

4.1.4 Phylogenetic analysis

A phylogenetic tree was created based on the 16S rDNA gene sequences of the obtained isolates and their respective reference sequences from GenBank. *Flavivirga algicola* and *Algibacter aestuarii* were used as the outgroup taxa, shown in Figure 4-5. There are three prominent Clades: Clade I, Clade II and Clade III.

All the Alphaproteobacteria and Bacilli are clustered together in Clade I with a support value of 0.89 at the co-joining (external) node, subdividing into two clades. These two sub-clades, A and B, have a support value of 1 and 1, respectively, at their internal nodes. Sub-clade A comprises of isolates that were closely related to *Rhizobium nepotum* (P21 and T37), *Rhizobium glycinendophyticum* (E6), *Rhizobium mayense* (T34), *Rhizobium leucaenae* (E1), *Mezorhizobim*

helmanticense (Ef44), *Agrobacterium fabrum* (P27) and *Sphingomonas leidyi* (E2 and P15). The support value of the node that separates *Agrobacterium fabrum*, that is closely related to isolate P27, and *Rhizobium nepotum*, that is closely related to isolates P21 and T36, was 1. The support value of the node that separates *Sphingomonas leidyi*, that is closely related to isolate E2 and P15, was 1. Sub-clade B comprises of isolates that were closely related to *Peribacillus simplex* (E11), *Peribacillus frigoritolerans* (E10, P25 and T33), *Bacillus inaquosorum* (E7), *Paenibacillus peoriae* (P31), *Paenibacillus tritici* (E4) and *Paenibacillus borealis* (P24). The support value of the node that clusters together the isolates closely related to the *Paenibacilus* genus was 1. The *Peribacillus* clustering showed that isolate E10, with the support value of 0.7, is closely related to both *Peribacillus simplex* and *Peribacillus frigoritolerans.*

All the Gammaproteobacteria and Actinomeycetia are clustered together in Clade II with a support value of 1 at the co-joining (external) node, subdividing into two clades. These two sub-clades, C and D, have a support value of 1 and 1, respectively, at their internal nodes. Sub-clade C comprises of isolates that are closely related to *Pseudomonas brassicacearum subsp. neoaurantiaca* (P18 and T36), *Pseudomonas vancouverensis* (Ef40), *Curtobacterium plantarum* (Ef46), *Enterobacter ludwigii* (T39), *Enterbacter mori* (E5 and P28) *and Enterobacter asburiae* (P17). The support value of the *Pseudomonas* cluster was 1 at the internal node. Isolates T36, P18 and Ef40 have all shown to be closely related to *Pseudomonas brassicacearum subsp. neoaurantiaca* as well as *Pseudomonas vancouverensis.* The support value of the node that separates isolate Ef46, which is closely related to *Curtobacterium plantarum* from the *Enterobacter* cluster, was 0.83. The cluster of the *Enterobacter* genus shows that isolates E5, P17 and P28 are closely related to *Enterobacter asburiae* and *Enterobacter mori*. Sub-clade D comprises of isolates that were closely related to *Xanthomonas maliensis* (T38) and *Xanthomonas arboricola pv. juglandis* (P22), and *Pseudoxanthomonas mexicana* (E3). The bootstrap value of the node that separates isolate E3, which is closely related to *Pseudoxanthomonas mexicana* is 1.

The Cytophagia forms the third Clade (Clade III) with a support value of 1 at the external node. This clade contains isolate P23, which is closely related to *Spirosoma sordidisoli*.

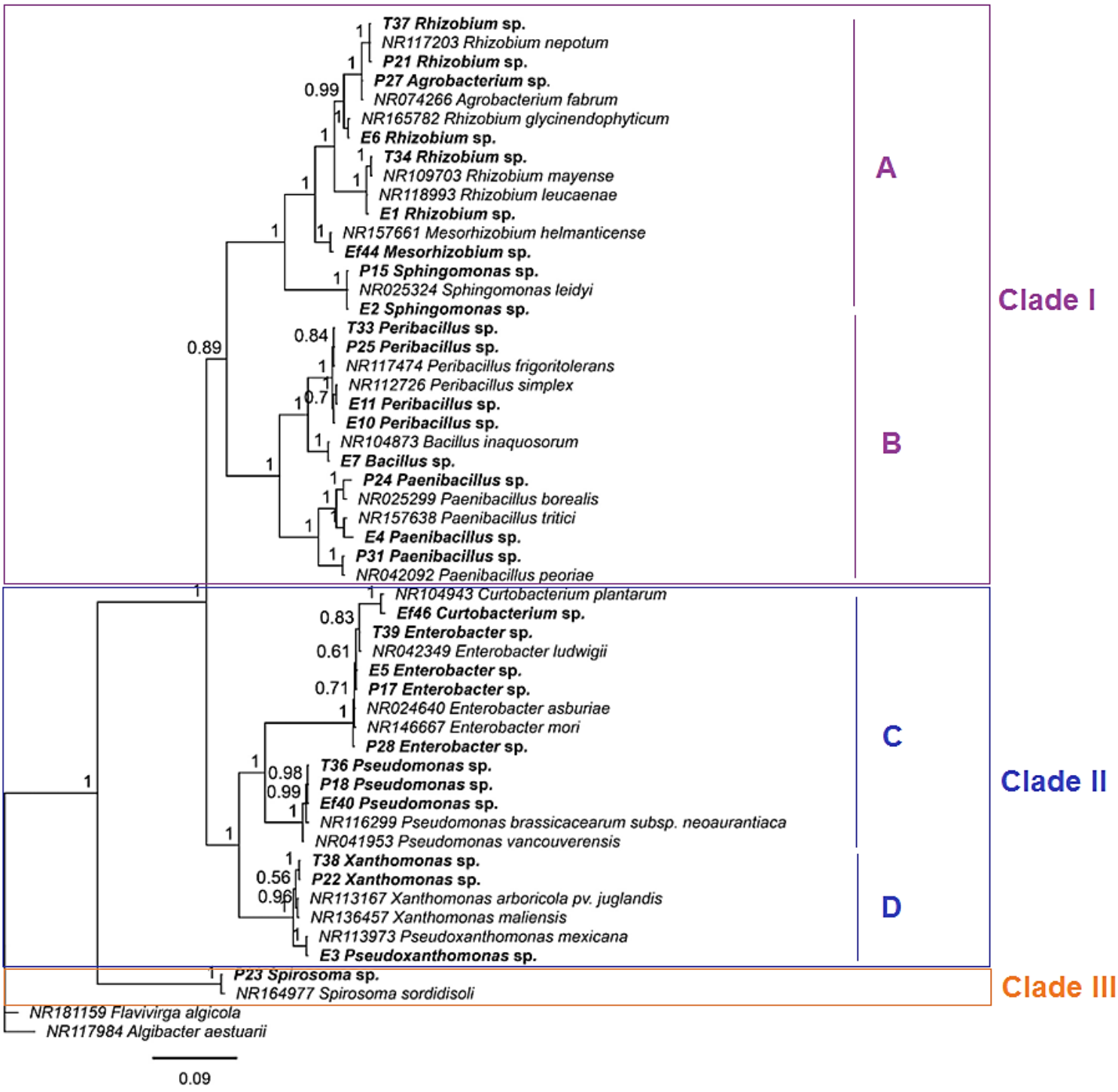

Figure 4-5: **Phylogenetic tree of identified isolates. *Flavivirga algicola* and *Algibacter aestuarii* were used as an outgroup. Reference sequences were obtained from NCBI (GenBank)**

4.1.5 Statistical analysis

Table 4-3 shows the Shannon Index (*H'*) of diversity calculated for each cultivar. The diversity index was calculated based on the occurrence of the different genera that were found to associate with each cultivar. The index value was the highest for the cultivar Perly and the lowest for the cultivar Esparsette (Ficksburg).

