Bacterial endophytes in the leaves of *Pavetta* spp. with a specific focus on those causing leaf nodules

by

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Declaration

I, the undersigned, hereby declare that the thesis submitted herewith for the degree **Magister Scientiae** to the University of Pretoria contains my own independent work. This work has not previously been submitted for any other degree at any other University.

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SUMMARY

Bacteria filled leaf nodules can be found on some plant species within the genus *Pavetta*. The identity of the bacterial endophytes within leaf nodules has been described for certain leaf nodulated plant species such as *Psychotria kirkii* and *Pavetta schumanniana*. These bacteria were found to belong to the genus *Burkholderia*. The bacterial endophyte population within the leaves of three *Pavetta* spp. indigenous to South Africa, viz. *P. lanceolata*, *P. edentula* and *P. schumanniana,* was investigated in this study with a focus on the species of bacterial endophytes that form the nodules on the leaves of these plants. To achieve this, a combination of culturing and Denaturing Gradient Gel Electrophoresis (DGGE) was used. The bacterial endophyte population within the leaves was found to be different between the three plant species but harboured bacterial genera that have been found in other plants. The nodule-forming bacterial endophyte in *P. edentula* and *P.*

schumanniana was found to be related to those *Burkholderia* spp. previously described. The nodule forming bacterial endophyte in *P. lanceolata* specimens growing in pots was found to be a different species, i.e. from the family Bradyrhizobiaceae. This was compared to *P. lanceolata* specimens that were growing in open soil where a bacterium belonging to the genus *Burkholderia* was identified within the nodules. Further sampling and experimentation is necessary to determine if this finding is a rare incident and why the bacterial species within the leaf nodules of *P. lanceolata* differed between the two sites.

Leaf nodule development and morphology has been well documented within the *Psychotria*, another plant genus that includes leaf nodulated species. The leaf nodule development in *Pavetta* spp. has not been documented. With the aid of electron microscopy, leaf nodule development was studied in the three indigenous *Pavetta* spp. Differences such as the number of bacteria at various leaf nodule ages and the shape of the bacteria were noted between the three plant species. The most notable difference was between the leaf nodules of *P. edentula* and *P. schumanniana* which were shown to be colonized by *Burkholderia* spp. in comparison to the *P. lanceolata* leaf nodules that were filled with a bacterium from the Bradyrhizobiaceae. Once again further analysis is required to determine if this difference in nodule morphology is due to the nodule-forming bacterial species or the species of *Pavetta*.

The presence of the nodule-forming bacteria has been observed within the leaf nodules and on rare occasions within the flowers and seeds of some *Pavetta* spp. but their presence within stem tissue and deciduous hosts during winter has not been reported. With the use of *Burkholderia* specific primers the presence of the nodule-forming bacterial endophyte was investigated in different tissue types of *P. schumanniana* in winter and summer and within *P. edentula*. A *Burkholderia* sp. was detected within the older leaf buds of both winter and summer samples of *P. schumanniana* as well as in the flowers. No *Burkholderia* spp. were detected in the stem tissue of either plant species or the leaf buds of *P. edentula*.

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PREFACE

Bacterial endophyte communities within plants in their natural environments have only recently become of interest to scientists (Reiter & Sessitsch, 2006). Various plant species within the Rubiaceae share a specialised symbiotic relationship with bacterial endophytes in the form of leaf nodules. There are three plant genera within this family that contain species that have bacterial nodules on their leaves, namely *Psychotria*, *Pavetta* and *Sericanthe* (van Wyk *et al*., 1990; Van Oevelen *et al*., 2001). *Pavetta* spp. that are indigenous to South Africa include *P. lanceolata* (Forest bride's bush), *P. edentula* (Gland-leaf bride's bush) and *P. schumanniana* (Poison bride's bush) (Coates Palgrave, 2002).

The fact that the leaf nodules are filled with bacteria was first discovered by Zimmerman in 1902. Since that time many scientists have attempted to identify the bacterial endophytes that form these leaf nodules but it was only in 2002 that Van Oevelen *et al*. were able to do so with the use of molecular techniques. This study found the nodule-forming bacterial endophyte in *Psychotria kirkii* to be closely related to the genus *Burkholderia* spp. However, these bacteria are to date unculturable and are classified under the provisional status of *Candidatus*. Once identification was successful it was not long thereafter that other leaf nodule-forming bacterial endophytes were identified in other nodulated plant species (Van Oevelen *et al*., 2004) amongst which was *Pavetta schumanniana* (Lemaire *et al*., 2011).

Leaf nodule development and morphology were studied in depth in *Psychotria* spp. with the use of electron microscopy (Lersten and Horner, 1967; Whitmoyer and Horner, 1970; Miller *et al*., 1983; Miller, 1990). Lersten and Horner (1967) found that the nodule-forming bacterial endophytes entered into the primordial leaf tissue through prematurely formed stomatal openings which thereafter closed as the bacteria began to divide and grow into the centre of the leaf blade. Other characteristics associated with leaf nodule development included unusual cell wall thickening and plant cell

distortion of the mesophyll cells that were in contact with the bacteria. Nodule development and morphology within the *Pavetta* spp. has not been studied in as much depth as has occurred with *Psychotria* spp.

The presence of the leaf nodule-forming bacteria has been found in low numbers within the seeds and flowers of various plant hosts (Miller, 1990). The process whereby the bacteria move from the leaf tissue to the flower and thereafter into the seed has, however, not been studied. *P. schumanniana* is a deciduous tree (Coates Palgrave, 2002) and should theoretically have a mechanism whereby the leaf nodule bacterial endophytes are stored within the plant during winter. The possible presence of the leaf nodule-forming bacterial endophytes within the stem tissue of nodulated hosts has not been explored.

This study focuses on the symbiotic relationship between bacterial endophytes and their plant hosts in the form of bacterial leaf nodules. The plant species to be investigated in this study are *Pavetta lanceolata*, *P. edentula* and *P. schumanniana*. Culturing and Denaturing Gradient Gel Electrophoresis (DGGE) will be used to identify the bacterial endophyte population within the leaves of these three plant species as well as to determine the identity of the nodule-forming bacterial endophytes. Electron microscopy will be used to observe characteristics associated with nodule development and morphology in the three *Pavetta* spp. Further studies will be conducted on the presence of the nodule-forming bacterial endophyte within the seeds and flowers of *P. schumanniana* as well as the stem and leaf bud tissues of *P. schumanniana* and *P. edentula*.

The first hypothesis of this research is that the nodule-forming bacterial endophytes will be related to the previously identified nodule-forming bacterial endophytes and will group with the genus *Burkholderia.* Secondly, the nodule development within the *Pavetta* spp. will resemble that found within nodulated *Psychotria* spp. Lastly, the nodule-forming bacterial endophytes will most likely be found within the flower, seed and leaf bud tissues but not within the stem tissue.

Identifying the bacterial endophyte population and the nodule-forming bacterial endophytes within the various *Pavetta* spp. will not only contribute to the existing knowledge of this plant species in South Africa but also to the general knowledge of this symbiotic relationship. Identification of the nodule-forming bacteria may give insight into how they may be cultured and the beneficial properties that they give to their plant hosts. Characteristics associated with nodule development combined with the knowledge of these bacterial species may also contribute to the understanding of this specific symbiotic relationship. Further research on leaf-nodule forming bacterial endophytes could lead to the use of the beneficial traits of this bacterial interaction in crop production and may shed light on the 'gousiekte' disease caused by various leaf-nodulated plant species when the leaves are eaten by domestic ruminants.

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Chapter 1

Bacterial endophytes in the leaves of the *Pavetta* spp. (Rubiaceae) with specific focus on those causing leaf nodules

Bacterial endophytes in the leaves of the *Pavetta* spp. (Rubiaceae) with specific focus on those causing leaf nodules

1.1) Introduction

The term endophyte stems from the Greek words '*endon*' meaning within and '*phyte*' meaning plant (Sturz *et al*., 2000). Therefore by definition a bacterial endophyte is a bacterium that can be found within plant tissue. Hallmann *et al*. (1997) defined a bacterial endophyte as any bacterium that can be isolated from surface sterilized plant material and that does not cause any visible harm to that plant. The study of bacterial endophyte populations are now carried out using the combination of culture-dependent and –independent techniques. The culture-independent approach provides a general overview of the bacterial endophyte community and the culture-dependent techniques give the opportunity to test the biochemical properties and Koch's postulates on those that can be cultured (Reiter and Sessitsch, 2006; Hardoim *et al*., 2008; Andreote *et al*., 2009).

Pavetta spp. is the largest plant genus that has bacterial nodules on their leaves (Miller, 1990). Three *Pavetta* spp. that are indigenous to South Africa include *P. edentula*, *P. lanceolata* and *P. schumanniana*. The latter plant species is poisonous to ruminants causing a disease known as "gousiekte" (Coates Palgrave, 2002).

The nodules or galls that are found on the leaves of *Pavetta* spp. are formed by endophytic bacteria (Zimmermann, 1902) that have formed a symbiotic relationship with the plants (Miller, 1990). This symbiotic relationship is crucial for the plants survival in that without the bacterial partner, growth of the plant is limited and results in death after approximately three years (Miller, 1990). The nodule bacteria were once thought to fix nitrogen but more recent studies have shown that this may not be the case (Miller, 1990). It is now hypothesized that the bacteria are involved in the production of a

cytokinin–like substance that enables the plant to grow and develop normally (Becking, 1971).

It has been suggested that the nodule-forming bacterial endophytes are acquired from the parent plant through the seed (Von Faber, 1912; Lersten and Horner, 1967). These bacterial endophytes then enter the primordial leaf tissue via precocious stomatal openings (Lersten and Horner, 1967). Leaf nodule development and morphology has been well documented for *Psychotria* spp. (Lersten and Horner, 1967; Whitmoyer and Horner, 1970; Miller *et al*., 1983; Miller, 1990) but less so for *Pavetta* spp. The presence of the leaf nodule-forming bacterial endophyte has been reported in the seed and flowers of various leaf nodulated plant species (Miller, 1990) but there is a lack of information on the presence of this bacterium in the stem and leaf bud tissue of their host.

Despite the many efforts to culture the nodule-forming bacteria, it has still not been achieved (Van Oevelen *et al*., 2004; Lemaire *et al*., 2011b; Verstraete *et al*., 2011). However, the identity of these bacteria have been discovered using 16S rRNA gene cloning and sequencing (Van Oevelen *et al*., 2002; Lemaire *et al*., 2011b; Verstraete *et al*., 2011). Van Oevelen *et al*. (2002) were the first to identify the nodule-forming bacterial endophytes in *Psychotria kirkii* as belonging to the genus *Burkholderia*. They classified the bacteria as '*Candidatus* Burkholderia kirkii' sp. nov. A further two nodule-forming bacterial endophytes were classified from *P. calva* and *P. nigropunctata* by Van Oevelen *et al*. in 2004 which were classified as '*Candidatus* Burkholderia calva' and '*Candidatus* Burkholderia nigropunctata'. Lemaire *et al*. (2011 b) were the first to describe the nodule-forming bacterial endophyte in *Pavetta* spp., namely *P. hispida*, *P. rigida* and *P. schumanniana*. The bacteria from this study were classified as '*Candidatus* Burkholderia hispidae', '*Candidatus* Burkholderia rigidae' and '*Candidatus* Burkholderia schumannianae' respectively.

The identification of the nodule-forming bacterial endophytes within the *Pavetta* spp. will not only aid in the quest to culture the bacteria but will also

allow for a breakthrough in research on the benefits that these bacteria give to the plant. The outcome of this research will also add to the knowledge of indigenous plants in South Africa. This review will focus on the various techniques used to study bacterial endophytes as well as what is already known about leaf nodule bacterial endophytes, specifically in *Pavetta* spp., and their role in nodule morphology and development.

1.2) Bacterial endophytes

The current definition of a bacterial endophyte is that it is a bacterium that can be isolated from surface sterilized plant material and can survive within plant tissue without visibly harming the plant in any way (Hallmann *et al*., 1997; Reiter and Sessitsch, 2006; Hardoim *et al*., 2008; Ryan *et al*., 2008). This definition can be separated further into obligate and facultative endophytes. Obligate endophytes are those that rely on the plant for survival and are transmitted from one plant to the next via seeds or vectors (Ryan *et al*., 2008). Facultative endophytes are those that exist within the plant for only part of their life-cycle, the rest of which is outside of the host plant (Hardoim *et al*., 2008).

Early studies reported bacterial endophytes as being contaminants although since then it has been realised that bacteria from the soil and phylloplane are able to move into the plant vascular system and spread through the plant forming an endophyte community within (Hallmann *et al*., 1997; Compant *et al*., 2008). Rosenblueth and Martinez-Romero (2006) state that the density of a bacterial population within a plant can be determined by many factors including the plant genotype, inoculum size, age of the host and the environmental conditions in which the plant is growing. Bacterial endophyte density can also differ between the various parts of the plant which is not only due to differences in nutrient availability but also competition between the various communities (Andreote *et al*., 2009).

The definition of symbiosis is two organisms of different species living closely together (The concise oxford dictionary, 1983). The relationship between a plant and its endophytic bacteria is a mutualistic one (Rosenblueth and Martinez-Romero, 2006) in which the bacteria receive a range of nutrients from the plant and in return they provide stress reduction, growth enhancement (Hardoim *et al*., 2008) and an increased resistance against certain phytopathogens (Hallmann *et al*., 1997). There are various ways in which bacterial endophytes can stimulate plant growth, for example, the solubilisation of phosphate or by making indole acetic acid (Ryan *et al*., 2008). It is because of these beneficial traits that bacterial endophytes have been considered as agents for biological control (Hallmann *et al*., 1997; Izumi *et al*., 2008).

Previous studies have isolated a variety of Gram negative and Gram positive bacterial endophytes from both monocotyledonous and dicotyledonous plants (Lodewyckx *et al*., 2002). Reiter and Sessitsch (2006) identified the bacterial endophytes found in the wildflower *Crocus albiflorus* using both culture-dependent and –independent techniques. In their study they found that this plant contained a diverse range of bacterial endophytes most of which were from the *γ-Proteobacteria* and *Firmicutes*. Some of these bacteria were known to be plant associated and some had not as yet been described as plant-associated.

Another example of a bacterial endophytic study is that of Garbeva *et al*. (2001) who studied the bacterial endophytes in potatoes and also found a range of bacterial endophytes that had already been identified and some that are yet to be identified. This study also attempted to re-introduce three of the bacterial endophytes, namely *Stenotrophomonas maltophilia* E241, *Bacillus* sp. and *Sphingomonas paucimobilis*, back into potato plants. The result was that only *S. maltophilia* E241 could successfully re-colonize the potato plants (Garbeva *et al*., 2001).

Many previous studies undertaken on bacterial endophytes focused on those that could only be cultured from plant material (Sturz *et al*., 2000). However,

this meant that there was an overrepresentation of the bacteria that could be cultured versus those that could not be cultured. The main reason why many bacteria cannot be cultured is the lack of knowledge about the growth requirements of many of these bacterial endophytes (Reiter and Sessitsch, 2006).

It was found using non-culturing techniques that those bacterial endophytes that can be cultured form only a small part of all of the bacterial endophytes that exist within the plant (Ryan *et al*., 2008). In more recent studies carried out on the identification of bacterial endophytic communities, culture-dependent and -independent techniques are used together as it enables scientists to determine both the possible extent of the endophyte community as well as test those that can be cultured (Reiter and Sessitsch, 2006; Hardoim *et al*., 2008). The various tests that are done on bacterial endophyte cultures include Gram staining and biochemical tests such as Biolog, oxidase test, catalase test and API 20E (Lodewyckx *et al*., 2002). The cultured bacteria can also be used to test Koch's postulates (Garbeva *et al*., 2001).

1.3) Techniques that are used to study bacterial endophytes

Culturing

Culturing is a simple and inexpensive technique that has been used to study bacterial endophytes for many years (Hallmann *et al*., 1997). Despite the disadvantages of culturing, such as overrepresentation of the faster growing bacterial endophytes and the exclusion of non-viable bacteria, it is still used for bacterial endophyte population studies (Androeote *et al*., 2009). Studies now tend to mimic the environment from which the bacteria are being isolated so as to increase the chance of culturing the endophytes. These include the incubation temperatures and atmospheric composition as well as the nutrients within the medium (Andreote *et al*., 2009).