Table 4-3: Table showing the calculation of the Shannon Index for diversity for the different sainfoin cultivars

Esparsette	**Genus**	**Number of genera in cultivar**	**pi**	**ln(pi)**	**pi*ln(pi)**
1	*Rhizobium* sp.	2	0,2222222	-1,504077397	-0,33423942
2	*Sphingomonas* sp.	1	0,1111111	-2,197224577	-0,24413606
3	*Pseudoxanthomonas* sp.	1	0,1111111	-2,197224577	-0,24413606
4	*Paenibacillus* sp.	1	0,1111111	-2,197224577	-0,24413606
5	*Enterobacter* sp.	1	0,1111111	-2,197224577	-0,24413606
6	*Bacillus* sp.	1	0,1111111	-2,197224577	-0,24413606
7	*Peribacillus* sp.	2	0,2222222	-1,504077397	-0,33423942
Sum		**9**	1		-1,88915916
H'					**1,889159164**
Perly					
1	*Sphingomonas* sp.	1	0,1	-2,302585093	-0,23025851
2	*Enterobacter* sp.	2	0,2	-1,609437912	-0,32188758
3	*Pseudomonas* sp.	1	0,1	-2,302585093	-0,23025851
4	*Rhizobium* sp.	1	0,1	-2,302585093	-0,23025851
5	*Xanthomonas* sp.	1	0,1	-2,302585093	-0,23025851
6	*Spirosoma* sp.	1	0,1	-2,302585093	-0,23025851
7	*Paenibacillus* sp.	1	0,1	-2,302585093	-0,23025851
8	*Peribacillus* sp.	1	0,1	-2,302585093	-0,23025851
9	*Agrobacterium* sp.	1	0,1	-2,302585093	-0,23025851
Sum		**10**	1		-2,16395566
H'					**2,163955657**
Taja					
1	*Peribacillus* sp.	1	0,1666667	-1,791759469	-0,29862658
2	*Rhizobium* sp.	2	0,3333333	-1,098612289	-0,3662041
3	*Pseudomonas* sp.	1	0,1666667	-1,791759469	-0,29862658
4	*Xanthomonas* sp.	1	0,1666667	-1,791759469	-0,29862658
5	*Enterobacter* sp.	1	0,1666667	-1,791759469	-0,29862658
Sum		**6**	1		-1,56071041
H'					**1,560710409**
Esparsette (Ficksburg)					
1	*Pseudomonas* sp.	1	0,3333333	-1,098612289	-0,3662041
2	*Mesorhizobium* sp.	1	0,3333333	-1,098612289	-0,3662041
3	*[Curtobacterium]* sp.	1	0,3333333	-1,098612289	-0,3662041
Sum		**3**	1		-1,09861229
H'					**1,098612289**

4.2 Discussion

4.2.1 Bacterial community composition in the extracted nodules

In this study, bacteria were isolated from root nodules of three different sainfoin (*Onobrychis viciifolia*) cultivars and their identities were determined using Sanger sequencing. The 16S rDNA gene is a conserved sequence that encodes for the expression of the bacterial ribosomal subunit. The 16S rDNA region is known to be specific for different species and this increases the chances of accurate identification (Moura et al., 2020). The agarose gels presented in Figures 4-1 and 4-2 show the successful amplification of extracted DNA. The presence of primer dimers is a result of access to primer in the PCR reaction. However, the use of 16S rRNA in bacterial identification studies is said to lack consistent accuracy when it comes to identification at the species level with closely related organisms (O'Callaghan et al., 2021), hence the identification of the isolates found in the study is based on similarity to those organisms found in the NCBI database.

Using 16S rDNA amplification, 13 genera of rhizobacteria were identified. These bacteria belong to the bacterial classes Proteobacteria (alpha- and gamma-), Bacilli, Actinomycetia and Cytophagia. Of these 13 genera, two can be classified as true rhizobial groups and 11 of the 13 can be classified as non-rhizobial groups. The rhizobial groups identified were the *Rhizobium* and *Mezorhizobium* genera; these two groups are classified as alpha-rhizobia (Alphaproteobacteria) (Lu et al., 2017). The non-rhizobial members identified were *Sphingomonas, Pseudoxanthomonas, Paenibacillus, Peribacillus, Pseudomonas, Enterobacter, Bacillus, Spirosoma, Agrobacterium, Xanthomonas* and *Curtobacterium*. The identified bacteria are known to be abundant in the rhizosphere and often closely associated with bacterial–root interactions through root colonisation or through symbiotic relationships (Pandya et al., 2013). However, the types of relationships that exist between the organisms and the plant must be further investigated to further explain the co-occurrence of certain organisms.

This study was carried out to identify rhizobia that form associations with the legume *Onobrychis viciifola* (sainfoin), and the results show that there is also a prevalence of non-rhizobial organisms in these associations. This correlates with the studies conducted by Pandya et al. (2013), which found the presence of non-rhizobial organisms in the root nodules of leguminous plants. Results from studies by Muresu et al. (2008) and Benito et al. (2017) also found rhizobial and non-rhizobial organisms in different species of *Hedysarum* and *Medicago* as well as *Trifolium* legume root nodules. Thus, non-rhizobial organisms might also be able to colonise root nodules of the sainfoin crop. It was observed that the different cultivars are colonised by different rhizobial and non-rhizobial organisms. The cultivar native to South African soils, Esparsette, was colonised by rhizobial organisms with high similarity (higher than 90%) to *Rhizobium leucanae, Rhizobium*

glycinedophyticum and *Mezorhizobium helmanticense.* The two exotic sainfoin cultivars, Perly and Taja, were colonised by *Rhizobium nepotum* and *Rhizobium mayense*. The identification of *Rhizobium* and *Mezorhizobium* species in legume studies is supported by Mukhtar et al. (2020), where nodule microbiome analysis was carried out on the legume, Cowpea. The non-rhizobial organisms identified from Esparsette belonged to the genera *Sphingomonas, Pseudoxanthomonas, Paenibacillus, Enterobacter*, *Curtobacterium, Bacillus* and *Peribacillus.* The non-rhizobial genera identified from Perly were *Sphingomonas, Enterobacter, Pseudomonas, Spirosoma, Agrobacterium, Xanthomonas, Paenibacillus* and *Peribacillus*. The non-rhizobial genera identified from Taja were *Peribacillus, Pseudomonas, Xanthomonas* and *Enterobacter*. A study by Lu et al. (2017) identified the non-rhizobial genera, *Bacillus, Pseudomonas, Xanthomonas* and *Sphingomonas* in legume nodules. Latif et al. (2013) identified *Paenibacillus* species co-existing with *Rhizobium* species within the nodules of wild legumes. Alemneh et al. (2021) isolated *Peribacillus* species from a study conducted that investigated chickpea symbiosis. Tapia-Garcia et al. (2020) isolated *Pseudoxanthomonas* species in a study conducted on wild *Phaseolus vulgaris* root nodules. The presence of *Enterobacter* species within the nodules is supported by Muresu et al. (2010), who also tested the possible virulence of these root colonising bacteria.

The difference in diversity between the cultivars that were observed during this study could be due to the plant–bacterial interactions that take place during root colonisation such as intricacies in the molecular dialog, signalling specificity and induction, and flavonoid secretions (Benito et al., 2017; Martínez-Hidalgo & Hirsch, 2017; Sharma et al., 2020). It can also be noted that the rhizobia identified vary between cultivars, as the selection of rhizobia by the plant is based on symbiotic traits; hence, not all cultivars are colonised by the same rhizobial species (Lardi & Pessi, 2018). Interestingly, this presence of rhizobia coupled with non-rhizobial rhizobacteria found in the nodule was found to enhance symbiosis and can facilitate nodule formation (Benito et al., 2017).

4.2.2 Proteobacteria

The bacterial class, Proteobacteria is sub-divided into three classes: alphaproteobacteria, betaproteobacteria and gammaproteobacteria. The members of this bacterial class are among the most abundant in soil environments (Mhete et al., 2020). Organisms belonging to this bacterial class are often associated with plant growth-promoting properties such as nitrogen fixation, phosphate solubilisation and increased nutrient availability in the soil (Mhete et al., 2020). The bacterial genera identified in this study that belong to the Alphaproteobacteria class are *Rhizobium, Sphingomonas, Agrobacterium* and *Mezorhizobium*. The occurrence of *Rhizobium* and *Mezorhizobium* in the nodules is expected, as organisms belonging to these genera are classified as true rhizobia. Beukes et al. (2019) also identified *Rhizobium* and *Mezorhizobium*

species in a study conducted on a leguminous plant grown in South Africa. These are the main organisms that are involved in the process of nodule formation and symbiotic nitrogen fixation in leguminous plants. The different *nod* and *nif* genes present within the organism are expressed in low-nitrogen environments and symbiotic nitrogen fixation occurs. The occurrence of *Agrobacterium* genera at first glance may be surprising, as it was previously stated that they can act as a pathogen when in soil communities and do not possess symbiotic genes (Goodner et al., 2001); however, it has been suggested by Abe et al. (1998) that if it does occur in nodule community populations, there may have been inter-specific symbiotic plasmid exchange which allows this once-pathogenic microorganism to be a beneficial endophyte. The presence of *Agrobacterium* species was found within legume nodules by Aserse et al. (2012). The presence of *Sphingomonas* species in the nodules has been reported by Ikeda et al. (2010) in hyper-nodulated soybeans. The bacterial genera identified in this study that belong to the Gammaproteobacteria class are *Pseudoxanthomonas, Xanthomonas, Pseudomonas* and *Enterobacter*. Bacterial organisms belonging to *Pseudomonas* and *Enterobacter* are reported to possess properties that can induce nodulation (Martínez-Hidalgo & Hirsch, 2017). The occurrence of *Pseudoxanthomonas* in the nodule is still novel and this is supported by Tapia-García et al. (2020), where this genus was identified in the nodules of legumes in Mexico. The role of *Xanthomonas* and *Pseudoxanthomonas* in nodule studies is not yet understood.

4.2.3 Bacilli

Members of this bacterial class occur in soil communities and often have the function of plant growth promotion through nutrient solubilisation or the secretion of metabolites lethal to plant pathogens (Engelbrecht et al., 2022). Identification of bacterial members belonging to this class must be done with caution, as the *Bacillus* species are closely related (Jeżewska-Frąckowiak et al., 2018). Organisms identified in this study that belong to this class are *Paenibacillus tritici, Paenibacillus borealis, Paenibacillus peoriae, Peribacillus frigoritolerans, Peribacillus simplex* and *Bacillus inaquosorum.* The presence of *Bacillus* species within the nodules of leguminous plants is supported by Stajković et al. (2009) and Pandya et al. (2013). The occurrence of these species was unexpected, as this organism is gram-positive and not known to be an endosymbiont. However, according to Tapia-García et al. (2020), *Bacillus* species in close proximities to symbiotic bacteria can test positive for the amplification of the symbiotic gene *nod*C. This presence may be explained by considering the acquisition of the symbiotic characteristics through lateral symbiotic gene transfer. The prevalence of this organism in the nodule is supported by Boukhatem et al. (2016), who noted the presence of *Bacillus* species in their study of nodular bacterial population diversity. According to Patel et al. (2020), *Peribacillus simplex* is a

reclassification of *Bacillus simplex.* The presence of *Paenibacillus* species within the nodule has been attributed to phytopathogenic antagonistic activity (Costa et al., 2022).

4.2.4 Cytophagia

The bacterial class Cytophagia falls under the phylum Bacteriodetes. Members of this phylum have been documented to occur near shores in the water sediments by Ul-Hasan et al. (2019). The organisms identified in this study belong to the genus *Spirosoma*. This genus has been isolated from soil environments by Li et al. (2018) in the Republic of Korea. Kang et al. (2020) reported the presence of isolates similar to *Spirosoma* in fresh water and tree bark samples. This is suggestive that *Spirosoma* species can be classified as endophytes due to their demonstrated ability to populate soil and vegetation. The symbiotic relationship of this genus with regard to vegetation is still being investigated. Therefore, its presence in the nodule is not yet understood.

4.2.5 Actinomycetia

The bacterial class Actinomycetia comprises microorganisms that are important for the decomposition of organic matter and production of extracellular enzymes and other compounds such as antibiotics and fungicides. Members of this bacterial class can be found in compost and soil habitats. The genus *Cutrobacterium* falls under the Actinomycetia bacterial class. Members of this genus have been regarded as either endophytes or plant pathogens (Pinto et al., 2022; Júnior et al., 2012). The organism identified in this study that falls under this bacterial class is *Curtobacterium plantarum*. Presence of this bacteria in the nodules has been reported by El-Batanony (2020) in a study conducted on wild legumes in Egypt. Bulgari et al. (2009) found that bacterial isolates belonging to this genus display endophytic symbiosis. Bacteria that belong to this genus are known to associate with plant roots and assist in plant growth promotion. Bacterial species in this genus can break down polysaccharides, which may explain its presence within the nodules as the cell wall of plants comprises of polysaccharides (Chase et al., 2016).

4.2.6 The phylogenetic relationship of identified isolates

Agrobacterium is grouped among the *Rhizobium* group. According to Farrand et al. (2003), the clustering of *Rhizobium* with *Agrobacterium* was a debatable issue, as they challenge the proposal made by Young et al. (2001), who suggested that *Agrobacterium* be included in the *Rhizobium* genus as the two genera have a common ancestor, and this was determined using 16S rDNA analysis. In this study, *Agrobacterium* was grouped and discussed with the non-rhizobial isolates. However, according to Kuzmanović et al. (2022), the genus *Agrobacterium* was reclassified under the Rhizobiaceae family which includes the genus *Rhizobium*. The *Paenibacullus* and *Bacillus* genus are grouped together, as *Paenibacillus* previously belonged to

the *Bacillus* genus but was then classified into its own genera, as microorganisms belonging to *Paenibacillus* have a conserved genome different to that of *Bacillus* (Grady et al., 2016; Ouyang et al., 2008). *Curtobacterium*, which falls under the bacterial class Actinocycetia, is grouped with the Gammaproteobacteria. This could be due to the ambiguity of the NCBI result shown by the square brackets in the reference for the isolate identification. The *Enterobacter* genus is not as defined as the other clusters. According to Hernandez-Alonso et al. (2021), classification of the *Enterobacter* genus at species level is challenging and may require a combination of polymerase chain reaction (PCR) and amplicon sequencing techniques involving housekeeping genes.