Due to the lack of knowledge on the availability of certain compounds within a plant various selective mediums have been created for the isolation of

bacterial endophytes (Bacon and Hinton, 2007). Plant metabolites that are commonly added to media when trying to isolate bacterial endophytes include various amino acids, organic acids, sugars, proteins and inorganic compounds (Bacon and Hinton, 2007). There are typically three kinds of media that are used when attempting to isolate bacterial endophytes, namely, R2A medium for the oligotrophic bacteria, Tryptic Soya Agar (TSA) for heterotrophic bacteria and SC medium for fastidious bacteria (Bacon and Hinton, 2007).

Selective media can also be used to study a subset of bacterial endophytes that may be present within the plant. Congo red (diphenyldiazo-bis-α-naphtylaminesulfonate) is a red dye that is commonly used in selective media or broth when testing for the presence of *Rhizobium* spp. *Rhizobium* spp. and *Bradyrhizobium* spp. are commonly known to be root nodule-forming bacteria (Willems and Collins, 1993: Verstraete *et al*., 2011). These bacteria are unable to use the red dye and under the correct growth conditions they will form white colonies on Congo red agar (Kneen and LaRue, 1983). Congo red has also been used as an indicator of cellulose-utilisation by bacteria. It was found that Congo red dye forms a complex with unhydrolyzed polysaccharides and, therefore, bacteria that can break down cellulose form clearing zones on Congo red cellulose agar (Hendricks *et al*., 1995).

Culture – independent

Examples of culture–independent techniques that are used to study bacterial endophytes include Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis, Denaturing Gradient Gel Electrophoresis (DGGE) and 16S rRNA gene cloning and sequencing (Reiter and Sessitsch, 2006; Ryan *et al*., 2008) and more recently pyrosequencing (Manter *et al*., 2010). There is, however, a limitation to culture-independent techniques. This limitation is the interference from plant organelle small-subunit rRNA such as that from mitochondria and chloroplasts (Reiter and Sessitsch, 2006). Chloroplasts originate from prokaryotes and therefore carry copies of the 16S rRNA gene. These copies compete with the 16S rRNA genes of bacteria during PCR

(Androete *et al*., 2009). Smalla *et al*. (2007) examined the bacterial diversity in different soil types using three culture-independent techniques, namely T-RFLP, DGGE and single strand conformation polymorphism (SSCP). The conclusion of this study was that the results of all of the techniques were similar (Smalla *et al*., 2007). Thus providing evidence that the choice of the technique used to study bacterial endophyte communities relies more on the availability of equipment for the study (Andreote *et al*., 2009).

16S rRNA gene sequencing

The 16S rRNA gene is one of the most important genes in bacterial ecology and phylogenetics (Andreote *et al*., 2009) and is commonly used as a marker gene in environmental microbial diversity studies (Armougom and Raoult, 2009). Despite the fact that the use of this gene as a phylogenetic tool has been criticized it is still widely used. The reason for the criticism is two-fold: firstly because there are often more than one copy of the 16S rRNA gene in a genome and these copies may differ, and secondly, because at species level the 16S rRNA gene does not supply enough resolution (Armougom and Raoult, 2009). Databases such as the Ribosomal Database Project (RDP) have been created to make the identification of 16S rRNA gene sequences more accurate (Nocker, 2007; Armougom and Raoult, 2009).

T-RFLP

Terminal Restriction Fragment Length Polymorphism (T-RFLP) is one of the culture-independent techniques that can be used to study bacterial endophyte communities (Reiter and Sessitsch, 2006; Ryan *et al*., 2008). Briefly this technique measures the size differences of terminal PCR fragments that have been digested with restriction enzymes. Typically the 16S rRNA gene is used because of the reasonably extensive database that has been created for this gene (Marsh, 1999; Nocker *et al*., 2007). A PCR is carried out with a set of primers, one of which is fluorescently labelled at the 5’ end. The PCR product is then digested with restriction endonucleases and run on an acrylamide gel (Nocker *et al*., 2007). With the use of automated systems such as ABI gels or capillary electrophoresis the fluorescent signals are recorded and the results are presented digitally as an electropherogram

(Marsh, 1999). The end result is a bacterial community being turned into a diagram of peaks (Andreote *et al*., 2009). T-RFLP is particularly useful for comparing communities (Marsh, 1999).

DGGE

Denaturing Gradient Gel Electrophoresis (DGGE) is a fingerprinting technique that separates PCR fragments that are of similar size but different in sequence using a polyacrylamide gel that has a low and a high denaturing gradient (Muyzer *et al*., 1993; Nocker *et al*., 2007). Once again the 16S rRNA gene is typically targeted for such diversity studies (Armougom and Raoult, 2009). A mixed DNA sample is first amplified by PCR using two primers, one of which has a GC clamp (Nocker *et al*., 2007). The PCR product is then run on a polyacrylamide gel that has a linear denaturing gradient consisting of formamide and urea (Muyzer, 1999; Nocker *et al*., 2007). The gel is run at a constant temperature of 60 ˚C and once a double stranded DNA band reaches a point in the gel where the denaturants are too high it splits, forming a “butterfly shape”. This characteristic shape is due to the GC clamp that holds the two strands together at one end. Once the double stranded DNA molecule has split it stops moving through the gel (Nocker *et al*., 2007).

One of the benefits of DGGE is that the bands can be cut out of the gel and subsequently be sequenced. Cloning the DNA product from the gel band prior to sequencing has been found to yield better quality sequences (Muyzer, 1999; Nocker *et al*., 2007). DGGE analysis is more of a qualitative technique than a quantitative one (Muyzer *et al*., 1993). Bacterial endophytic diversity in plants has been found to be low when compared to other plant-associated bacterial communities (Andreote *et al*., 2009) and DGGE analysis is best used for communities of low diversity (Nocker *et al*., 2007).

Pyrosequencing

According to Armougom and Raoult (2009), pyrosequencing is different to Sanger sequencing in that only one dNTP is added to the sequencing reaction at a time. If one of the dNTP’s is added to the growing DNA chain then the DNA polymerase cleaves off a pyrophosphate which is then used by

ATP sulfurylase to produce ATP. The ATP is subsequently used in another reaction where luciferin is converted to oxyluciferin by the enzyme luciferase and the light produced by this reaction is measured by a Charge-Coupled Device (CCD) (Edwards *et al*., 2006). Once the reaction has finished the left over nucleotides and ATP are degraded by an enzyme such as apyrase before a different dNTP is added. 454 Life Sciences has designed a way for many reactions to occur simultaneously in one picotiter plate (Edwards *et al.*, 2006). Pyrosequencing has allowed for approximately 300, 000 reads of 200 – 400 bp in length to be produced in 5 hours (Andreote *et al*., 2009).

The first study published on the use of pyrosequencing to determine bacterial endophyte diversity was that of Manter *et al*. (2010). This study looked at the bacterial endophyte population within the roots of various potato cultivars. A variable region of the 16S rRNA gene was used to identify the bacterial endophytes to genus level in the roots of 12 potato cultivars using pyrosequencing. The result was around 477 ± 71 bacterial operational taxonomic units were identified and the majority of these had not yet been described as bacterial endophytes of potatoes. Manter *et al*. (2010) also found a difference in the bacterial endophyte communities between the different cultivars of potato plants. Despite the accuracy of pyrosequencing and the ability to detect bacterial endophytes at low numbers it is more expensive in terms of coverage and repeat experiments (Manter *et al*., 2010).

1.4) *Pavetta* spp.

The Rubiaceae are the fourth largest plant family of angiosperms (van Wyk *et al*., 1990) and within this family there are three genera namely, *Pavetta*, *Psychotria* and *Sericanthe* that have bacterial nodules on their leaves (van Wyk *et al*., 1990; Van Oevelen *et al*., 2001). There is approximately 353 nodulated *Pavetta* spp. making it the largest nodulated genus of plants (Miller, 1990). Plants belonging to this genus are found in Asia, Africa and Australia (Herman *et al*., 1987; Miller, 1990) of which approximately 41

species can be found in southern Africa (Grobbelaar *et al*., 1971). The position of the bacterial nodules on the leaves is commonly used as an identification tool of the host. The nodules can either be scattered randomly over the surface of the leaf or found along the midrib (Boon, 2010). These nodules can also be different in shape i.e. they can be spherical, rod-like, branched or unbranched (Miller, 1990).

In South Africa, *Pavetta* spp. are mainly found in summer rainfall areas (Herman *et al*., 1987). Most *Pavetta* spp. are considered to be shrubs such as *P. harborii* and *P. catophylla* but other *Pavetta* spp. can grow into trees such as *P. edentula* and *P. schumanniana* (Schmidt *et al*., 2007). *P. edentula*, commonly known as the gland-leaf bride's bush, grows mainly in the bushveld in the north-east region of South Africa on steep rocky hillsides (Coates Palgrave, 2002). *P. edentula* is a deciduous or semi-deciduous tree with hairless, bright green leaves that are approximately 250 x 80 mm in size (Schmidt *et al*., 2007). *P. lanceolata* is commonly known as the weeping or forest bride's bush and is one of the most noticeable *Pavetta* spp. as it has large clusters of white flowers in summer and grows on the edges of evergreen forests found in coastal and some bushveld areas. *P. lanceolata* can be found growing along the east coast of South Africa from Kwa-Zulu Natal to inland northern Limpopo (Coates Palgrave, 2002). The weeping bride's bush is a shrub or small tree that has leaves of approximately 50-70 mm x 9-15 mm in size and is evergreen (Schmidt *et al*., 2007). It is believed that the leaves of *P. lanceolata* and *P. edentula* are edible (Fox and Young, 1982). The native people of Venda use the roots of *P. lanceolata* as a medicine to cure nausea and vomiting (Schmidt *et al*., 2007).

Pavetta schumanniana is one of the *Pavetta* spp. that causes a disease known as 'gousiekte' in ruminants and hence it is called the poison bride's bush (Coates Palgrave, 2002). This plant is a deciduous tree (Schmidt *et al*., 2007) that grows in the north-east corner of South Africa, Zimbabwe and Botswana amongst rocks and on termite mounds in open woodlands and bushveld areas (Coates Palgrave, 2002). The leaves of *P. schumanniana* are 60-140 x 20-60 mm in size (Schmidt *et al*., 2007) and are unlike the other

Pavetta spp. mentioned here as it has hairs on the surface of the leaf (Coates Palgrave, 2002). Being deciduous the question of where the nodule symbiont resides during the winter season can be investigated. Despite its lethality towards ruminants *P. schumanniana* is used in Zimbabwe to treat a variety of medical conditions in humans such as abdominal pain, coughing, pneumonia and infertility in woman (Schmidt *et al*., 2007).

1.5) Bacterial nodules

Studies that have been done on leaf nodules and their bacterial symbiont have mostly focused on the *Psychotria* spp. (Becking, 1971; Edwards and LaMotte, 1975; Van Oevelen *et al*., 2002). The *Pavetta* spp. are significant because they are one of the few plant genera that have species that form bacterial nodules in their leaves which when held against the light can be seen as black spots (Herman *et al*., 1986). In *P. lanceolata*, *P. edentula* and *P. schumanniana* the bacterial nodules are randomly scattered over the leaf and are generally not found near the main vein (Herman *et al*., 1987). The nodules are surrounded by layers of thin-walled, elongated plant parenchyma cells (Herman *et al*., 1986). Miller (1990) stated that relatively early in the development of a leaf the bacterial endophytes in the nodules enter a stage at which they become non-viable meaning that these bacteria cannot be cultured from mature leaves.

The nodule-forming bacteria of the Rubiaceae do not “infect” the host plant in the same manner as that of *Rhizobia* or *Frankia* spp. but rather they are confined to their host and the host’s life-cycle (Miller, 1990). The bacteria that form leaf nodules may differ greatly from those plant-associated bacteria that live freely in the environment but they are possibly ancestrally related (Miller, 1990). Leaf nodule bacteria may have entered into this symbiotic relationship several million years ago and have since adapted to the controlled and stringent conditions that exist within a plant (Miller, 1990). Lemaire *et al*. (2011a) recently showed that this may not be the case. This study showed

that the nodule-forming bacterial endophytes within various nodulated hosts were closely related to bacteria found within the environment. This finding suggests that what was previously thought to have been a single 'infection' event several million years ago may have been multiple 'infection' events in a more recent time frame (Lemaire *et al*., 2011a).

One of the first studies done on nodule formation was performed on a *Pavetta* sp. and was carried out by Zimmermann in 1902. He was the first to conclude that the nodules were formed by bacteria. Since this finding many studies have been done in order to isolate, culture and identify the nodule-forming bacterial endophytes (von Faber, 1912; Zeigler, 1958; Knösel, 1962 cited in Miller, 1990; Centifanto and Silver, 1964; Lersten and Horner, 1967). One of the reasons that there is much focus on the culturing of the leaf nodule-forming bacterial endophyte is so that Koch's postulates can be performed. This would be the final step in proving that the identified bacterium is the causal agent of the leaf nodules.

Bacteria-free plants are scarce and this is due to the fact that the plants do not grow well without the bacterial symbiont. Nodule-free plants, also known as 'cripples', do occur naturally but in very low numbers. According to Miller (1990) the 'crippled' seedling will develop two to three leaves and then the shoot tip will turn to callus tissue and growth and differentiation of the plant will stop. A nodule-free seedling can survive for up to three years but typically the plant will gain its bacterial symbiont 6 months to a year after it has started growing. One theory behind the plants eventually developing leaf nodules is that the bacteria are not at optimal numbers within the seed at the time of germination. However, after several months a functional bacterial colony size is reached in order for the symbiotic benefits of the bacteria to take effect and for nodules to develop on the leaves. Techniques that have been used to try and create nodule-free seedlings include heat treatment, irradiation, cold and tissue culture but none have been successful as the seedlings usually revert back to a nodulated state or die (Miller, 1990).

The nodule-forming bacterial endophytes were found in the seeds of *Pavetta zimmermanniana* by von Faber (1912) between the embryo and the endosperm. These observations lead von Faber to believe that these bacteria could be considered as being acquired vertically (Miller, 1990). Von Faber (1912) also discovered that bacteria were present in the mucilage in which the flowers developed and reasoned that the bacteria entered into the embryo sac of the seed through the pollen tube. He, however, could not detect the bacterial symbionts at critical points in the life cycle of the plant meaning that his reasoning can only be considered as possibilities (Miller, 1990). The bacterial symbiont is thought to be maintained in a protein and carbohydrate mucilage in the developing shoot tip which leads to the inoculation of new leaves with this bacterial partner (Miller, 1990). Verstraete *et al*. (2011) found that the same bacterial endophyte was isolated from a specimen of *Fadodia homblei* grown from seed as that detected from the leaves of wild specimens. This result further strengthens the idea that bacterial endophytes can be vertically transmitted through the seed.

Leaf nodule development within the nodulated *Psychotria* spp. has been well documented with the use of electron microscopy (Lersten and Horner, 1967; Whitmoyer and Horner, 1970; Miller *et al*., 1983; Miller, 1990). The only study done on leaf nodule development in *Pavetta* spp. was carried out by Ziegler in 1958 with the use of low magnification electron microscopy. Within *Psychotria bacteriophila* leaf nodule development begins with the entrance of the bacterial endophyte into the primordial leaf tissue through a stomatal opening. The bacteria then begin to multiply and grow and migrate away from the stomatal opening into the leaf mesophyll tissue (Lersten and Horner, 1967). Whilst the bacterial endophytes increase in number, surrounding plant cells are separated from each other by a proteolytic action of enzymes that are thought to be secreted by the bacteria. These plant cells then move away from the other plant cells and end up floating within the bacterial mass (Miller *et al*., 1983).

With the use of Transmission Electron Microscopy, Lersten and Horner (1967) noticed the plant cells that were in contact with the bacterial

endophytes had unusually thicker cell walls. The reasoning given by this study was that this cell wall thickening was a form of nutrient transfer from the plant host to the bacteria. Whitmoyer and Horner (1970) also investigated the levels of ribonucleic acid (RNA) during leaf nodule development in *Psychotria bacteriophila*. They found that the levels of RNA were highest during the initial stages of nodule development indicating that the host plant receives beneficial substances from the bacterial endophytes during this period.