4.2.7 Similarity and differences in bacterial community structure between the different cultivars used in this study

At the class level, four bacterial classes were identified in nodules extracted from the different cultivars. Two classes were shared by all the cultivars, as indicated in Table 4-2. The predominant classes among all the cultivars were Alphaproteobacteria followed by Gammaproteobacteria. This difference in diversity could be due to bacterial community selection by the plant (Sohn et al., 2021). At the genus level, a total of 13 classified genera were obtained from the extracted nodules. Three genera were common among the cultivars and two genera were unique to different cultivars. The common bacterial genera among the cultivars were *Rhizobium, Peribacillus* and *Enterobacter. Spirosoma* and *Agrobacterium* were the only bacterial genera that was peculiar to the Perly cultivar. *Curtobacterium* and *Mesorhizobium* were the only bacterial genera peculiar to the Esparsette (Ficksburg) cultivar, and *Pseudoxanthomonas* as well as *Bacillus* were the only bacterial genera unique to the Esparsette cultivar. Common bacterial genera between Esparsette and Perly were *Sphingomonas* and *Paenibacillus*. The common bacterial genus between Perly and Taja was *Xanthomonas.* According to Busby et al. (2016), this difference in isolate identification could be due to the introduction of exotic microorganisms into the environment by the exotic plant species to enhance host compatibility. Observational analysis of similarities and differences in bacterial diversity between the cultivars showed that the dominant microorganisms identified in this study were rhizobacterial microorganisms. This is similar to results reported by Tokgoz et al. (2020) in a study conducted on legume nodules.

4.2.8 Microbial associations within the environment surrounding the roots

There is a consistent co-occurrence of two rhizobacterial and rhizobial classes between the three cultivars. These classes are Gammaproteobacteria and Bacilli, which have consistently occurred side by side with true rhizobia throughout this study. This suggests that although the factors of specificity come into play in community structure composition, there are core similarities between the cultivars, be it common signalling patterns or common flavonoid secretions, that attract

common microbial members (Sohn et al., 2021). The co-occurrence of these microbial members could be due to their beneficial attributes to the plants. The Proteobacteria group is known for organic matter decomposition and producing glycosyl hydrolases such as cellulase, amylase and chitinase, as well as oligosaccharides and aromatic alcohols which can be used as a carbon source by other bacteria such as *Bacillus* (Berlemont and Martiny, 2016). These metabolic products and organic matter decomposition patterns can be used as an indication of the members involved in the bacterial community defined by *Onobrychis viciifolia* in the rhizosphere. The reason for the occurrence of these members could also be due to competent symbiosis. It can therefore be stated conclusively that the non-symbiotic endophytes naturally occur with symbiotic bacteria, as supported by Lu et al. (2017), where rhizobial and non-rhizobial microorganisms were found to co-habitat soybean nodules.

4.2.9 Nodule community population discussion and impact of plant cultivar on the variation of bacterial taxa

Many factors such as symbiotic genes, signalling, and flavonoid secretions influence nodule microbial diversity. Different root exudate compositions from the different cultivars can attract distinct microbial populations in the rhizosphere to colonise the root and thus be present in the nodule (Sohn et al., 2021). Such can be considered valid when considering the results found in this study. Nodules from three different sainfoin cultivars were extracted and from the results indicated in Table 4-2 and the dendogram shown in Figure 4-5, it can be noted that microbial population composition specificity is not only related to plant species but goes a step further to the cultivar of the particular plant species. The results shown in Table 4-2 suggest that even though the cultivars are all from the same plant species, there is still microbial diversity even within the different cultivars. This suggestion is supported by Afzal et al. (2019), whereby a study was conducted between cultivars that also resulted in a varied microbial population between the cultivars.

The implications of the findings of this study suggest that it is not only true rhizobia that colonise the nodules, as there is also a possibility that other organisms are able to populate and colonise the root nodules (Sohn et al., 2021). Different members of rhizobia colonise the nodules, as each cultivar has been shown to have different species of the rhizobium genus. This highlights the importance and specificity of signalling and flavonoid secretions during plant root colonisation. The co-occurrence of rhizobacteria with rhizobia is indicative of the benefits which can be achieved by co-inoculation of isolates that are found in natural bacterial populations that could facilitate and induce nodule formation and assist in environmental stress management. In a study conducted by Enebe and Babalola (2018), the importance of the co-occurrence of rhizobacteria is highlighted, where a leguminous plant capable of forming symbiotic relationships with rhizobia

was affected by drought. Even though the nitrogen fixation was secured, the plant was affected by the drought. Without the drought-resistant trait of the rhizobacteria (non-rhizobial), the plant would not survive. This highlights the invaluable importance of rhizobacteria–plant symbiosis.

4.2.10 Statistical analysis

The statistical measure of bacterial diversity used in this study was the Shannon Index of diversity. According to Ortiz-Burgos (2015), the Shannon Index of diversity values range from 1.5 to 3.5. When used in ecological studies, a high Shannon Index value is indicative of a high community diversity (Yin et al., 2019). This is conclusive with the results found in this study. The exotic cultivar, Perly, had the highest value (2.16) for the diversity index and also the highest diversity in terms of its bacterial community population (nine bacterial genera). This was followed by the endemic cultivar, Esparsette, which had a value of 1.88 for the diversity index. The exotic cultivar, Taja, had the second lowest diversity index value (1.56), and the lowest diversity index belonged to the endemic cultivar, Esparsette, with a diversity index of 1.09. The diversity indices were in proportion to the microbial communities associated with each cultivar, as Perly had the highest index and microbial community followed by Esparsette (seven bacterial genera), then Taja (five bacterial genera), and, finally, Esparsette (Ficksburg) (three bacterial genera).

CHAPTER 5: EVALUATION OF PLANT GROWTH-PROMOTING PROPERTIES

5.1 Results

After molecular identification of bacterial isolates from three different sainfoin cultivars, one endemic – Esparsette, and two exotic – Perly and Taja, these isolates were screened for plant growth-promoting traits. These traits were: phosphate solubilisation, siderophore production, indole acetic acid (IAA) production and nitrogen fixation (Table 5-1). The positive results for nitrogen fixation detected in the *Rhizobium* and *Mesorhizobium* was followed by a literature-based comparison of the amount of nitrogen fixed by each of the genera, using two quantification methods (^{15}N isotopic dilution and ^{15}N natural abundance).

5.1.1 Screening for plant growth-promoting properties

Table 5-1 shows the results obtained from the screening of the plant growth-promoting properties of isolates according to the different cultivars. Esparsette and Perly had the higher numbers of isolates positive for phosphate solubilisation and siderophore production. Perly showed the highest number of isolates that showed positive for nitrogen fixation. All the isolates from the Esparsette and Taja cultivars tested positive for siderophore production. All the isolates from the Esparsette (Ficksburg) cultivar tested positive for phosphate solubilisation and nitrogen fixation.

Table 5-1: Table showing plant growth-promoting properties evaluation

Plant growth-promoting trait:		**Cultivar:**			
		Esparsette	**Esparsette (Ficksburg)**	**Perly**	**Taja**
Phosphate solubilisation	**positive**	*E1* *E2* *E3* *E5* *E6* *E7* *E11*	*Ef40* *Ef44* *E46*	*P17* *P21* *P23* *P25* *P27* *P28* *P31*	*T34* *T37* *T38*
	negative	*E10* *E4*	*None*	*P15* *P18* *P22* *P24* *P25*	*T33* *T39* *T36*

Siderophore production	**positive**	*E1* *E2* *E3* *E4* *E5* *E6* *E7* *E10* *E11*	*Ef40* *E46*	*P15* *P17* *P18* *P21* *P22* *P25* *P27* *P28* *P31*	*T33* *T34* *T36* *T37* *T38* *T39*
	negative	*None*	*Ef44*	*P23* *P24*	*None*
Nitrogen fixation	**positive**	*E1* *E4* *E5* *E6* *E7* *E10* *E11*	*Ef40* *Ef44* *E46*	*P17* *P18* *P21* *P22* *P23* *P25* *P27* *P28* *P31*	*T33* *T34* *T36* *T37* *T38*
	negative	*E2* *E3*	*None*	*P15* *P24*	*T39*

Figure 5-1 shows the concentrations of IAA produced by isolates from the different sainfoin cultivars, one endemic – Esparsette, and two exotic – Perly and Taja. The concentrations varied according to cultivar. Esparsette had three bacterial isolates that showed IAA concentrations below 1 µg/ml and six that showed concentrations greater than 2 µg/ml, with the highest concentration being 19.81 by isolate E6 (*Rhizobium glycinendophyticum*). Esparsette (Ficksburg) had one bacterial isolate that showed IAA concentration below 0 µg/ml and two that showed concentrations greater than 9 µg/ml, with the highest concentration being 20.36 µg/ml by isolate Ef44 (*Mesorhizobium helmanticense*). Perly had four bacterial isolates that showed IAA concentration below 0 µg/ml and seven that showed concentrations greater than 2 µg/ml, with the highest concentration being 17.69 µg/ml by isolate P31 (*Paenibacillus peoriae*). Taja had three bacterial isolates that showed IAA concentration below 0 µg/ml and three that showed concentrations greater than 3 µg/ml, with the highest concentration being 21.97 µg/ml by isolate T39 (*Enterobacter ludwigii*).

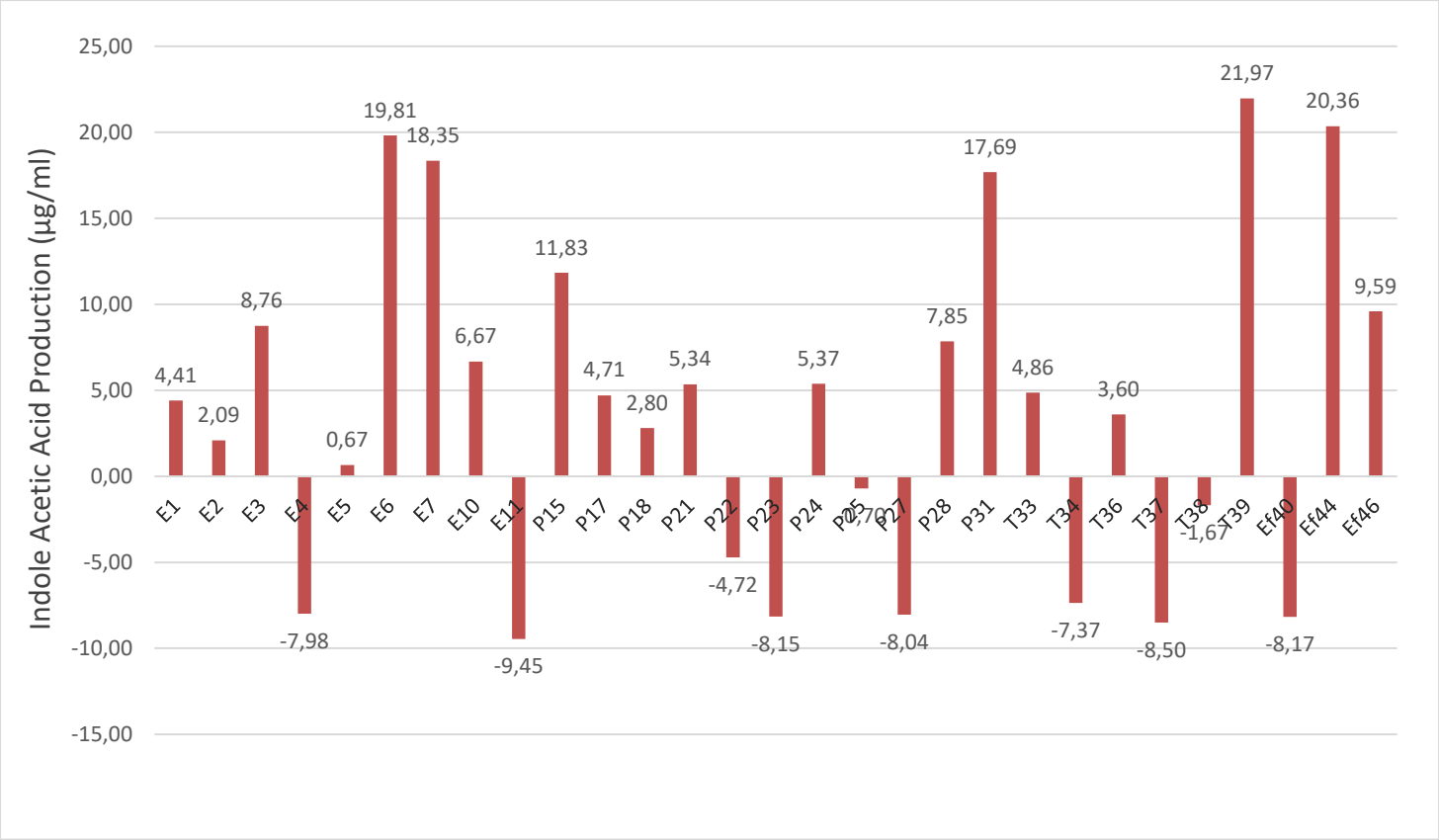

Figure 5-1: Graph showing Indole Acetic Acid production of the isolates from different sainfoin cultivars

5.1.2 Literature based comparison of the two rhizobial genera in terms of quantification of nitrogen fixation

Table 5-2 shows the literature-based comparison of the amount of nitrogen fixed by the identified genera, *Rhizobium* and *Mesorhizobium*. For both genera, studies utilising ^{15}N isotopic dilution and ^{15}N natural abundance was included.