Von Faber (1912) observed that the bacterial nodules on *Pavetta* spp. had a pore on the adaxial surface of the nodule. This finding then begs the question of whether these nodule-forming bacteria can be considered as bacterial endophytes or not. Bacterial endophytes are defined by Hallmann *et al*. (1997) as bacteria that can be isolated from surface sterilized plant material and do not visibly harm the plant in any way. Therefore according to this definition the nodule-forming bacteria can be classified as bacterial endophytes as long as they can survive the surface sterilization process. In order to survive surface sterilization the bacteria must not be exposed to the environment and hence will not be affected by the surface sterilant. Herman *et al*. (1986) mentioned a tube that is found above the bacterial nodule in *Pavetta* spp. which may be seen as a pore but when looking at the figure below (Fig. 1) one can see that the tube does not come into contact with the bacterial nodule, indicating that the bacteria are not exposed to the environment.

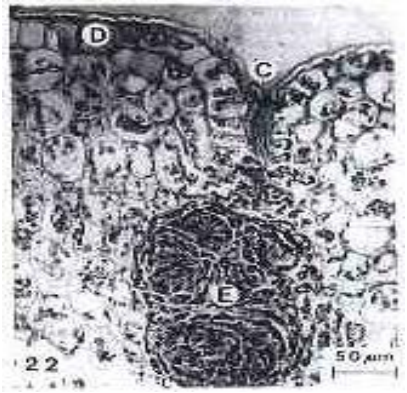

Fig. 1 Transverse section through bacterial leaf nodule (from Herman *et al*., 1986)

Horner and Lersten (1972) define a bacterial nodule as internal cavities within the leaves that are only open to the exterior by way of stomatal pores when the leaf is in the early stages of development. Using this definition it can be said that the nodule-forming bacteria can only be classified as endophytes once the leaf has reached late development or maturity. Despite the confusion as to whether the nodule-forming bacteria are in fact bacterial endophytes, scientists still refer to them as such (Miller, 1990; Van Oevelen *et al*., 2004).

Some examples of bacteria that have been proposed to be the nodule-forming endophytes in *Pavetta* spp. are *Chromobacterium lividim*, *Mycobacterium rubiacearum*, *Bacterium rubiacearum, Phyllobacterium rubiacearum* and *Bacillus* sp. (Miller, 1990). The above suggested nodule-forming bacteria in *Pavetta* spp. have been rejected by either Horner and Lersten (1972) or Miller (1990). The proposed nodule-forming bacteriuim *Bacterium rubiacearum,* has been excluded because the rules of nomenclature were not followed and *Mycobacterium rubiacearum* is not considered because it is Gram-positive whereas the nodule–forming bacteria are thought to be Gram-negative (Horner and Lersten, 1972). The *Bacillus* sp. has also been excluded due to the fact that they form endospores (Miller, 1990). According to Miller (1990) *Chromobacterium lividum* is rejected because it produces pigments, usually purple in colour, which would be picked up at some point during the bacterium's life cycle in the plant but this has not as yet been observed. Lastly the proposed bacterium *Phyllobacterium rubiacearum* has been questioned by Miller (1990) due to the fact that it was isolated from mature leaves which are believed to harbour inactive nodule bacteria.

Van Oevelen *et al*. (2002) were the first to identify the nodule-forming bacteria in *Psychotria kirkii* using culture-independent techniques. It was found using 16S rRNA gene cloning and sequencing that the bacteria found within nodule tissue grouped closely to the genus *Burkholderia*. The proposed name for this bacterium is '*Candidatus* Burkholderia kirkii' sp. nov.

(Van Oevelen *et al*., 2002). In 2004, Van Oevelen *et al*. identified a further two nodule-forming bacterial endophytes as being closely related to the genus *Burkholderia*. The two new species were isolated from *Psychotria calva* and *P. nigropunctata* and their proposed names are '*Candidatus* Burkholderia calva' and '*Candidatus* Burkholderia nigropunctata', respectively. Despite many efforts to culture the bacterial endophytes that form these nodules in *Psychotria* spp., Van Oevelen *et al*. (2004) have not yet managed to do so.

Lemaire *et al*. (2011b) used a similar technique to that adopted by Van Oevelen *et al*. (2002) to identify the nodule-forming bacterial endophytes in three *Pavetta* spp., namely *P. hispida*, *P. rigida* and *P. schumanniana*. This study also used the *recA* and *gyrB* genes for identification of the nodule-forming bacterial endophyte. The result was that all three *Pavetta* spp. harboured bacteria from the genus *Burkholderia* within the leaf nodules. These bacteria have been described under the provisional status of *Candidatus* namely; '*Candidatus* Burkholderia hispidae', '*Candidatus* Burkholderia rigidae' and '*Candidatus* Burkholderia schumannianae'. To date the leaf nodule-forming bacteria have not been cultured and this is most likely due to an inability to mimic the precise conditions and nutrients that the bacteria need to grow (Reiter and Sessitsch, 2006). A study conducted by Verstraete *et al*. (2011) also used molecular techniques to identify the leaf nodule-forming bacterial endophyte within *Pavetta harborii* as belonging to the genus *Burkholderia*.

Recent studies have found that there are a number of plant associated bacteria belonging to the genus *Burkholderia* that live freely in the soil environment, inhabit the rhizosphere, the phyllosphere or even internal tissues of the plant (endosphere). The identification of the nodule-forming bacterial endophyte as *Burkholderia* spp. is realistic in that bacteria from this genus are commonly found in endophyte communities (Compant *et al*., 2008). Various species of *Burkholderia* have been identified as endosymbionts as they have been isolated from root nodules of *Mimosa* spp. and it has been shown that some of these bacterial endophytes have the

capacity to fix atmospheric nitrogen or promote plant growth (Compant *et al*., 2008). The characteristics that have been found in this diverse genus in some ways match the known characteristics of the nodule-forming bacteria indicating that Van Oevelen *et al*. (2002; 2004) and Lemaire *et al*. (2011b) have come the closest to truly identifying the nodule-forming bacteria.

The question of where the nodule-forming bacterial endophytes go to when *P. schumanniana* drops its leaves in winter as well as if the leaf nodule-forming bacteria can be found within other non-reproductive plant tissues has not yet been addressed in literature. *P. schumanniana* forms buds during winter and when opened the primordial leaves can be seen. *Burkholderia* specific primers could be used to detect these nodule-forming bacteria within the leaf buds of *P. schumanniana* and possibly in other plant tissues as well.

1.6) Benefits of leaf nodules

The bacterial symbiont that forms these leaf nodules was previously thought to play a role in nitrogen fixation as is the case with *Rhizobacteria* (Boodle, 1923; Miller, 1990) but more recent studies have found that it may be more involved in growth stimulation or growth regulation of the plant (Herman *et al*., 1986; Miller, 1990). This train of thought is due to the presence of a green ring around nodules during leaf senescence (Becking, 1971; Miller, 1990). It was Becking (1971) that noticed this characteristic on the leaves of *Psychotria mucronata* and investigated it further by placing sliced nodule discs onto the surface of young oat leaves. The oat leaves remained green underneath the nodule discs indicating that a cytokinin–like substance was being produced (Becking, 1971). Using bioassays, Edwards and LaMotte (1975) discovered that the bacterial nodules on the leaves of *Psychotria punctata* produced high levels of cytokinin**.** They reasoned that because non-nodulated areas of the leaf showed significantly less cytokinin levels it must be the bacteria within the nodules that produce the cytokinin (Edwards and LaMotte, 1975). This theory has as yet not been proven conclusively.

1.7) Conclusions

The leaf nodule-forming bacterial endophytes in various nodulated plant species has been identified as belonging to the genus *Burkholderia* (Van Oevelen *et al*., 2002, 2004; Lemaire *et al*., 2011b). The questions that now remain are how will one be able to culture this bacterium so as to test Koch's postulates and is this result the same for all leaf nodulated plant species.

The development and morphology of leaf nodules has been well documented for plants from the genus *Psychotria* (Lersten and Horner, 1967; Whitmoyer and Horner, 1970; Miller *et al*., 1983; Miller, 1990). The leaf nodule morphology and development within *Pavetta* spp. using electron microscopy has not been as well documented. Would the leaf nodule development and morphology of the *Pavetta* spp. be different to that found for *Psychotria* spp. and if so, what might the differences be?

The nodule-forming bacterial endophytes have been seen within the flowers and seeds of various *Pavetta* spp. (von Faber, 1912; Miller, 1990). However, the issue of where leaf nodule bacteria reside within deciduous *Pavetta* spp. has not been addressed. This also leads to the question of whether the leaf nodule-forming bacterial endophytes can be found within the stem tissue of their hosts or are they limited to the leaf and reproductive tissues of the host plant?

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Chapter 2

Identification of the bacterial endophytes found within the leaves of three *Pavetta* spp. with a focus on those causing nodules

Identification of the bacterial endophytes found within the leaves of three *Pavetta* spp. with a focus on those causing nodules

2.1) Abstract

Pavetta is one of several plant genera within the Rubiaceae that harbour leaf nodulating bacterial endophytes. The identity of these bacteria in some plant species has only recently been confirmed as belonging to the genus *Burkholderia*. Research on the overall bacterial endophyte population within the leaves of nodulated plant hosts has, however, not been investigated. The aim of this chapter was to identify the nodule-forming bacterial endophytes in three indigenous *Pavetta* spp., namely, *P. lanceolata*, *P. edentula* and *P. schumanniana*, as well as to characterise the bacterial endophyte population within their leaves. A culture-dependent and -independent approach was adopted in this study. The culture-independent technique that was used was Denaturing Gradient Gel Electrophoresis (DGGE). Both nodulated as well as non-nodulated leaf tissues were sampled. Based on results from the initial *P. lanceolata* samples that were collected, a *Burkholderia* specific PCR was used to investigate a second set of *P. lanceolata* samples that had been collected from another source. The bacterial endophyte population of the leaf tissue was found to be diverse within *P. edentula* but less so in *P. lanceolata* and *P. schumanniana*. The nodulated tissue of *P. lanceolata* yielded bacteria that were found to be related to the family Bradyrhizobiaceae in both the culture-dependent and –independent analyses. The DGGE results from the nodule tissue of *P. edentula* and *P. schumanniana* indicated that the nodule-forming bacterial endophytes are closely related to the previously described leaf nodule-forming bacterial endophyte, *Candidatus* Burkholderia schumannianae. The *Burkholderia* specific PCR was able to detect a *Burkholderia* sp. within the nodulated tissue of the second set of *P. lanceolata* samples that had been collected from specimens growing in open soils.

2.2) Introduction

It was previously thought that bacterial growth from surface sterilized plant tissue was due to contamination. However, scientists later realised that bacteria can inhabit the internal tissues of plants and hence the term "bacterial endophytes" was created (Hallmann *et al.*, 1997). There are several definitions of a bacterial endophyte. Hallmann *et al.* (1997) refers to a bacterial endophyte as a bacterium that can be isolated from surface sterilized plant tissue and that does not visibly cause harm to the host plant. The relationship between endophytic bacteria and their host plant is one of symbiosis where the bacteria receive a protective environment filled with nutrients and the plant gains growth enhancement and stress reduction (Hardoim *et al*., 2008). To date studies on bacterial endophytes have focused on agricultural crops, however, recently these studies have diversified and now include plants that grow in their natural habitats (Reiter and Sessitsch, 2006).

Due to the lack in knowledge of the growth requirements of bacterial endophytes and that not all bacteria are culturable, a combined approach of culture-dependent and culture-independent techniques are used to study them (Reiter and Sessitsch, 2006). For culture analysis of bacterial endophytes, typically three types of media are used. A minimal media is used to select for oligotrophic bacteria, nutrient rich medium is used for heterotrophic bacteria and SC medium is used for fastidious bacteria (Bacon and Hinton, 2007). There are various culture-independent techniques that have been adopted in order to study bacterial endophyte diversity. These include Denaturing Gradient Gel Electrophoresis (DGGE), Terminal Restriction Fragment Length Polymorphism (T-RFLP), 16S rRNA gene cloning and sequencing (Reiter and Sessitsch, 2006; Ryan *et al*., 2008) and more recently pyrosequencing (Manter *et al*., 2010).

The Rubiaceae are the fourth largest plant family (van Wyk *et al*., 1990) and there are three genera within this family that have a specialised symbiotic relationship with bacteria, namely *Pavetta* spp., *Psychotria* spp. and

Sericanthe spp. (van Wyk *et al*., 1990; Van Oevelen *et al*., 2001). This symbiotic relationship is found in the leaves of certain species within these three genera. There are approximately 353 nodulated *Pavetta* species (Miller, 1990). Three such species include *P. lanceolata*, *P. edentula* and the 'gousiekte' toxin producing *P. schumanniana*.

Pavetta lanceolata is an evergreen tree that can be found growing along the east – coast of South Africa and inland towards northern Limpopo. This plant is more commonly known as the weeping or forest bride's bush and is one of the *Pavetta* spp. that grows as a bush or a small tree and can typically be seen growing along the edges of forests (Coates Palgrave, 2002; Schmidt *et al*., 2007). The leaves of *P. lanceolata* are typically 50-70 mm x 9-15 mm in size (Schmidt *et al*., 2007) and the bacterial nodules are circular in shape and scattered over the leaf blade (Herman *et al*., 1987). The roots of *P. lanceolata* are believed to have a medicinal purpose and are eaten by the native people of Venda as a cure for nausea and vomiting (Schmidt *et al*., 2007). The leaves of *P. lanceolata* are also considered to be edible and are eaten as a vegetable (Fox and Young, 1982).

Pavetta edentula is commonly known as the gland-leaf bride's bush. This species of *Pavetta* is a semi-deciduous tree with large bright green leaves and can be found growing on rocky hillsides in the bushveld areas of the north-east region of South Africa (Coates Palgrave, 2002). The leaves of *P. edentula* are hairless and approximately 250 x 80 mm in size (Schmidt *et al*., 2007) and are edible (Fox and Young, 1982). The bacterial nodules are scattered over the leaf blade and are varied in shape compared to that of *P. lanceolata* and *P. schumanniana* (Herman *et al*., 1987).

Pavetta schumanniana is a deciduous tree and can be found growing naturally in the north-east corner of South Africa typically growing amongst rocks and on top of termite mounds in open woodlands and bushveld areas (Coates Palgrave, 2002). The leaves of *P. schumanniana* are generally 60-40 x 20-60 mm in size (Schmidt *et al*., 2007) and have hairs present on the adaxial surface (Coates Palgrave, 2002). The bacterial nodules are scattered

over the leaf blade and are round in shape (Herman *et al*., 1987). The common name given to *P. schumanniana* is the poison bride's bush and as the name suggests this species is toxic, causing a disease in domestic ruminants known as 'gousiekte' (Coates Palgrave, 2002). Despite the name of the plant and its lethality towards ruminants it is used by the native people of Zimbabwe to treat various illnesses such as abdominal pains, coughing, pneumonia and infertility in woman (Schmidt *et al*., 2007).

Psychotria is another plant genus from the family Rubiaceae that has various species with bacterial nodules occurring on their leaves (Miller, 1990). In 2002, Van Oevelen *et al.* used gene cloning and sequencing to identify the nodule forming bacterial endophyte in *Psychotria kirkii*. This study identified these bacteria as belonging to the genus *Burkholderia*. In 2004, Van Oevelen *et al*. identified a further two *Burkholderia* spp. in the nodule tissue of *Psychotria calva* and *P. nigropunctata*. Despite all efforts, the nodule-forming bacterial endophytes are yet to be grown *in vitro* (Van Oevelen *et al.*, 2004; Verstraete *et al*., 2011). Due to the unculturability of the bacterium, the new bacterial species identified from *Psychotria* spp. were described under the provisional status of *Candidatus*, namely, '*Candidatus* Burkholderia kirkii' sp. nov. (Van Oevelen *et al*., 2002), '*Candidatus* Burkholderia calva' and '*Candidatus* Burkholderia nigropunctata' (Van Oevelen *et al*., 2004).

Recently, a study was undertaken by Lemaire *et al.* (2011b) to determine the identity of the nodule-forming bacterial endophytes in three *Pavetta* spp., specifically *P. hispida*, *P. rigida* and *P. schumanniana*. Using gene cloning and sequencing this study identified the nodule-forming bacterial endophytes in the three *Pavetta* spp. as belonging to the genus *Burkholderia*. The three proposed species identified were '*Candidatus* Burkholderia hispidae', '*Candidatus* Burkholderia rigidae' and '*Candidatus* Burkholderia schumannianae' (Lemaire *et al.*, 2011b). Further investigation found that the nodule-forming bacterial endophytes found in the three tested *Pavetta* spp. grouped closely to those identified in the *Psychotria* spp. (Lemaire *et al*., 2011b).