Table 5-2: Table showing the literature based comparison of identified rhizobia according to N_2 fixation in legume studies

Legume studied:	*Rhizobial strains*		Amount of nitrogen fixed (kg. ha^{-1})	Method of analysis for nitrogen quantification	References:
	Rhizobium sp.	***Mesorhizobium sp.***			
Vicia faba	*Rhizobium sp.*		76–125	^{15}N isotopic dilution	(Carranca et al., 1999)
Pisum sativum	*Rhizobium sp.*		34–112	^{15}N isotopic dilution	(Kelstrup et al., 1996)
Lens culinaris	*Rhizobium sp.*		37–55	^{15}N natural abundance	(Usukh et al., 2010)
Anythyllis sp.		*Mesorhizobium sp.*	78.6	^{15}N isotopic dilution	(Mahieu, 2014)
Cicer arietinum		*Mesorhizobium sp.*	32.1–62.1	^{15}N natural abundance	(Tena et al., 2016)
Lotus pedunculatus		*Mesorhizobium sp.*	99.2	^{15}N isotopic dilution	(Casanova-Lugo et al., 2014)

5.2 Discussion

Isolates identified in this study (Chapter 4) were screened for plant growth-promoting properties. These isolates were screened for these traits through the use of culture-based/ plating methods. Phosphorous is an important nutrient required for increased crop yield, therefore, the ability for plant-associated microorganisms to solubilise phosphorus present in the soil is important (Amanullah & Muhammad, 2015). For phosphate solubilisation, isolates identified as rhizobia from endemic – Esparsette, and exotic – Perly and Taja cultivars showed positive results. These isolates were identified as belonging to the genera *Rhizobium* and *Mesorhizobium*. This is supported by a study conducted by Sridevi and Mallaiah (2009), where positive phosphate solubilisation was exhibited by *Rhizobium* isolates from legume roots and legume nodules. Positive phosphate solubilisation by *Mesorhizobium* is supported by Brigido et al. (2019), where *Mezorhizobium* species known to nodulate chickpeas showed positive results for phosphate solubilisation. For the rhizobacterial isolates extracted from the endemic cultivar, isolates belonging to the genera *Sphingomonas*, *Pseudomonas*, *Paenibacillus*, *Enterobacter* and *Bacillus* displayed positive results. For the rhizobacterial isolates extracted from the exotic cultivars, isolates belonging to the genera *Spirosoma* and *Agrobacterium* exhibited positive results. The ability for rhizobacteria to solubilise phosphates is supported by Sashidhar and Podile (2010), where the organic acids released by the different rhizobacteria are documented. Isolates extracted from exotic cultivars that belong to the genera *Pseudomonas*, *Xanthomonas*, *Bacillus*,

Enterobacter and *Sphingomonas* exhibited varying results in terms of phosphate solubilisation ability. This could be due to the contrasting solubilisation capacity among members within the same genera due to the release of different organic acids such as formate, acetate, lactate, glycolate, fumarate and succinate. According to Orozco-Masqueda et al. (2021), the genera *Agrobacterium*, *Bacillus*, *Enterobacter,* and *Pseudomonas* produce phosphatases that mineralise phosphates found in the soil. The variance in organic acid synthesis is highlighted by culture-based methods. Hence, there is a possibility that the phosphate solubilising ability can be present in some isolates but cannot be detected via plating techniques. This variance within the same bacterial genera is observed in the exotic cultivars.

Siderophores promote plant growth through the supply of iron to the plants (Ahemad & Kibret, 2014). For siderophore production, the rhizobial strains from the exotic cultivars showed positive results. For the endemic cultivar, one out of the two identified rhizobial strains exhibited a positive result; the other exhibited a negative result. The genus *Rhizobium* showed a positive result, while the *Mesorhizobium* showed a negative result. This negative result is suggestive that: I) this particular isolate does not have the genes necessary for the expression of proteins needed for siderophore biosynthesis, or, II) this isolate produces siderophores that can be detected using different culture media. This is supported by a study conducted by Brigido et al. (2017), where it is shown that siderophore production of the genus *Mesorhizobium* varied between species depending on the culture media used. The ability to produce siderophores by the isolates identified as *Rhizobium* isolates is supported by Berraho et al. (1997), where different *Rhizobium* isolates produced different siderophore compounds. The rhizobacterial isolates of the endemic and exotic cultivars showed positive results for the production of siderophores for all isolates excluding *Spirosoma* and *Paenibacillus* from the exotic cultivar, Perly. The ability of rhizobacterial strains to produce siderophores is supported by Joseph et al. (2007), laslo et al. (2012) and Ghavami et al. (2017). The negative results shown by *Spirosoma* and *Paenibacillus* could be due to molecular defects. Najimi et al. (2008) suggest that bacterial isolates with deletions in target genes (asbG, asbD and asbC) are unable to effectively synthesise siderophores. The mechanism of siderophore production evaluation on CAS agar involves the uptake of iron from the culture media, resulting in a halo forming around the bacterial inoculation area. In some cases, this halo can be colourless or can have red shading. This red shading can be interpreted as a pathogenic indicator. According to Ahemad and Kibret (2014), phytopathogens can establish themselves through the sequestration of iron from the environment. Several isolates identified in this study exhibited a red-hued halo result on the CAS agar. These isolates were identified as belonging to the genera *Agrobacterium*, *Pseudomonas* and *Enterobacter*. The possibility of these isolates being phytopathogens is documented by da Silva et al. (2018).

Nitrogen is thought to be the most important nutrient needed by plants, as it is required for plant growth and plant productivity. The ability to fix atmospheric nitrogen was tested using Burk's media. From the results obtained in this study, a large number of isolates showed positive results. The rhizobial isolates, *Rhizobium* and *Mesorhizobium*, from the exotic and endemic cultivars all showed positive for nitrogen-fixing ability. This is a mark of true rhizobia microbes, in that the rhizobial members in rhizosphere studies are known as mainly nitrogen fixers (Yousef, 2018). The rhizobacterial isolates showed varied results for nitrogen-fixing ability. Isolates belonging to the genera *Bacillus*, *Argobacterium*, *Pseudomonas*, *Xanthomonas* and *Spirosoma* have shown the ability to fix atmospheric nitrogen. This is supported by Verma et al. (2013) in a study to increase the nodulation in chickpeas using plant growth-promoting rhizobacteria. This ability to fix atmospheric nitrogen is attributed to the expression of *nif* genes that encode for the expression of nitrogenase. The rhizobacterial isolates that exhibited a negative result belong to the genera *Sphingomonas*, *Enterobacter* and *Pseudoxanthomonas*, and this negative result can be attributed to the absence of the necessary *nif* genes. Their presence can be attributed to the premise that they may be nitrogen scavengers instead of nitrogen fixers, in that they feed off the nitrogen fixed by neighbouring microbial population members (Orozco-Mosqueda et al., 2021). The table showing the literature-based comparison of *Rhizobium* and *Mesorhizobium* in terms of nitrogen fixation by different legume crops that form symbiosis with the genera *Rhizobium* and *Mesorhizobium* were compared. The quantification of nitrogen fixation using the ^{15}N natural abundance technique involves the consideration of differences in nitrogen sources available for plant use in the soil and atmospheric nitrogen, whereas the use of ^{15}N isotopic dilution technique involves a comparison of fixed nitrogen between N_2 fixing and non-N_2 fixing plants (usually called 'reference plants') grown in ^{15}N enriched fertilizer over a certain growth period (Boddey et al., 2000). The nitrogen fixation was quantified through the ^{15}N isotope dilution and the ^{15}N natural abundance analysis, and the results from the different studies show that the genus *Rhizobium* is the better symbiont in terms of nitrogen fixation. According to Lindström and Mousavi (2020), biological N2 fixation rates vary under different sites and environmental conditions, and this is evidently observed in Table 5-1.

Indole Acetic Acid is an auxin, which means it is a plant hormone, and one that regulates the growth and development of plants. The processes of cell division and tissue differentiation – and hence root and shoot growth, are regulated by IAA (Prusty et al., 2004). Indole Acetic Acid stimulates the growth of root hairs and in that process releases nutrients such as saccharides from root cell walls (Etesami et al., 2015). From the results obtained for the screening of the production of IAA, it can be observed that most of the isolates from all three of the cultivars were able to produce IAA. For the endemic cultivar, Esparsette, it can be observed that the rhizobial strains identified, *Rhizobium* and *Mesorhizobium* were able to produce IAA. The production of

IAA by *Mesorhizobium* is supported by a study of Mendez et al. (2020), in which the positive IAA production of different *Mesorhizobium* strains was reported. The IAA production of *Rhizobium* isolate is supported by Ghosh et al. (2007), where positive IAA production of rhizobial strains was reported. For the exotic cultivars, Perly and Taja, it can be observed that of the rhizobial strains identified, two strains of *Rhizobium* showed varied results. The rhizobial isolate from the Perly cultivar was able to produce IAA, however, the isolate from the Taja cultivar was unable to produce IAA. According to Gopalakrishnan et al. (2015), the biosynthesis of IAA in *Rhizobium* species is carried out through the indole-3-pyruvic acid; this requires tryptophan as a precursor, which may not have been available for use by the isolate. For the rhizobacterial isolates of the endemic cultivar, Esparsette, isolates from the genera *Sphingomonas, Pseudoxanthomonas, Bacillus* and some *Peribacillus* isolates were able to produce IAA. Isolates from the genera *Paenibacillus, Enterobacter* and some *Peribacillus* isolates were not able to produce IAA. For the rhizobacterial isolates from the exotic cultivars, Perly and Taja, isolates from the genera *Sphingomonas*, *Enterobacter*, *Pseudomonas*, *Paenibacillus* and some *Peribacillus* isolates were able to produce IAA. Isolates from the genera *Xanthomonas*, *Spiromsoma*, *Agrobacterium* and some *Peribacillus* isolates were not able to produce IAA. Indole Acetic Acid production by *Pseudomonas*, *Enterobacter* and *Bacillus* is supported by Chase et al. (2016). The variance in results observed for isolates of the same genera can be a result of gene transfer in the bacterial community pool. The lack of IAA production can be attributed to a lack of genes that encode for the proteins needed for IAA biosynthesis and to the lack of precursors needed for the IAA synthesis pathway (Leontovyčová et al., 2020; Spaepen et al., 2007). At first glance, the ‘negative’ production of IAA that is exhibited by certain isolates could be seen as a malfunction of laboratory equipment or the inability of the equipment to measure IAA levels or the isolates. However, this might be explained by a phenomenon described by Ghosh et al. (2008), namely, as the antagonistic effect that occurs when a microorganism produces enzymes that break down the IAA produced by the same microorganism. To confirm this, one would need to evaluate the effect of enzyme production on plant hormones for these isolates.

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

6.1 General conclusion

The aim of this study was to identify and characterise root nodule bacteria (rhizobia) associated with three sainfoin cultivars, one endemic to South Africa, Esparsette, and two exotic cultivars, Perly and Taja. The study commenced with a review of literature, in Chapter 2, on soil bacterial communities associated with legumes, the functions of rhizobia and rhizobacteria – which highlighted the importance of biological nitrogen fixation, the symbiotic relationship that exists between bacteria and legumes, and the benefits of this relationship in terms of plant growth promotion and the molecular methods used to identify the bacterial community (Luciński et al., 2002; Liu et al., 2018). Sainfoin plants (three cultivars) were then extracted from the greenhouse after 90 days from planting and root nodules were separated from the root for laboratory analysis (Chapter 3). The second sampling was done at a sainfoin farm in Ficksburg, Free State, where the sainfoin plants (Esparsette cultivar) were uprooted and the roots were examined for the presence of nodules and prepared for laboratory analysis. The laboratory analysis involved sterilisation of the root nodules, and isolation, cultivation, and purification of the bacteroids. Thereafter, the molecular analysis of the bacteroids was done using DNA-based identification followed by phylogenetic analysis (Chapter 4). Following on from here, the plant growth-promotion traits (Chapter 5) of the identified bacteria were investigated by testing for nitrogen fixation on Burk's media, phosphate solubilisation on glucose yeast media, siderophore production on CAS agar and indole acetic acid (IAA) production using Kings B media and the Salkowski reagent. The literature-based comparison of the nitrogen fixation by the identified rhizobial microorganisms was done through comparing the amount of the nitrogen fixed by the identified genera calculated through N_2 isotopic dilution and the ^{15}N natural abundance methods.

To achieve the aim of this study, three specific objectives were put forward at the onset of the investigation. The major findings were as follows:

6.1.1 Identifying the rhizobia and rhizobacteria associated with exotic, Perly and Taja, and endemic, Esparsette, O. *viciifolia* cultivars by means of DNA-based molecular techniques

The first objective of this study was to identify and characterise rhizobial and rhizobacterial microorganisms associated with exotic and endemic sainfoin cultivars. This was achieved through the isolation and cultivation of root nodule bacteroids. Polymerase chain reaction and 16S region sequencing were used to determine the phylogeny of the extracted isolates. After the elimination of repetitions, a total of 29 bacterial isolates were identified. Six out of the 29 identified bacterial

isolates were rhizobial microorganisms and 23 were rhizobacterial microorganisms. The cultivars Esparsette and Taja had two rhizobial microorganisms each. Esparsette (Ficksburg) and Perly had one rhizobial microorganism each. The endemic cultivar, Esparsette, grown during this study in a greenhouse, comprised of more isolates than the Esparsette obtained from a farm in Ficksburg. This can be attributed to soil microbial population and the molecular dialogue between the rhizospheric bacteria and the plant (discussed in Chapter 4). The bacterial classes associated with the endemic cultivar, Esparsette, were Alphaproteobacteria (34%), Bacilli (33%), Gammaproteobacteria (25%) and Actinomycetia (8%), with the Alphaproteobacteria group being the dominant bacterial class. For the Esparsette (Ficksburg) cultivar, the bacterial classes identified were Alphaproteobacteria (34%), Gammaproteobacteria (33%) and Actinomycetia (33%), with the Alphaproteobacteria group being the dominant bacterial class.

The bacterial classes associated with the exotic cultivar, Perly, were Gammaproteobacteria (37%), Alphaproteobacteria (27%), Bacilli (27%) and Cytophagia (9%), with the Gammaproteobacteria being the dominant bacterial class. For the second exotic cultivar, Taja, the bacterial classes identified were Gammaproteobacteria (50%), Alphaproteobacteria (33%) and Bacilli (17%), with the Gammaproteobacteria being the dominant bacterial class. The genera associated with each cultivar varied and were discussed in Table 4-2. The results revealed that over and above the presence of rhizobia in the nodule, the presence of rhizobacteria was also observed. This was an interesting finding, which highlighted the specificity of plants to cultivar level and leads to questions about the function of the rhizobacteria found inside the nodules.