Lemaire *et al.* (2011a) carried out an evolutionary study on the bacterial nodule symbionts and found that within all of the bacterial nodules tested, including *P. lanceolata* and *P. edentula*, the nodule-forming bacterial endophytes belonged to the genus *Burkholderia*. This study also concluded that despite the leaf nodule-forming bacterial endophytes being passed from parent to offspring through the seed, the relationship between bacteria and plant is not as old as previously thought. Lemaire *et al*. (2011a) reasoned that the initial symbiotic relationship of leaf nodules was first formed by many species of bacteria that were horizontally transferred from sources such as soil or an insect vector. They suggested that recently an event caused this symbiotic relationship to be narrowed down to bacteria from the genus *Burkholderia* (Lemaire *et al*., 2011a).

The aim of this study is to identify the bacterial endophyte diversity within the leaves of three *Pavetta* spp. and identify the nodule-forming bacterial endophytes in each species.

2.3) Materials and methods

2.3.1) Collection of plant material

Initially samples of *Pavetta lanceolata* were obtained from a nursery in Pretoria. Two specimens were kept in pots in a greenhouse at 28 ˚C. A second set of *P. lanceolata* samples were collected at a later stage from the National Botanical Gardens in Pretoria. *P. edentula* samples were obtained from an open plot located on a hillside in the Barberton area. The summer samples of *P. schumanniana* were collected from a tree growing in the toxicology gardens at Onderstepoort. All samples were placed into plastic bags and processed in the laboratory within 24 hours of collection.

2.3.2) Isolation of bacteria from leaves

The leaves of each *Pavetta* spp. were surface sterilized by using the following procedure: washed with distilled water to remove surface dirt, placed into an 5.25 % sodium hypochlorite solution containing 0.2 % Tween 20 for 1 minute, 100 % ethanol for 1 min and then finally rinsed with double distilled water 3 times (adapted from Garbeva *et al.*, 2001). Separate leaves were prepared for the various incubation media.

Before further processing, the leaves were tested for successful surface sterilization by plating 100 µl of the final double distilled water used to wash the leaves onto Tryptone Soya Agar (TSA) plates (Hallmann *et al*., 1997). These plates were incubated at 28 ˚C and were examined every 24 hours for signs of bacterial growth.

2.3.3) Inoculation of media

The media used in this experiment included Congo Red Agar (CRA), Tryptone Soya Agar (TSA) and R2 Agar (R2A). Congo Red agar was prepared as follows; Mannitol, 10 g, K_2HPO_4, 0.5 g, $MgSO_4 \cdot 7H_2O$, 0.2 g, NaCl, 0.1 g, Yeast extract, 0.5 g, 2.8 ml 0.1 M H_2SO_4, agar 15 g, distilled water to 1 litre and 25 µg ml^{-1} filter sterilized Congo red dye (Somasegaran and Hoben, 1994). The TSA and R2A were prepared according to the manufacturer's instructions (Biolab). All media contained 100 µg ml^{-1} cyclohexamide (Garbeva *et al.,* 2001). Once the leaves of the three *Pavetta* spp. had been surface sterilized they were used to inoculate the three types of media. The leaves were processed by first punching out the nodules using a modified hypodermic needle that was 0.5 mm in diameter. The nodule discs were then cut up into smaller pieces with a sterile scalpel and were used to inoculate one of each of the three types of media mentioned above in duplicate. The same procedure was then carried out for the non-nodulated areas of the leaf. The same amount of nodulated and non-nodulated leaf tissue was used to inoculate each of the media. Once inoculated the agar plates were incubated at 28 ˚C for up to 72 hours.

Three types of broth solutions were also used in this experiment. These included Congo red broth (CRB), Tryptic Soya Broth (TSB) and Sodium Phosphate (NAP) buffer. All solutions contained 100 µg ml^{-1} cycloheximide (Garbeva *et al.*, 2001). The CRB was prepared as above excluding the agar. The TSB was made according to the manufacturer's instructions. Each broth solution was inoculated in duplicate with nodule and non-nodulated tissue in the same manner as the media. The broth solutions were incubated for 72 hours at 28 ˚C with shaking to dislodge the bacteria from the plant tissue (Garbeva *et al.*, 2001). Every 24 hours for at least 3 days, 1 ml aliquots were taken from each broth solution in duplicate. One set of the 1 ml aliquots were used to make a serial dilution up to 10^{-3}. These serial dilutions were used for culture-dependent analysis by plating out 100 µl of each dilution onto the respective medium i.e. TSB onto TSA, CRB onto CRA and NAP onto R2A. The media plates were incubated at 28 ˚C for 72 hours. The other set of aliquots were used for culture-independent analyses.

2.3.4) Culture dependent approach

Any bacterial growth which developed on the agar was streaked onto fresh media in order to obtain pure cultures. DNA was extracted from all cultures using the Genomic DNA™ extraction kit (Zymo Research) used according to the manufacturer's instructions. Each culture was identified to genus level by sequencing the 16S rRNA gene region. The universal primers pA (5' AGA GTT TGA TCC TGG CTC AG 3') and pH (5' AAG GAG GTG ATC CAG CCG CA 3') (Edwards *et al*., 1989) (Inqaba biotechnologies) were used for gene amplification. Each 50µl PCR reaction contained 1 x Reaction buffer, 1.5 mM $MgCl_2$, 250 µM of each nucleotide (dATP, dCTP, dGTP, dTTP), 10 pmol of each primer (forward and reverse), 1.5 U Taq DNA polymerase (Southern Cross Biotechnologies), 33.7 µl nuclease free water (Qiagen) and 1 µl genomic DNA. Amplification was carried out in a Veriti Thermal Cycler (Applied Biosystems). The cycling conditions included denaturation at 94 ˚C for 10 minutes, 30 cycles of denaturation at 94 ˚C for 1 minute, primer binding at 58 ˚C for 1 minute, elongation at 72 ˚C for 1 minute followed by a

final elongation step at 72 ˚C for 5 minutes. A negative water control was added to every PCR to ensure that there was no contamination.

2.3.5) Culture independent approach: Denaturing Gradient Gel Electrophoresis (DGGE)

The broth aliquots that had been collected from each plant were pelleted and DNA was extracted using the Genomic DNA™ extraction kit (Zymo Research) according to the manufacturer's instructions. The resulting DNA was amplified using the primers pA8f with a GC clamp (5' AGA GTT TGA TCC TGG CTC AG 3') (5' CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G 3') (Fjellbirkeland *et al.*, 2001) and PRUN518r (5' ATT ACC GCG GCT GCT GG 3') (Muyzer *et al.*, 1993) (Inqaba biotechnologies). Each 50 µl PCR reaction contained 1 x Reaction buffer, 250 µM of each nucleotide (dATP, dCTP, dGTP, dTTP), 10 pmol of each primer (forward and reverse), 1.5 U Taq Gold polymerase (Southern Cross Biotechnologies), 37.7 µl nuclease free water (Qiagen) and 1 µl genomic DNA. A negative water control was included in all PCR reactions to ensure that there was no contamination. All reactions were run using a Veriti Thermal Cycler (Applied Biosystems). The cycling conditions were as follows: denaturation at 95 ˚C for 10 minutes, 30 cycles of denaturation at 94 ˚C for 30 seconds, primer binding at 60 ˚C for 30 seconds, elongation at 72 ˚C for 1 minute followed by a final elongation step at 72 ˚C for 10 minutes.

The DGGE gel was run in a DCode Universal Detection System (Bio-Rad) containing 1 x TAE buffer. 45 µl of the amplified product was run on a linear gradient gel containing 8 % acrylamide of 30 – 55 % denaturant (100 % denaturant = 7 M Urea and 40 % formamide). Electrophoresis was carried out at 60 ˚C for 5 hours at 200 V. Once the run was complete the gel was stained for 30 min at room temperature with 1:10 000 (v/v) SYBRgold (Invitrogen, Molecular Probes Inc.) (Fjellbirkeland *et al.*, 2001). The resulting bands were excised from the gel using a dark reader and incubated in 30 µl of nuclease free water (Qiagen) at 4 ˚C overnight. The gel band product was re-amplified as was done before running the DGGE gel, however, the

forward primer used was pA8f (5' AGA GTT TGA TCC TGG CTC AG 3') (Edwards *et al*., 1989) (Inqaba biotechnologies) which did not have a GC clamp.

To avoid sequencing of samples that had more than one copy of the 16S rRNA gene in each gel band, the samples were cloned prior to sequencing (Muyzer, 1999; Nocker *et al*., 2007) using the pGEMTeasy cloning kit (Promega) according to the manufacturer's instructions. The vector with insert was cloned into *Escherichia coli* DH5α cells and then plated onto LB agar containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). The resulting white colonies were used in a colony PCR using the primers SP6 (5' ATT TAG GTG ACA CTA TAG AAT 3') and T7 (5' TAA TAC GAC TCA CTA TA 3') (Promega) (Inqaba biotechnologies). Each 50 µl reaction contained 1.5 U Taq DNA polymerase (Southern Cross Biotechnologies), 1x reaction buffer, 1.5 mM $MgCl_2$, 250 µM of each nucleotide (dATP, dGTP, dCTP, dTTP), 10 pmol of each primer (forward and reverse), 33.7 µl of nuclease free water (Qiagen) and 1 µl colony DNA. A negative water control was included. The cycling conditions included denaturation at 94 ˚C for 2 minutes, 30 cycles of denaturation at 94 ˚C for 30 seconds, primer binding at 52 ˚C for 30 seconds, elongation at 72 ˚C for 2 minutes and a final elongation step at 72 ˚C for 7 minutes.

2.3.6) *Burkholderia* specific PCR

The *P. lanceolata* samples that were collected from the National Botanical Gardens were surface sterilized and incubated in the various broth solutions as mentioned above. DNA was extracted from the pellets, using the Genomic DNA™ extraction kit (Zymo Research) according to the manufacturer's instructions. *Burkholderia* specific primers, namely GB-F (5' AGT AAT ACA TCG GAA CRT GT 3') (Perin *et al*., 2006) (Inqaba biotechnologies) and GBN2-R (5' GCT CTT GCG TAG CAA CTA G 3') (Perin *et al*., 2006) (Inqaba biotechnologies) were used to determine if the nodule tissue of these samples contained bacteria belonging to the genus *Burkholderia*. The PCR reagent quantities and cycling conditions used were the same as used by

Perin *et al.* (2006). DNA from an authentic *Burkholderia* sp. culture was used as a positive control and DNA from a *Bradyrhizobium* sp. culture previously isolated from leaf nodules was added as a negative control for each PCR run. A negative water control was also tested. Apart from the above mentioned samples, the *Burkholderia* specific primers were also used to test the nodule samples of *P. edentula* and *P. schumanniana* as well as the *P. lanceolata* trees that were obtained from the nursery.

2.3.7) Sequencing

Once all PCR reactions were completed, 5 µl of each PCR product was mixed with 1 µl gel red loading dye (Biotium) and was run on a 1 % agarose gel in 1 x TAE buffer for 30 minutes at 80 V. Each gel was run with a 1kb DNA marker (Fermentas). The gels were viewed under UV light to confirm that the product was amplified and that there were no contaminants.

All PCR products were cleaned using 4 U FastAP Thermosensitive Alkaline Phosphatase (Fermentas), 20 U Exonuclease I (Fermentas) and 45 µl of PCR product. The samples were mixed and incubated at 37 ˚C for 15 minutes after which they were incubated at 85 ˚C for 15 minutes so as to stop the reaction. The resulting cleaned PCR product was then used as template for the sequencing PCR.

The PCR product that resulted from the cultures that had been isolated directly from the leaf tissue as well as from the incubation solutions were sequenced using the primer *pD (5' CAG CAG CCG CGG TAA TAC 3') (Edwards *et al*., 1989) (Inqaba biotechnologies). Certain cultures that were isolated form *P. lanceolata* specimens growing in pots were also sequenced with the primers pA (5’ AGA GTT TGA TCC TGG CTC AG 3’) and pH (5’ AAG GAG GTG ATC CAG CCG CA 3’) (Edwards *et al*., 1989) (Inqaba biotechnologies) so as to obtain the full 16S rRNA gene sequence. The cloned PCR fragments were sequenced using the primer SP6 (5’ ATT TAG GTG ACA CTA TAG AAT 3’) (Promega) (Inqaba biotechnologies). Samples that indicated the presence of *Burkholderia* spp. were sequenced using the

forward primer GB-F (5' AGT AAT ACA TCG GAA CRT GT 3') (Perin *et al*., 2006) (Inqaba biotechnologies). The primer sequences used for PCR amplification and sequencing the different regions of the 16S rRNA gene are listed in Table 1.

Each 12 µl sequencing reaction contained 0.5 µl BigDye Terminator v3.1 reaction premix (Applied Biosystems), 2.5 µl BigDye sequencing buffer (Applied Biosystems), 3 pmol primer, 4.7 µl nuclease free water (Qiagen) and 4 µl cleaned PCR product. Sequencing PCR conditions included denaturation at 96 ˚C for 5 seconds, 25 cycles of denaturation at 96 ˚C for 10 seconds, primer binding at 55 ˚C for 5 seconds, elongation at 60 ˚C for 4 minutes. The resulting PCR products were sequenced with an ABI Prism DNA Automated Sequencer (Perkin-Elmer).

The sequences that were obtained were viewed and edited using BioEdit Sequence Alignment Editor v 7.0.9.0 (Hall, 1999). The edited sequences were then used for BLAST analysis (Altschul *et al*., 1990) against the National Center for Biotechnology Information (NCBI) database to identify the most similar 16S rRNA gene sequences. The most similar 16S rRNA gene sequences were used to assign each sequence to a genus. The sequences obtained from certain cultures isolated from *P. lanceolata* nodule tissue were aligned using CLC Genomics Workbench v.6 (CLC bio, University of Pretoria, South Africa) and the consensus sequence was used for phylogenetic analysis.

Phylogenetic analyses were carried out on the sequences showing close association to either *Burkholderia* spp. or the Bradyrhizobiaceae. The sequences of interest as well as the most similar BLAST sequences were identified and obtained from the NCBI database. Closely related strains including type strains from the All-Species Living Tree (Yarza *et al*., 2010) were also included. The selected sequences were aligned using MAFFT (Version 6) online alignment tool (Katoh *et al*., 2002). The aligned sequences were trimmed in BioEdit Sequence Alignment Editor v 7.0.9.0 (Hall, 1999) and the most suitable model was assigned using the jModelTest program v

0.1.1. (Posada, 2008). Maximum Likelihood trees (Felsenstein, 1981) with a 1000 bootstrap replicates were drawn using PhyML 3.0 software (Guidon *et al*., 2010).

2.4) Results

2.4.1) Culture-dependent

Fifteen bacterial cultures were isolated from *P. lanceolata* of which seven were from the plant material and the other eight were from the serial dilutions of the different incubation solutions. The BLAST results and percentage similarity for each culture were recorded (Table 2). Twelve of these cultures were identified as belonging to the family Bradyrhizobiaceae, all of which had been isolated from the nodule tissue. The complete 16S rRNA gene sequence was obtained for these cultures and a Maximum Likelihood tree was constructed (Fig. 1). All twelve cultures grouped together in a highly (100 % bootstrap) supported cluster. The closest sequence that grouped with this cluster was one that was obtained from a culture tentatively identified as a *Bradyrhizobium* sp., obtained when performing a BLAST analysis against the NCBI database. The closest type strain identified during the same BLAST analysis was that of *Oligotropha carboxidovorans* (Accession number CP001196).

Pavetta edentula yielded 14 bacterial cultures all of which originated from the serial dilution of the various broth solutions. The cultures were identified using BLAST analysis and the resulting closest match as well as the percentage similarity were recorded for each culture (Table 3). *Bacillus* spp. were isolated from both the nodule and non-nodulated leaf tissues. NAP buffer was the only solution that yielded bacterial cultures from the nodule tissue. These bacteria included a *Massilia* sp. and a *Curtobacterium* sp. Cultures that were isolated from the non-nodulated leaf tissue included *Sphingomonas* sp., *Methylobacterium* sp., *Enhydrobacter* sp., Arthrobacter sp. and *Microbacterium* sp.