6.1.2 Screening for potential plant growth-promoting properties of the rhizobia and rhizobacteria identified from the exotic, Perly and Taja, and endemic, Esparsette, *O. viciifolia* cultivars

The second objective of this study was to screen the identified isolates from the exotic and endemic cultivars for plant growth-promoting traits. This was done using culture-based methods. The results of the plant growth-promoting properties by the rhizobia and rhizobacteria varied between the three cultivars – refer to Table 5-1. For the endemic cultivar, Esparsette, the identified rhizobia isolates showed positive results for phosphate solubilisation and siderophore production. The identified rhizobacteria isolates showed positive results for siderophore production; moreover, only isolate E10 showed negative results for phosphate solubilisation. For the Esparsette (Ficksburg) cultivar, the identified rhizobia isolate showed positive results for phosphate solubilisation but showed negative results for siderophore production. The identified rhizobacteria isolates tested positive for phosphate solubilisation and siderophore production. It was observed that the rhizobia for Esparsette (Ficksburg) are unable to produce siderophores, unlike the rhizobia associated with Esparsette.

For the exotic cultivar, Perly, the identified rhizobia isolate showed positive results for phosphate solubilisation and siderophore production. The identified rhizobacteria isolates showed varied results (positive and negative) for phosphate solubilisation. The rhizobacteria isolates tested positive for siderophore production, except for P23 and P24. For the exotic second cultivar, Taja, the identified rhizobia isolates showed positive results for phosphate solubilisation and siderophore production. The rhizobacteria isolates showed varied results. For phosphate solubilisation, only isolate T38 showed positive results. For siderophore production, all rhizobacteria isolates were positive.

For IAA production, the rhizobia and rhizobacteria isolates were able to produce IAA. Some of the rhizobacteria showed negative values for IAA production (Figure 5-1). These negative values are a possible outcome of the enzymatic breakdown of IAA by indole oxidase (Ghosh et al., 2008).

The results revealed that for the exotic cultivars, the rhizobial isolates were able to solubilise phosphates, produce siderophores and fix nitrogen. For the rhizobacterial isolates, most were able to solubilise phosphates, produce siderophores and fix nitrogen, except for non-phosphate solubilising *Sphingomonas*, *Bacillus*, *Pseudomonas*, *Xanthomonas*, and *Enterobacter*, non-nitrogen fixing *Sphingomonas* and *Paenibacillus* as well as non-siderophore producing *Spirosoma* and *Peribacillus*.

6.1.3 Evaluation of nitrogen-fixing ability of the rhizobia and rhizobacteria identified from exotic, Perly and Taja, and endemic, Esparsette, *O. viciifolia* cultivars

The results of the nitrogen fixation by the rhizobia and rhizobacteria varied between the three cultivars (Table 5-2). For the endemic cultivar, Esparsette, the rhizobia isolates showed positive results for nitrogen fixation. The identified rhizobacteria isolates showed positive results for nitrogen fixation, except for E2 and E3. For the Esparsette (Ficksburg) cultivar, the rhizobia and rhizobacteria isolates showed positive results for nitrogen fixation. For the exotic cultivar, Perly, the rhizobia isolate showed positive results for nitrogen fixation. The identified rhizobacteria isolates tested positive for nitrogen fixation, except for P15 and P24. For the second exotic cultivar, Taja, the rhizobia showed positive results for nitrogen fixation. For the rhizobacteria isolates, only T39 showed negative results for nitrogen fixation.

The root nodule is a unique specialised structure established in the roots of leguminous plants via a highly specific symbiosis between legumes and nodule-inducing bacteria (Benizri et al., 2001). Unlike previous assumptions that rhizobia were the only group of bacteria inhabiting legume root nodules, the results of this study document the isolation and identification of bacterial species belonging to other bacterial genera (Figure 6-1). These bacteria can be regarded as

nodule endophytes, non-rhizobia endophytes or nodule-associated bacteria. These non-rhizobia bacteria now bring about the need for the exploration of their role or potential benefits (Sharma et al., 2020). By understanding their roles and exploiting their potential benefits, non-rhizobial bacteria may provide new opportunities to enhance agricultural production through their capability of plant growth promotion.

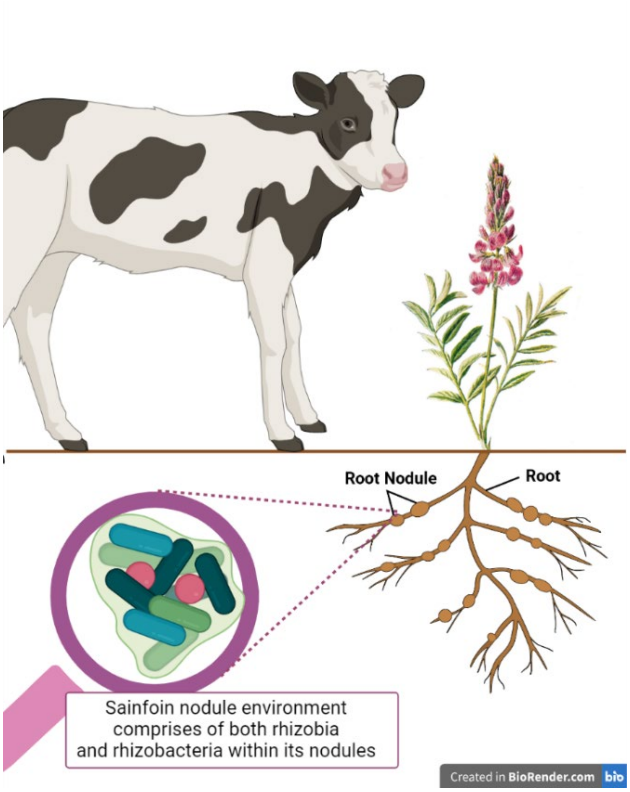

Figure 6-1: Schematic representation of livestock grazing on sainfoin (*Onobrychis viciifolia*) harbouring rhizobia and rhizobacteria

6.2 Recommendations

The first objective involved the identification of the different rhizobial and rhizobacterial symbiotic microorganisms. From the results obtained, agriculturally important isolates have been identified. For this, a whole genome sequencing analysis or a metagenomic survey of these isolates is recommended.

Sequencing the isolates with known housekeeping genes for the separate groups of rhizobia and rhizobacteria for a more defined evolutionary analysis is also recommended, as this will allow the possible determination and indication of genes obtained by the isolates from the environment. This will also give a better understanding as to which bacterial populations are the best for optimised plant growth and establishment (Puławska et al., 2022).

The molecular underpinnings of the plant growth-promoting rhizobia and rhizobacteria are not yet fully understood. By coupling culture-based methods with gene screening of the genes responsible for a particular plant growth-promotion trait, a more defined understanding of the mechanism used by the microbes may be achieved.

Culture-based methods used to screen for the different plant growth-promoting traits need to be optimised through the inclusion of more than the use of a universal media, as some isolates have plant growth-promoting traits, however, the screening methods are unable to detect these abilities. The screening of the plant growth-promoting effects should be coupled with enzyme production tests to further define the scope of the bacterial plant growth-promoting abilities (Fahsi et al., 2021).

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