The bacterial cultures isolated from *P. schumanniana* consisted of three plant material isolates and four serial dilution isolates. The BLAST results and percentage similarity for each isolate were recorded (Table 4). Majority of the isolates were identified as belonging to the genus *Bacillus*. A *Pantoea* sp. and a *Micrococcus* sp. were isolated from the non-nodulated leaf tissue by means of a serial dilution.

2.4.2) Culture-independent (DGGE)

Each clone for the DGGE analysis was sequenced and the BLAST results as well as percentage similarities were recorded in Table 5 for *P. lanceolata*, in Table 6 for *P. edentula* and in Table 7 for *P. schumanniana.* The sequences that were identified as chloroplast or mitochondrial DNA were not included and some of the cloned colonies did not yield a sequence. The bacteria that were identified as belonging to the Bradyrhizobiaceae from the DGGE analysis of *P. lanceolata* were used in combination with those Bradyrhizobiaceae isolated in culture to construct a Maximum Likelihood tree (Fig. 2). The DGGE sequences and the culture sequences grouped together in a largely undefined cluster together with a number of known strains amongst them. One cloned sequence, TN1.81LD, was found to group away from the other sequences with a strain of *Afipia clevelandensis*.

The bacteria from the DGGE analysis that were identified as belonging to the genus *Burkholderia* were used to construct a Maximum Likelihood tree (Fig. 3). The *Burkholderia* spp. from *P. edentula* and *P. schumanniana* clustered together and grouped closely with the *Burkholderia* spp. previously identified by Lemaire *et al*. (2011a, b) with bootstrap support of 88 %. The *Burkholderia* spp. identified from the *P. lanceolata* samples growing in pots did not cluster with the other nodule-forming *Burkholderia* spp., including the *Burkholderia* sp. identified from *P. lanceolata* by Lemaire *et al*. (2011a).

2.4.3) *Burkholderia* specific PCR

Burkholderia specific primers (Perin *et al*., 2006) were able to detect the presence of a *Burkholderia* sp. in two samples of the nodule tissue collected from the *P. lanceolata* trees growing in the National Botanical Gardens. The BLAST results for the sequences obtained from these samples indicated that the closest match for both samples was *Candidatus* Burkholderia schumannianae. The *P. edentula* and *P. schumanniana* samples also yielded sequences that closely matched the sequence of *Candidatus* Burkholderia schumannianae from the NCBI database. The *P. lanceolata* samples from the specimens kept in pots did not show any amplification even though the positive control had a band. The sequences obtained from the *Burkholderia* specific PCR were used to construct a Maximum Likelihood tree (Fig. 4). The sequence obtained from *P. edentula* grouped with the *Burkholderia* sp. identified by Lemaire *et al*. (2011a) from *P. edentula.* The sequence obtained from the nodule tissue of *P. schumanniana* grouped with the *Candidatus* Burkholderia schumannianae sequence (Lemaire *et al*., 2011b) and the *P. edentula* 16S rRNA gene sequences in a well supported group with a bootstrap value of 83 %. The *Burkholderia* sp. sequences obtained from the Botanical Gardens samples of *P. lanceolata* grouped with the *Burkholderia* sp. identified from *P. lanceolata* by Lemaire *et al*. (2011a).

2.5) Discussion

The bacteria isolated and identified from the *P. lanceolata* trees from the nursery are known endophytes in other plants (Lodewyckz *et al*., 2002; Bacon and Hinton, 2007). Only two bacterial species were cultured from the leaf tissue of these *P. lanceolata* plants, namely, *Bacillus* sp. and *Paenibacillus* sp. These cultures were only isolated when an initial enrichment step was included. This result may indicate a low presence of bacterial endophytes within the leaf tissue of these *P. lanceolata* trees that could only be detected after enrichment (Reiter and Sessitsch, 2006). An alternate reason for the presence of these bacterial species may be due to their spore-forming ability (Nicholson, 2002) and the spores were not

removed from the surface of the leaf during the surface sterilization procedure. Only one bacterial genus was identified from the leaf tissue of *P. lanceolata* using DGGE namely, *Burkholderia*. As with the culture-dependent results this may indicate that there are few bacterial endophytes within the leaf tissue of these *P. lanceolata* trees (Reiter and Sessitsch, 2006).

The majority of bacteria isolated from the *P. lanceolata* trees grown in pots were from the nodule tissue which was dominated by bacteria identified as *Afipia* sp. This observation may indicate that the nodule-forming bacterial endophyte within these *P. lanceolata* specimens is an *Afipia* sp. However, two cultures isolated from the nodule tissue were closely matched to the genus *Bradyrhizobium*. This genus has been found to be linked to the development of nodules and nitrogen fixation on the roots of legumes (Jordan, 1982). The leaf nodules of the Rubiaceae were also initially thought to fix nitrogen but further studies could not prove this hypothesis (Miller, 1990). The results of the Maximum Likelihood tree indicated that the Bradyrhizobiaceae cultures isolated from the *P. lanceolata* samples obtained from the nursery may belong to the same species. The closest type strain found within the BLAST results for these sequences was *Oligotropha carboxidovorans* but the isolates did not cluster with this sequence during the phylogenetic analysis. This may indicate the presence of a new species within the nodule tissue of these two *P. lanceolata* plants.

The results for the DGGE analysis of the *P. lanceolata* specimens obtained from the nursery were significant in that the nodule tissue samples were dominated by genera belonging to the family Bradyrhizobiaceae. This is in contrast to the results by Lemaire *et al*. (2011a) who found the nodule-forming bacteria in *P. lanceolata* to be a *Burkholderia* sp. The bacteria from the nodule tissue of the *P. lanceolata* samples obtained from the nursery grouped mostly to *Nitrobacter* sp. (41 %). Another 24 % was found to group closely to Nitrite – oxidising bacteria, 24 % grouped with *Bradyrhizobium* sp. and lastly 7 % grouped with *Rhodopsedomonas* sp. Once again the presence of a *Bradyrhizobium* sp. is not surprising due to the implication that the leaf nodules of the *Pavetta* sp. fix nitrogen (Miller, 1990). Lemaire *et al*.

(2011a) suggested that the nodule-forming bacteria could have been introduced from different environments such as insects and soil and that the initial colonization of the nodulated hosts was by a diverse range of bacterial species. This initial colonization process was then believed to be narrowed down to one specific bacterial genus namely, *Burkholderia,* by a recent specialization process (Lemaire *et al*., 2011a). This recent specialization may, however, not be fixed and it could still be possible for other bacteria from the Bradyrhizobiaceae to form nodules on the leaves.

Using DGGE, a *Burkholderia* sp. was detected within the nodule and non-nodulated leaf tissue of the *P. lanceolata* trees that were obtained from the nursery. The Maximum Likelihood tree, however, indicates that these *Burkholderia* spp. sequences do not group close to the *Candidatus* Burkholderia spp. sequences identified previously from *P. lanceolata* or other *Pavetta* spp. (Lemaire *et al*., 2011a, b). This indicates that the *Burkholderia* sp. detected within the *P. lanceolata* nursery samples is not related to the previously identified nodule-forming bacteria.

The majority of the bacterial endophytes cultured from *P. edentula* were isolated from the non-nodulated leaf tissue. Most of these cultured bacterial endophytes have been identified previously in other plants (Lodewyckz *et al*., 2002; Bacon and Hinton, 2007). No bacterial endophytes were isolated directly from the plant tissue of *P. edentula*. This result may be due to the fact that the bacterial endophytes are at low concentrations within the leaf and are only given the opportunity to increase in numbers during the enrichment step (Reiter and Sessitsch, 2006).

The bacterial endophytes that were identified in the leaf tissue of *P. edentula* from DGGE analysis were more diverse that that found in the *P. lanceolata* trees growing in pots. The genera found have generally been identified in other plants as bacterial endophytes (Lodewyckz *et al*., 2002; Bacon and Hinton, 2007). The fact that none of these genera, except *Bacillus* sp., was present in the cultures that had been isolated from the non-nodulated leaf tissue of *P. edentula* indicates that they are most likely unculturable.

The bacteria cultured from the nodule tissue of *P. edentula* included *Massilia* sp., *Bacillus* sp. and *Curtobacterium* sp. none of which have been previously implicated in nodule development. These bacteria were, however, only cultured from the serial dilutions of the NAP buffer solutions which suggests that these bacterial endophytes are oligotrophic (Bacon and Hinton, 2007).

The nodule forming bacterial endophyte in *P. schumanniana* has already been identified as *Candidatus* Burkholderia schumannianae (Lemaire *et al*., 2011b). Out of the cloned sequences obtained from the nodule tissue of *P. edentula*, 79 % were closely matched to the species *Candidatus* Burkholderia schumannianae. This result infers that the nodule forming bacterial endophytes found in *P. edentula* and *P. schumanniana* are closely related. The same result was found by Lemaire *et al*. (2011a) who carried out an evolutionary study on the leaf nodule-forming bacterial endosymbionts.

The bacterial endophytes cultured from *P. schumanniana* were dominated by the genus *Bacillus.* The low isolation frequency of bacterial endophytes from this species of *Pavetta* may be due to the fact that only one tree was sampled and it was not growing in its natural environment. Rosenblueth and Martinez-Romero (2006) stated that the population density of bacterial endophytes is dependent on the host-developmental stage, inoculum density as well as environmental conditions. Thus if the *P. schumanniana* tree that was sampled was stressed due to the environmental conditions, this may explain the low numbers of bacterial endophytes that were cultured.

As stated above the nodule-forming bacterial endophyte in *P. schumanniana* has previously been identified (Lemaire *et al*., 2011b). In this study only one clone from the nodule tissue of *P. schumanniana* was found to be a *Candidatus* Burkholderia schumannianae. This result may again be due to the plant sampled in this study being under stress (Rosenblueth and Martinez-Romero, 2006). The *Nitrobacter* sp. and *Bradyrhizobium* sp. that were identified in the nodule tissue of *P. schumanniana* can be excluded as being the nodule-forming bacterial endophytes as they were found within leaf tissue as well. However, the question of whether there is a link between

these Bradyrhizobiaceae found in *P. schumanniana* and the nodule-forming bacterial endophyte found in the *P. lanceolata* samples obtained from the nursery still remains unanswered.

The presence of *Bacillus* spp. was high in both the culture-dependent and –independent analysis of the three *Pavetta* spp. This result may be due to these bacterial endophytes being favoured, especially if they are fast growing, during enrichment. This allows them to grow to high numbers even though there numbers *in vivo* may be low. Further reasoning is that despite all efforts to surface sterilize the leaf surface, bacterial DNA or endospores may still be present which is then detected during the use of culture-independent methods (Nicholson, 2002; Reiter and Sessitsch, 2006).

The *Burkholderia* specific primers were able to detect the nodule-forming bacterial endophyte within the nodule tissue of the *P. lanceolata* trees sampled from the National Botanical Gardens. The phylogenetic analysis of the *Burkholderia* spp. found within the above samples along with those found in *P. edentula* and *P. schumanniana* confirmed the results of Lemaire *et al*. (2011a). This result raises the question of why no proposed nodule-forming *Burkholderia* spp. could be detected within the nodule tissue of the *P. lanceolata* trees obtained from the nursery. Once again this could be explained by the leaf nodule colonization and specialisation theory suggested by Lemaire *et al*. (2011a). The results from the nodule tissue of the *P. lanceolata* samples suggest that the relationship between plant and bacteria is more complex and the species of nodule-forming bacterial endophytes within a plant may be influenced by the environmental conditions. It may be possible that because the nursery samples of *P. lanceolata* were planted into and kept within heat sterilized potting soil, a different bacterial endophyte was given the opportunity to colonize the leaf nodules.

2.6) Conclusions

- Overall the bacterial endophyte diversity within the leaves of the three *Pavetta* species resembles that of other plants.
- The culture-dependent results revealed that the included enrichment step may have enabled certain bacterial species to increase in number and hence be detected.
- DGGE analysis of the nodule tissue of *P. edentula* and *P. schumanniana* revealed that the nodule-forming bacteria in these *Pavetta* spp. is from the genus *Burkholderia*.
- The identification of a Bradyrhizobiaceae as the nodule-forming bacterial endophyte within *P. lanceolata* samples growing in pots indicates that the symbiotic relationship of leaf nodules may not be as strict as originally thought.
- The presence of a *Burkholderia* sp. within the nodule tissue of *P. lanceolata* growing in open soils suggests that the environmental conditions in which the plant grows may play a role in the selection of the species of nodule-forming bacterial endophyte by the plant.
- It may be possible that all leaf nodulated plants harbour both a *Burkholderia* sp. and bacteria from the Bradyrhizobiaceae hence the conditions during germination may dictate which bacterial species will colonise the leaf tissue and form nodules.
- Despite the fact that the *Burkholderia* spp. found within the nodule tissue are not culturable, it appears that the Bradyrhizobiaceae bacteria found within leaf nodules can be cultured. This may be an indication that the more tolerant bacteria will colonise the leaf nodules when the host plant is placed in an abnormal environment.

2.7) References

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Table 1: Sequences of primers used for amplification and sequencing.

Primer name	Sequence 5' – 3'	Reference
pA	AGA GTT TGA TCC TGG CTC AG	Edwards *et al*., 1989
pH	AAG GAG GTG ATC CAG CCG CA	Edwards *et al*., 1989
pA8f-GC	AGA GTT TGA TCC TGG CTC AG CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G	Fjellbirkeland *et al.*, 2001
pA8f	AGA GTT TGA TCC TGG CTC AG	Edwards *et al*., 1989
PRUN518r	ATT ACC GCG GCT GCT GG	Muyzer *et al.*, 1993
SP6	ATT TAG GTG ACA CTA TAG AAT	Promega
T7	TAA TAC GAC TCA CTA TA	Promega
GB-F	AGT AAT ACA TCG GAA CRT GT	Perin *et al*., 2006
GBN2-R	GCT CTT GCG TAG CAA CTA G	Perin *et al*., 2006
*pD	CAG CAG CCG CGG TAA TAC	Edwards *et al*., 1989

Table 2: BLAST results for bacterial cultures isolated from *P. lanceolata*.

Inoculum source: Plant material / Broth	Nodule or leaf	Culture name	BLAST result	Similarity (%)
Plant material	Nodule	P1TN2LC	*Micrococcus* sp.	100 %
		P1RN2LC	*Afipia* sp.	98 %
		P2CN1LC	*Oligotropha* sp.	98 %
		P2CN2LC	*Afipia* sp.	99 %
		P2TN2LC	*Afipia* sp.	99 %
		P2TN3LC	*Afipia* sp.	98 %
		P2TN4LC	*Afipia* sp.	98 %
Broth	Nodule	B2CN1.1LC	*Afipia* sp.	98 %
		B2CN1.2LC	*Afipia* sp.	98 %
		B2CN2LC	*Afipia* sp.	99 %
		B2TN1.1LC	*Afipia* sp.	98 %
		B2NN2.1LC	*Bradyrhizobium* sp.	99 %
		B2NN2.2LC	*Bradyrhizobium* sp.	99 %
	Leaf	B1TL2LC	*Paenibacillus* sp.	99 %
		B2TL1.2LC	*Bacillus* sp.	100 %

Table 3: BLAST results for bacterial cultures isolated from *P. edentula*.

Inoculum source: Plant material / Broth	Nodule or leaf	Culture name	BLAST result	Similarity (%)
Broth	Nodule	BNN2.1EC	*Bacillus* sp.	100 %
		BNN2.2EC	*Massilia* sp.	99 %
		BNN2.3EC	*Bacillus* sp.	100 %
		BNN2.4EC	*Curtobacterium* sp.	100 %
	Leaf	BCL1.1EC	*Sphingomonas* sp.	98 %
		BCL1.2EC	*Methylobacterium* sp.	99 %
		BTL2.1EC	*Bacillus* sp.	100 %
		BTL2.2EC	*Enhydrobacter* sp.	99 %
		BNL1.1EC	*Bacillus* sp.	100 %
		BNL1.2EC	*Bacillus* sp.	100 %
		BNL1.3EC	*Bacillus* sp.	100 %
		BNL2.1EC	*Arthrobacter* sp.	99 %
		BNL2.2EC	*Arthrobacter* sp.	100 %
		BNL2.3EC	*Microbacterium* sp.	100 %

Table 4: BLAST results for bacterial cultures isolated from *P. schumanniana*.

Inoculum source: Plant material / Broth	**Nodule or leaf**	**Culture name**	**BLAST result**	**Similarity (%)**
Plant material	Nodule	PCN1SC	*Bacillus* sp.	100 %
	Leaf	PCL2SC	*Bacillus* sp.	99 %
		PCL3SC	*Bacillus* sp.	100 %
Broth	Nodule	BTN1SC	*Bacillus* sp.	99 %
		BTN2SC	*Bacillus* sp.	100 %
	Leaf	BCL1SC	*Pantoea sp.*	100 %
		BNL1SC	*Micrococcus* sp.	100 %

Table 5: BLAST results for culture-independent analysis of *P. lanceolata*.

Nodule or Leaf material	Sample name	Clone number	BLAST result	Similarity (%)
Nodule	CN1.2LD	1	*Bradyrhizobium* sp.	97 %
		3	*Burkholderia* sp.	99 %
		4	*Nitrobacter* sp.	97 %
		5		
	CN1.3LD	1	*Nitrobacter* sp.	97 %
		2		
		3		
		4		
		5		
	CN1.4LD	1	*Nitrobacter* sp.	97 %
	CN1.5LD	1	*Nitrobacter* sp.	97 %
		2	Nitrite-oxidising bacteria	97 %
		5		
	CN1.6LD	1	Nitrite-oxidising bacteria	97 %
		3	*Nitrobacter* sp.	97 %
		5		
	CN1.8LD	5	*Nitrobacter* sp.	98 %
		6		
	CN1.9LD	1	Nitrite-oxidising bacteria	97 %
		2		
		4	*Nitrobacter* sp.	98 %
		5		
	CN1.10LD	1	*Nitrobacter* sp.	98 %
		2		
		3		
	TN1.3LD	1	*Nitrobacter* sp.	98 %
		2	*Bradyrhizobium* sp.	99 %
		3	*Nitrobacter* sp.	98 %
	TN1.4LD	1	*Nitrobacter* sp.	97 %
		2		
	TN1.5LD	1	*Bradyrhizobium* sp.	99 %
	TN1.6LD	1	Nitrite- oxidising bacteria	99 %
	TN1.7LD	1	*Bradyrhizobium* sp.	99 %
	TN1.8LD	1	*Rhodopseudomonas* sp.	99 %
		2		
		3		
		4	*Bradyrhizobium* sp.	98 %
	TN1.9LD	1	Nitrite-oxidising bacteria	97 %
		2	*Bradyrhizobium* sp.	98 %
		3	*Bradyrhizobium* sp.	97 %
	TN1.10LD	1	*Rhodopseudomonas*	99 %

		2	sp.	
		4		
		5		
	NN1.2LD	3	Nitrite-oxidising bacteria	94 %
		4		
		5		
	NN1.3LD	3	Nitrite-oxidising bacteria	98 %
	NN1.12LD	2	*Nitrobacter* sp.	97 %
		3		
Leaf	CL1.8LD	4	*Burkholderia* sp.	100 %
		5		
		6		

Table 6: BLAST results for culture-independent analysis of *P. edentula*.

Nodule or Leaf material	Sample name	Clone number	BLAST result	Similarity (%)
Nodule	CN1.1ED	5	*Candidatus* Burkholderia schumannianae	98 %
	CN1.3ED	2	*Curtobacterium* sp.	97 %
		3	*Candidatus* Burkholderia schumannianae	99 %
		4		
		5		
	CN1.4ED	1	*Erwinia* sp.	99 %
		2	*Candidatus* Burkholderia schumannianae	99 %
		4		
	CN1.6ED	1	*Candidatus* Burkholderia schumannianae	99 %
		2		
		3		
		4		
		5		
	CN1.7ED	1	*Candidatus* Burkholderia schumannianae	99 %
		3		98 %
	CN1.8ED	4	*Bacillus* sp.	98 %
	TN1.1ED	2	Variovorax sp.	96 %
		5	*Candidatus* Burkholderia schumannianae	99 %
	TN1.2ED	3	*Candidatus* Burkholderia schumannianae	99 %
	TN1.4ED	5	Variovorax sp.	99 %
	TN1.5ED	1	*Candidatus* Burkholderia schumannianae	98 %
		3		99 %
		4		
	NN1.3ED	1	*Candidatus* Burkholderia schumannianae	99 %
Leaf	CL1.1ED	3	*Leuconostoc* sp.	100 %
	CL1.2ED	1	*Acinetobacter* sp.	99 %
		2		98 %
	CL1.3ED	1	*Sporosarcina* sp.	96 %
	CL1.4ED	4	*Comamonas* sp.	99 %
	CL1.5ED	2	*Bacillus* sp.	100 %
	TL1.3ED	1	*Acinetobacter* sp.	99 %
		2		
	TL1.5ED	5	*Paenibacillus* sp.	99 %
	NL1.1ED	2	*Pseudomonas* sp.	99 %

Table 7: BLAST results for the culture-independent analysis of *P. schumanniana*.

Nodule or Leaf material	Sample name	Clone number	BLAST result	Similarity (%)
Nodule	CN1.2SD	1	*Candidatus* Burkholderia schumannianae	99 %
	CN1.2SD	2	*Bradyrhizobium* sp.	97 %
	CN1.3SD	3	*Nitrobacter* sp.	98 %
		5	*Propionibacterium* sp.	99 %
	CN1.4SD	1	*Propionibacterium* sp.	99 %
	TN1.1SD	1	*Bacillus* sp.	99 %
		2		
	TN1.2SD	1	*Bacillus* sp.	97 %
		2		
		3		
	TN1.4SD	1	*Bacillus* sp.	100 %
Leaf	CL1.3SD	1	*Nitrobacter* sp.	97 %
		3		
	TL1.4SD	2	*Propionibacterium* sp.	92 %
		3		
		4	*Bacillus* sp.	100 %
	TL1.5SD	5	*Bacillus* sp.	97 %
	NL1.1SD	1	*Bradyrhizobium* sp.	97 %
		2	*Nitrobacter* sp.	97 %
	NL1.2SD	2	*Nitrobacter* sp.	97 %
		3	*Propionibacterium* sp.	99 %
	NL1.3SD	4	*Bradyrhizobium* sp.	91 %

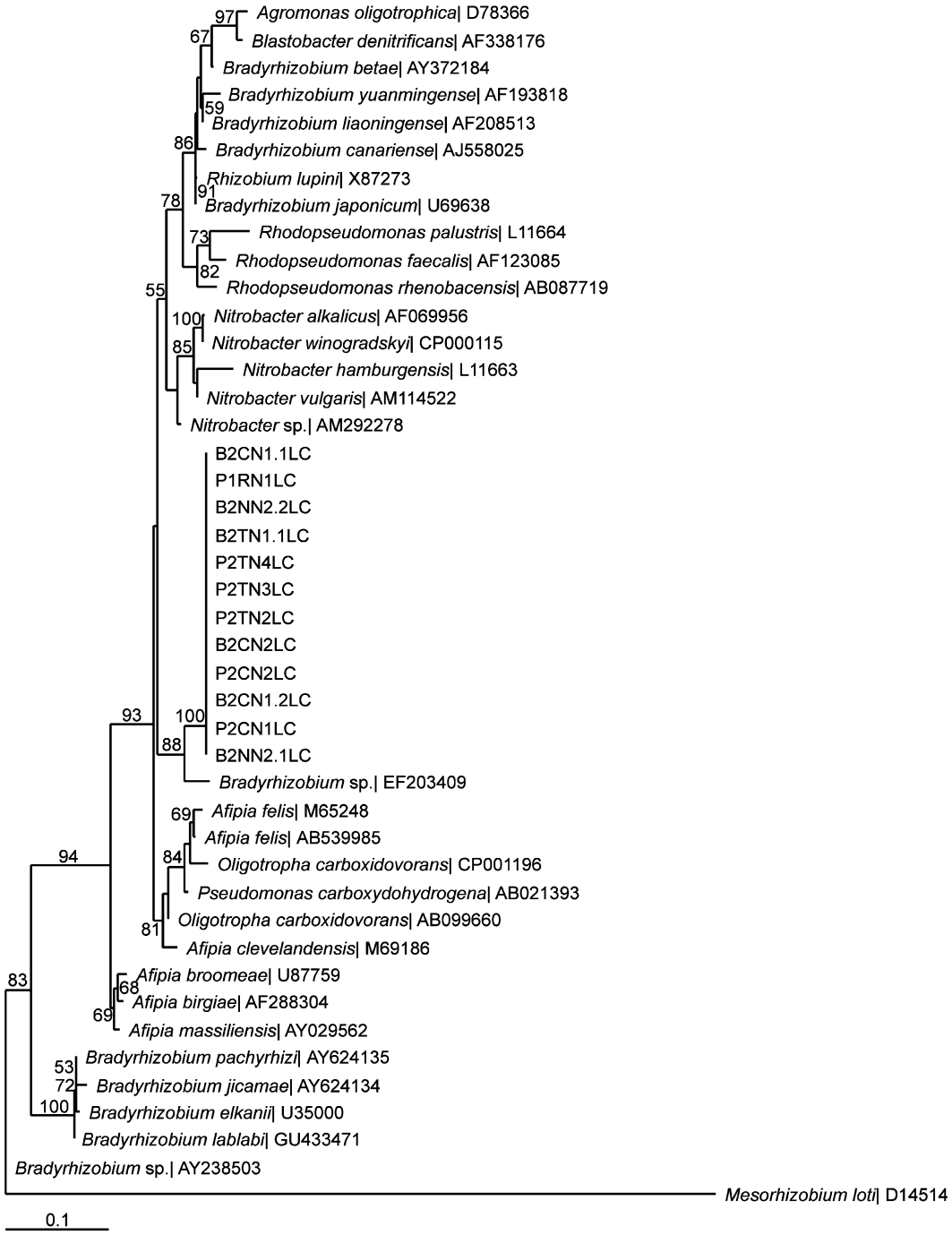

Figure 1: Maximum Likelihood tree of 16S rRNA gene region obtained from Bradyrhizobiaceae cultures isolated from *P. lanceolata* nursery samples. The sequence length was 1182 bp. Bootstrap values of a 1000 replicates are represented as a percentage. Values lower than 50 % are not shown. *Mesorhizobium loti* was used as the outgroup.

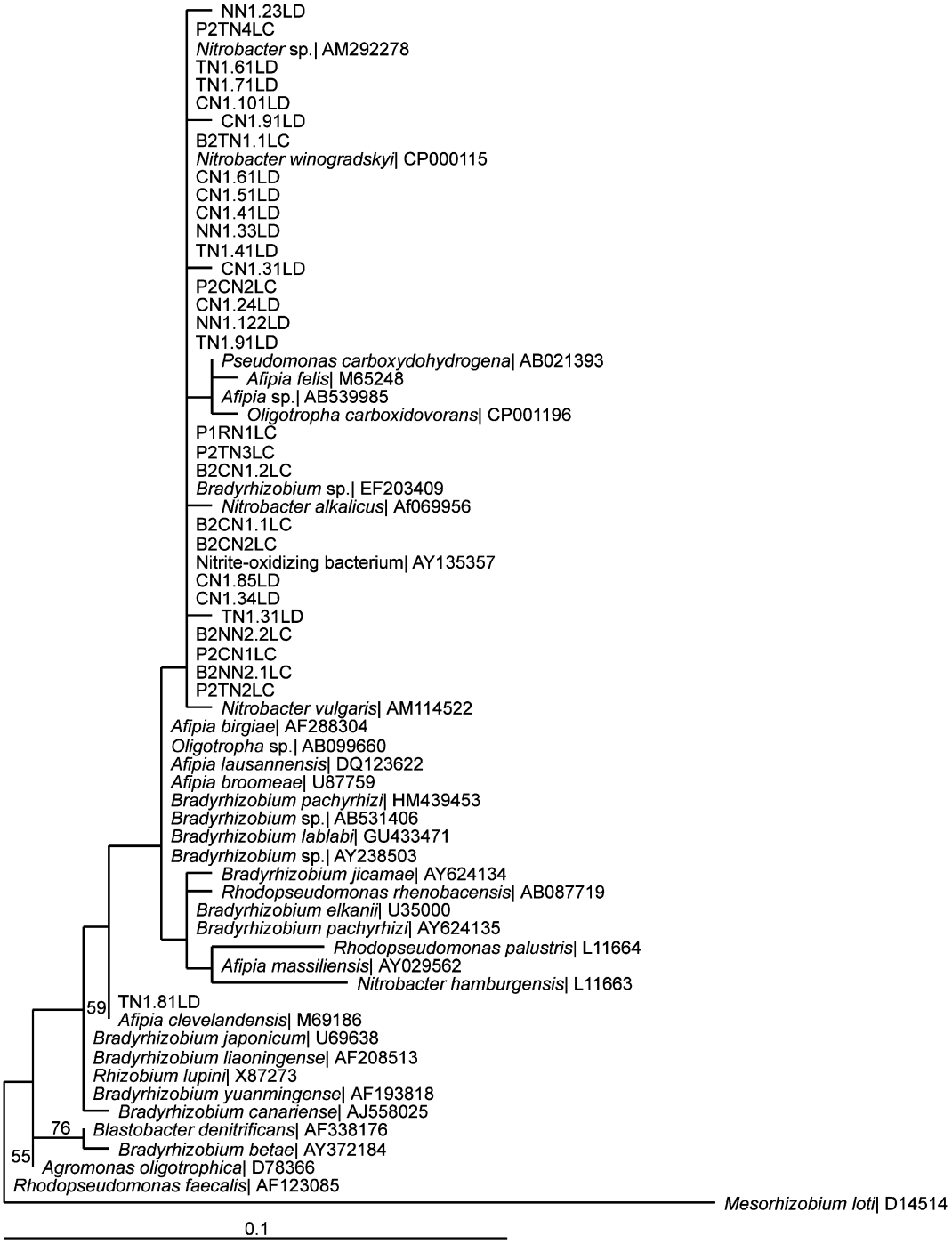

Figure 2: Maximum Likelihood tree of Bradyrhizobiaceae 16S rRNA gene region identified in *P. lanceolata* nursery samples using culturing (LC) and DGGE (LD). The sequence length of was 208 bp. Bootstrap values represented as a percentage of a 1000 replicates. *Mesorhizobium loti* was used as the outgroup.

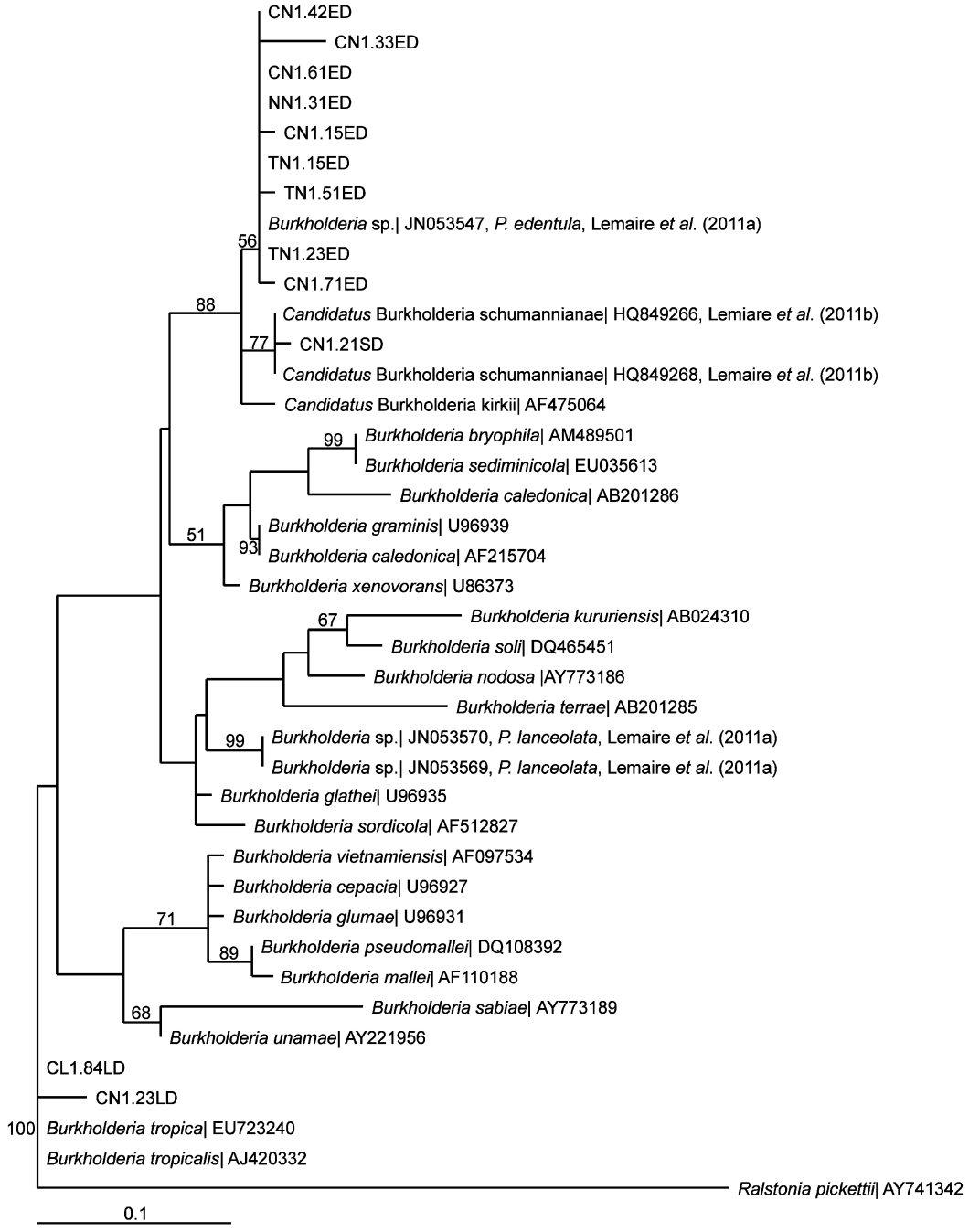

Figure 3: Maximum Likelihood tree of 16S rRNA gene region of *Burkholderia* species detected with DGGE analysis in nodule and leaf tissue of three *Pavetta* spp. The sequence length was 282 bp. Bootstrap values are presented as a percentage of 1000 replicates. Values lower than 50 % were not included. *Ralstonia picketti* was used as the outgroup. ED – *P. edentula*, SD – *P. schumanniana* and LD – *P. lanceolata* nursery samples.

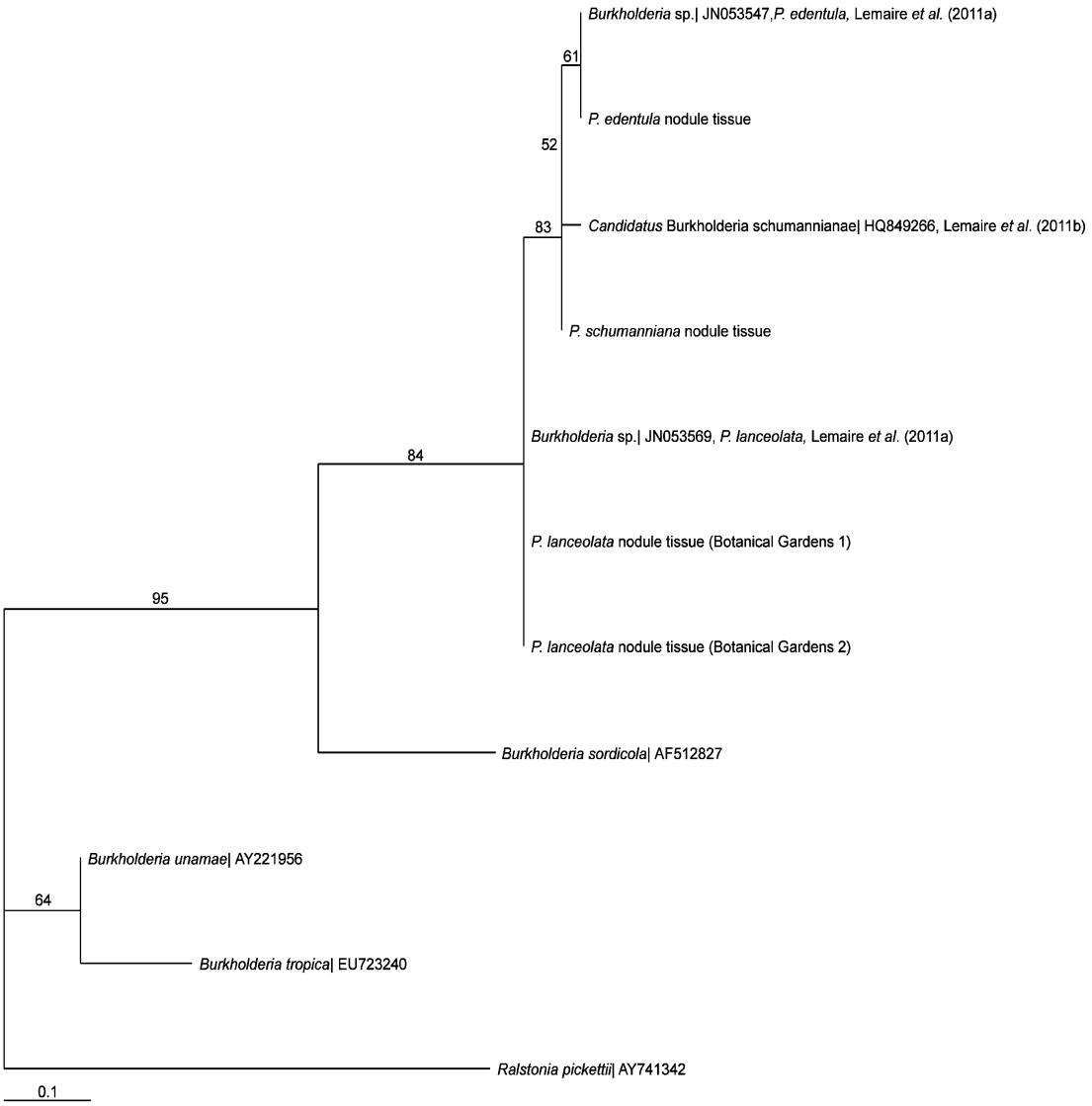

Figure 4: Maximum Likelihood tree of *Burkholderia* specific PCR on 16S rRNA gene region of *P. lanceolata* Botanical Garden samples, *P. edentula* and *P. schumanniana*. The sequence length was 286 bp. Bootstrap values for a 1000 replicates are indicated as percentages. Values lower than 50 % were not shown. *Ralstonia picketti* was used as the outgroup.

Chapter 3

Electron microscopy of leaf nodule development in three indigenous *Pavetta* spp.

Electron microscopy of leaf nodule development in three indigenous *Pavetta* spp.

3.1) Abstract

Bacterial leaf nodules occur in some members of the Rubiaceae. The manner in which these leaf nodules form has been studied in certain plant species for many years with the aid of light and electron microscopy. One plant in particular that has been studied in depth using this technology is *Psychotria bacteriophila*. *Pavetta*, another genus within this family, also have bacterial filled leaf nodules. The manner in which the leaf nodules form within these species has not been described in depth. In this study, nodule formation in three indigenous *Pavetta* spp., viz. *P. lanceolata*, *P. edentula* and *P. schumanniana*, was investigated by examining nodules of different ages using light and electron microscopy. It was evident from the results that the manner in which the nodules form is unique to each *Pavetta* spp. which is most likely due to the complex relationship between the plant and the different bacteria capable of nodulation. Some of the differences that were most notable were the plant cell wall thickening present in *P. edentula* and *P. schumanniana* but not in *P. lanceolata* as well as the high bacterial numbers present in the leaf nodule tissue of *P. edentula* and *P. schumanniana* compared to *P. lanceolata*. Certain characteristics that were associated with nodule development in all these *Pavetta* spp. were the production of extracellular mucilage in which the bacteria reside and an increase in bacterial numbers as the nodule matured.

3.2) Introduction

Leaf nodules have been reported in approximately 400 species of plants within the Rubiaceae (van Wyk *et al.*, 1990). The position of these nodules can be used as a host identification tool as they are either scattered over the leaf blade or found along the midrib of the leaf (Boon, 2010). The shape of the nodules can also vary from spherical to rod-like and can be branched or unbranched (Miller, 1990). One genus in particular that has approximately 353 nodulated species is *Pavetta* (Miller, 1990). Three such *Pavetta* spp. indigenous to South Africa include *P. lanceolata*, commonly known as the forest bride's bush, *P. edentula*, the gland-leaf bride's bush and *P. schumanniana*, the poison bride's bush.

The leaves of *P. lanceolata* are narrowly elliptic and generally 50 – 70 mm in length and 9 – 15 mm in width. An interesting characteristic found on the leaves of *P. lanceolata* are the small hair-fringed domatia that are located on the underside of the leaves alongside the main vein (Schmidt *et al.*, 2007). *P. edentula* has narrowly elliptic or oblanceolate leaves that are typically 250 x 80 mm in size (Schmidt *et al.*, 2007). The leaves of *P. schumanniana* are obovate in shape and usually 60 – 140 mm in length and 20 – 60 mm in width. These leaves differ from that of *P. lanceolata* and *P. edentula* in that they have hairs on the adaxial and abaxial surfaces and venation is very prominent on the underside of the leaves (Schmidt *et al*., 2007). The leaf nodules of the above three *Pavetta* spp. are scattered over the leaf lamina and are generally not found near the midrib (Herman *et al.*, 1987).

Psychotria is another genus within the Rubiaceae. Various species within this genus also show nodules on their leaves and they have been studied extensively in the past (Miller, 1990). The development of the leaf nodules in this host has been relatively well documented over the years (Lersten and Horner, 1967; Whitmoyer and Horner, 1970; Miller *et al.*, 1983; Miller 1990). The manner in which leaf nodules are initiated was first proposed by

Zimmermann (1902) and has since been expanded upon (Von Faber, 1912; Lersten and Horner, 1967; Miller, 1990).

Before the development of electron microscopy, leaf nodule development studies were limited to the use of light microscopy (Miller, 1990). Electron microscopy is, however, a powerful tool in detecting the microorganisms within plant tissue and despite the lengthy preparation process it is worth the effort due to the high resolving power (Hallmann *et al.*, 1997). The first study to use electron microscopy on leaf nodule development in the Rubiaceae was that of Zeigler (1958) who viewed the leaf nodules of *P. zimmermanniana* under low magnification electron microscopy.

Nearly a decade later Lersten and Horner (1967) used electron microscopy at higher magnification to identify characteristics associated with leaf nodule development in *Psychotria bacteriophila*. They identified certain characteristics associated with the mesophyll cells that were situated within and around the bacterial masses that were different to those not in contact with the bacteria. Two such characteristics were that the mesophyll cells located within the bacterial mass were often distorted in shape and that these cells had unusually thick primary cell walls. They proposed that the cell wall thickening may be due to the plant supplying the bacteria with a constant source of carbohydrates. Despite these differences found in the mesophyll cells, chloroplasts that contained numerous starch granules were present within the cells indicating that normal photosynthetic activity was taking place. Whitmoyer and Horner (1970) took this study one step further and used a combination of acridine-orange fluorescence and ribonuclease extractions to detect ribonucleic acid (RNA) concentrations during nodule growth in *P. bacteriophila*. This study indicated that RNA concentrations were high during the young developing stages of the nodule but reduced as nodules got older. They concluded that reduction in RNA concentration was due to bacterial degeneration and that the beneficial traits of the bacteria were most likely received by the plant during the early stages of nodule development.

In recent years, studies on bacterial leaf nodules have become more focused on the identity of the nodule forming-bacterial endophytes and on how they enter the tissue during leaf development. However, apart from the work done by Zeigler (1958), the development of the nodule once the leaf tissue has been 'inoculated' is not very well documented for *Pavetta* spp. Recently Lemaire *et al.* (2011) examined leaf nodules of *P. schumanniana* under Scanning Electron Microscopy (SEM) and found that the bacteria within the nodule were rod-shaped, non-flagellated and approximately 1 – 2 µm in size. No further research was carried out on the nodule morphology of *P. schumanniana* during this study.

The aim of this study is to identify morphological changes which occur in leaf nodules of different ages in three indigenous *Pavetta* spp. using a combination of light and electron microscopy.

3.3) Materials and methods

3.3.1) Collection and preparation of samples

Samples of *P. lanceolata* were obtained from two plants that had been purchased form a nursery. Samples of *P. edentula* were collected from an open plot that was located on the slope of a hill in Barberton. Samples of *P. schumanniana* were collected from a plant growing in the toxicology gardens at Onderstepoort.

Before the leaf samples were processed, stereo-microscope images were taken of the adaxial and abaxial surface of each *Pavetta* spp. that was sampled. For the Scanning Electron Microscopy (SEM) samples, 1 cm^2 segments that included the bacterial nodules were cut out of the leaf tissue. For the Transmission Electron Microscopy (TEM) samples, 1 mm^2 segments were cut out of the leaf tissue ensuring that a bacterial nodule was present in the centre of each segment. For each plant species four different leaves of various ages (very young, young, mature and old) were sampled. The very young leaf tissue that had been sampled for each plant was that of the leaf

primordia which was still enclosed by the stipules. At the young leaf stage, leaves that had been exposed to the atmosphere but were still very soft were sampled. The old nodule tissue was taken from leaves that were near to abscission.

3.3.2) Fixation of samples

All segments were placed into a 2.5 % gluteraldehyde in 0.075 M phosphate buffer (pH 7.4) solution containing 1 % caffeine for 1.5 hours at room temperature. The solution was then discarded and the segments were washed 3 times for 10 minutes in 0.075 M phosphate buffer. The samples were then fixed in 0.5 % aqueous osmium tetroxide for 2 hours after which they were rinsed 3 times in distilled water. Lastly the samples were dehydrated with successive washes in varying concentrations of ethanol (30 % EtOH for 10 minutes, 50 % EtOH for 10 minutes, 70 % EtOH for 10 minutes, 90 % EtOH for 10 minutes, 100 % EtOH for 10 minutes, 100 % EtOH for 10 minutes, 100 % EtOH overnight).

3.3.3) Scanning Electron Microscopy (SEM)

The segments prepared for SEM were dried using critical point drying in liquid CO_2. The various plant species were then mounted onto separate stubs and sputtered with gold before being viewed in the SEM.

3.3.4) Light microscopy and Transmission Electron Microscopy (TEM)

Each sample prepared for TEM was first removed from the 100 % ethanol solution and placed into a solution containing 500 µl 100 % ethanol and 500 µl 2, 2 dimethyloxypropane for 10 minutes. The ethanol / 2, 2 dimethyloxypropane solution was then replaced with 100 % 2, 2 dimethyloxypropane for 15 minutes to remove any residual ethanol from the samples (Luft, 1961). A 10.234 g solution of the embedding resin Embed 812 was prepared by first adding 5.234 g Embed 812, 3.534 g methyl nadic anhydride (MNA) and 1.366 g dodecenyl succinic anhydride (DDSA) which

were mixed well before adding 0.1 g S1. The MNA and DDSA were added as hardening agents (Luft, 1961).The samples were then incubated for 30 minutes, with rotation, in a 1:1 mixture of Embed 812 mix and 2, 2 dimethyloxypropane. This solution was then replaced with a 100 % Embed 812 mixture and the samples were incubated for 4 hours with rotation. The samples were polymerized at 60 ˚C for 48 hours in 100 % Embed 812 mixture to obtain a hard resin block containing the sample for further processing with the microtome (Luft, 1961).

The resulting resin blocks were then cut into ultrathin sections using a microtome. For viewing under the light microscope, 0.5 µm – 1 µm thick sections of each sample were cut with a glass knife. The sections were placed into a drop of water on a glass slide and then dried on a heating block. Once dry the sections were stained for 30 seconds with toluidine blue and viewed under the light microscope. The light microscope images of each nodule age for each plant were taken to ensure that the sections made for the TEM were of the correct area of the nodule. Once viewed under the light microscope, ultrathin sections of the sample were prepared using a diamond knife. These sections were picked up with 200 mesh grids and were stored until being viewed with the TEM. Once the samples had been viewed unstained in the TEM, fresh sections were stained as follows: 5 minutes in uranyl acetate, rinsed 3 times in distilled water, stained for 3 minutes in Reynold's lead citrate and finally rinsed 3 times in distilled water before blotting dry. The stained samples were then again viewed in the TEM.

3.4) Results

3.4.1) P. lanceolata

The stereo-microscope images of *P. lanceolata* showed nodules that were generally round in shape and raised on both the adaxial and abaxial surfaces (Fig. 1). The average size of the nodules was 0.5 mm in diameter. The adaxial view of the nodule showed a slight indent or pit in the middle of the nodule (Fig. 1A). It can be seen from the abaxial view of the nodule that they

were generally situated amongst a network of tertiary veins (Fig. 1B). The characteristic domatia were also viewed under the stereo-microscope (data not shown).

The leaf primordia of *P. lanceolata* had no visible nodules and no stomatal pores or openings could be seen in the epidermal layers using the light microscope (Fig. 4, A1). A gap could be seen between the two leaves that stained blue/purple indicating that this space was filled with a mucilaginous-like substance. At a young age the nodules of *P. lanceolata* were difficult to see with the naked eye due to their size and the fact that the epidermal layer had not yet been raised as can be seen under the light microscope (Fig. 4, A2). No bacterial mass could be seen within the nodule under the light microscope. However, a light purple stain was noticed around the mesophyll cells situated in the middle of the nodule tissue. These cells also appeared to be denser, with no intercellular spaces, compared to the surrounding leaf tissue on the left and right sides of the nodule.

In the mature nodule tissue of *P. lanceolata* a darker purple stain within the intercellular spaces was detected (Fig 4, A3). The intercellular spaces within the nodule and non-nodulated leaf tissue also appeared larger than that found in the young leaf tissue. There was still no bacterial mass evident within the mature nodule tissue of *P. lanceolata.* The old nodule tissue of *P. lanceolata* was similar to that seen in the mature nodule tissue in that no bacterial mass was evident and only a dark purple stain could be detected within the intercellular spaces (Fig. 4, A4). A difference that could be seen in the old leaf tissue of *P. lanceolata* compared to the younger tissues was the large intercellular spaces found in the mesophyll tissue adjacent to the nodule.

The Scanning Electron Microscope (SEM) images obtained from the leaf nodule tissue of *P. lanceolata* were inconclusive. As can be seen from the SEM image (Fig. 5A) no distinct separation between plant cells and the bacterial mass was observed. At a higher magnification, areas of the nodule showed clumps of spherical structures measuring between 0.5 – 2.5 µm (Fig.

5B). Despite not being able to conclusively find the bacterial endophytes within the nodule tissue using the SEM, the bacteria could be found using TEM.

The nodule-forming bacterial endophytes could only be found within small intercellular spaces spread sporadically through the primordial leaf tissue of *P. lanceolata* (Fig. 6A). The intercellular spaces were relatively large compared to that of surrounding leaf tissue but these spaces were however not completely filled with bacteria. The bacteria seemed to be suspended in a mucilage-like substance that may either have been produced by the bacteria or the plant. The plant cells that surround these bacterial 'pockets' did not appear to be affected in that the cell wall thickness was the same as those plant cells not found around the bacteria and the contents of the plant cells resembled that of a healthy, active plant cell.

Within the young nodule tissue of *P. lanceolata* the bacteria-filled intercellular spaces were more numerous than that of the very young nodule tissue (Fig. 6B). The intercellular spaces were filled with bacterial endophytes and in some cases it appeared that the intercellular spaces were starting to join. The chloroplasts within the surrounding plant cells were filled with starch grains indicating healthy activity of the plant cells. The cell walls and shape of the plant cells were still relatively normal.

A similar image to that found in the young nodule tissue was found in the mature nodule tissue of *P. lanceolata* (Fig. 6C). The intercellular spaces were filled with bacterial endophytes and these bacteria filled 'pockets' appeared to be coalescing. The plant cell contents in the mature nodule still indicated a healthy state.

The intercellular spaces in the old nodule tissue where bacteria were still present appeared to be more filled with the mucilage-like substance than bacterial cells (Fig. 6D). These intercellular spaces occurred less frequently than in the mature nodule tissue. The plant cells were no longer filled with starch granules and did not seem to be photosynthetically active.

3.4.2) *P. edentula*

The stereo-microscope images of *Pavetta edentula* showed rod-like shaped nodules which vary from 2.5 – 5 mm in length and 0.5 – 1.5 mm in width and were raised on both the adaxial and abaxial surfaces (Fig. 2). The adaxial surfaces of some of the nodules also showed a slight indent in the middle (Fig. 2A). The abaxial view of the nodule showed a tertiary vein running through the nodule (Fig. 2B).

The very young leaf tissue of *P. edentula* consisted of a single leaf and under the light microscope a small very light purple mass on the left leaf blade indicated the presence of a relatively small leaf nodule starting to develop (Fig. 4, B1). Once again a mucilaginous substance could be seen outside of the leaf tissue as a blue/purple stain. For the young nodule tissue of *P. edentula* no bacterial mass could be seen in the centre of the nodule however, a darker purple/ blue stain was present in the central region of the nodule (Fig. 4, B2).

Under the light microscope the bacterial endophytes in the mature nodule tissue of *P. edentula* could be seen as a dark purple mass in the centre of the nodule (Fig 4, B3). The intercellular spaces in *P. edentula* also appeared to be larger than that found in the young nodule tissue. The bacterial mass within the old nodules of *P. edentula* could clearly be seen by the purple stained area in the centre of the nodule (Fig. 4, B4).

Under the SEM the location of the bacterial endophytes within the leaf nodule tissue of *P. edentula* could be seen as a dense mass surrounded by plant mesophyll cells (Fig. 7A). At a higher magnification the mass of rod shaped bacteria could be seen tightly packed in an abundant amount of mucilage-like substance (Fig. 7B). The plant cells also appeared to be distorted in shape at a higher magnification.

Under the TEM the very young leaf tissue of *P. edentula* (Fig. 8A) was found to be similar to *P. lanceolata* in that the bacteria could only be found spread

sporadically, within intercellular spaces, throughout the leaf tissue. The bacteria once again appeared to be suspended in a mucilage-like substance. The amount of bacteria within the intercellular spaces however, was much greater than that found in *P. lanceolata*. The shape of the bacteria within the intercellular spaces varied from being rod-shaped in some places to pleomorphic in other areas. The plant cells situated around the bacteria appeared to still be active and were not distorted nor were the cell walls any thicker than those cells in the non-nodulated leaf tissue.

Within the young nodule tissue of *P. edentula* there seemed to be an increase in bacterial numbers and the intercellular spaces appeared to have coalesced to form larger areas for the bacterial endophytes (Fig. 8B). There also seemed to be an increase in mucilage in between individual bacterial cells. As with the SEM image it appeared that the plant cells within the bacterial mass were distorted. A further discovery under the TEM was the thickening of the plant cell walls. The chloroplasts within the plant cells contained starch granules indicating normal photosynthetic activity and the presence of other plant cell organelles implied that these cells were functional.

The characteristics seen in the young nodule tissue were also seen in the mature nodule tissue of *P. edentula*. There were still a high number of bacteria seen within the mature nodule tissue, the plant cell shape was abnormal and the plant cell walls were thickened (Fig. 8C). Two differences that were noted in the mature nodule tissue were that the plant cell contents indicated a drop in activity and despite the bacterial numbers being high they appeared to be less than that seen in the young nodule.

The old nodule tissue of *P. edentula* is representative of all of the differences that were found within the younger leaf nodule tissues (Fig. 8D). The plant cell walls were unusually thick and the bacteria found within the intercellular spaces appeared greatly varied in shape. The plant cells showed signs of photosynthetic activity but much less so than that of the younger nodules. The ratio of bacteria to mucilage was also different but it is not clear as to

whether this was due to more mucilage or fewer bacteria. Regions of the intercellular spaces could be seen that did not contain bacterial endophytes but rather lighter areas of mucilage.

3.4.3) *P. schumanniana*

The stereo microscope images showed that nodules on the leaves of *P. schumanniana* were similar in shape and size to that of *P. lanceolata* in that they were generally round and commonly 0.5 mm in diameter (Fig. 3). The *P. schumanniana* leaf nodules were raised on both the adaxial and abaxial surfaces of the leaf. On the abaxial surface of the leaves, the characteristic trichomes could clearly be seen (Fig. 3B). As with the other *Pavetta* spp. in this study the nodules were situated close to tertiary veins which were prominent on the underside of the leaf.

Under the light microscope the leaf primordia of *P. schumanniana* was different in morphology to that of *P. lanceolata* and *P. edentula* (Fig. 4, C1). There was no mucilaginous substance surrounding the very young leaf tissue of *P. schumanniana* but the development of the trichomes was clear. The young nodule tissue of *P. schumanniana* was different to that of *P. lanceolata* and *P. edentula* under the light microscope in that a large bacterial mass could immediately be identified in the middle of the nodule area (Fig. 4, C2).

In the mature nodule tissue of *P. schumanniana* more mesophyll cells were evident within the purple mass in the centre of the nodule and the characteristic elongated parenchyma cells could be seen surrounding the potential bacterial endophytes (Fig. 4, C3). Once again the possible bacterial mass within the old nodule of *P. schumanniana* could clearly be seen as a large purple stained area in the centre of the nodule (Fig. 4, C4).

As with *P. edentula* the area within the leaf tissue that the bacterial endophytes of *P. schumanniana* were situated could clearly be seen under the SEM (Fig. 9A). At a higher magnification (Fig. 9B) the rod-shaped

bacteria were non-flagellated and appeared to be sitting within a mucilage-like substance. High numbers of bacteria could also be seen indicating that these were most likely the nodule-forming bacterial endophytes.

Under the TEM, the very young leaf tissue of *P. schumanniana* (Fig. 10A) was a mix of what was found for *P. lanceolata* and *P. edentula*. The bacteria were found in the intercellular spaces and these spaces were few within the leaf tissue. In some cases these bacteria filled 'pockets' were small like that found in *P. lanceolata* but in other areas the 'pockets' were as large as those observed in *P. edentula*. The bacterial endophytes were all similar in shape and the mucilage-like substance was evident around the individual bacterial cells. The plant cells surrounding the bacterial endophytes were not distorted in shape and seen to be healthy and with no clear cell wall thickening.

Under the TEM the young nodule tissue of *P. schumanniana* (Fig. 10B) showed a high number of bacteria no longer situated within small intercellular spaces but rather in large masses. The mesophyll cells situated amongst the bacterial mass were distorted in shape but the cell wall thickening was not as noticeable as that seen in *P. edentula*.

The characteristics that were found in the mature nodule tissue of *P. schumanniana* were similar to that found in the young nodule tissue except for the cell wall thickening being more obvious in certain areas (Fig. 10C). Photosynthetic activity was still indicated in the plant cells by the presence of starch granules in the chloroplasts.

From the TEM images obtained from the old nodule tissue of *P. schumanniana* (Fig. 10D) the bacterial endophytes did not appear to be dying or being covered in mucilage-like substance as was the case with *P. lanceolata* and *P. edentula*. The plant cells seemed to be less active than those found in the mature nodule and the cell wall thickness was not as striking as that in the old nodule tissue of *P. edentula*.

3.5) Discussion

Research carried out on leaf nodule development has been focused on that found in *Psychotria* spp. (Lersten and Horner, 1967; Whitmoyer and Horner, 1970; Miller *et al*., 1983). This research has been useful in identifying some of the characteristics associated with nodule development in *Pavetta* spp. There were, however, many differences found not only between *P. bacteriophila* and the three *Pavetta* spp. but between the three *Pavetta* spp. as well.

In this study, the stereo-microscope images of the leaf nodules of three different *Pavetta* spp. showed an indent on the surface of the nodule (data not shown). Herman *et al.* (1986) noted the presence of a tube above a leaf nodule of *P. revoluta* which could be seen as a pore on the adaxial surface of the leaf. According to this study, this tube was formed by an indentation in the epidermal layer above the bacterial nodule. Under the light microscope no pores in the epidermal layer such as that described by Herman *et al.* (1986) could be found in any of the *Pavetta* spp. studied, except a slight indent in the upper epidermal layer of the old nodule tissue of *P. edentula*.

The close proximity of the nodules to tertiary veins was also observed for all three *Pavetta* spp. under the stereo-microscope. Herman *et al.* (1986) also noted that the bacterial leaf nodules on *Pavetta* spp. were often found near the main or secondary veins. This may have an implication in the transport of various beneficial substances to and from the bacteria within the nodule.

The primordial leaf tissue from each *Pavetta* spp. in this study varied in morphology. The small region of bacteria found within the very young leaf blade region of *P. edentula* was indicative of how early leaf nodule development begins. Lersten and Horner (1967) found precocious stomatal openings within the epidermal layer of the leaf primordia of *P. bacteriophila* from which the bacterial endophytes supposedly gained entry into the leaf tissue. No such openings could be seen in the *Pavetta* spp. samples under

both the light microscope and the TEM. This absence of stomatal gaps in the epidermal layer of each leaf specimen in this study was different to that found by Lersten and Horner (1967) and may suggest an alternate mode of entry for the nodule-forming bacterial endophytes into the leaf tissue compared to that found in *Psychotria* spp.

The light microscope images of the three *Pavetta* spp. showed that there was a difference between *P. lanceolata* and the other two *Pavetta* spp. in that a large purple mass was never seen within the centre of the nodule of *P. lanceolata* at any age. This outcome was most likely due to the nodule forming bacteria found within *P. lanceolata* being a different species to that found in *P. edentula* and *P. schumanniana* as discussed in Chapter 2. It could also be seen from the light microscope images that leaf nodule development in *P. schumanniana* was the fastest as the bacterial endophytes could already be detected in high numbers at a young age. The absence of a large purple/blue stain in the young nodule tissue of *P. lanceolata* and *P. edentula* compared to that of *P. schumanniana* already indicated that the initiation of nodule development is different for each *Pavetta* spp.

The absence of elongated parenchyma cells and a clear idea of where the bacteria were situated within the nodule tissue of *P. lanceolata* made the identification of the bacteria under the SEM challenging. The circular structures that were found within the leaf tissue could possibly be the nodule-forming bacterial endophytes or merely plant organelles that were moved out of the plant cells during preparation of the nodule for viewing under the SEM. Once again this finding may be explained by the nodule-forming bacterial endophyte in *P. lanceolata* being a different species (Chapter 2).

The TEM images of the very young leaf tissue of *P. lanceolata* showed that the intercellular spaces were not completely filled with bacteria. Miller *et al*. (1983) suggests that nodule development includes a process whereby the middle lamellae of the plant cells are weakened by the action of pectolytic enzymes that are secreted by the bacteria. These enzymes degrade the

middle lamella and eventually the plant cells separate from each other and float into the bacterial mass. The results from very young leaf tissue of *P. lanceolata* suggested otherwise. It may be possible that in *P. lanceolata* it is not the bacteria forcing the plant cells to separate but rather that the plant cells were forming larger spaces into which the bacteria were able to grow.

The TEM images of the young and mature nodule tissue of *P. lanceolata* explained why a large purple/blue mass was never seen in the centre of the nodule under the light microscope. The bacterial endophytes appeared only in expanded intercellular spaces and not in such large quantities like that seen in *P. edentula* and *P. schumanniana*. The shape of the surrounding plant cells never appeared distorted and there was no sign of cell wall thickening. These results again could be due to the bacterial endophyte being a different species (Chapter 2) and hence the reason why the manner of nodule development in *P. lanceolata* may be different.

The old nodules sampled from *P. lanceolata* show a situation similar to that described by Whitmoyer and Horner (1970) in that the bacteria appear to have died. The intercellular spaces were filled with what look like remnants of bacteria i.e. lighter areas in the mucilage where the bacteria were located in younger nodule tissue (data not shown). The observation of more mucilage-like substance than bacteria within the intercellular spaces was in line with what Whitmoyer and Horner (1970) suggested i.e. a decrease in bacterial numbers could appear as a higher mucilage content.

The presence of unusually shaped mesophyll cells amongst bacteria in the nodules of *P. edentula* as seen under the SEM was the same as that found in *P. bacteriophila* by Lersten and Horner (1967). This characteristic may play a role in increasing the surface area of the plant cell so as to interact more efficiently with the bacteria. An alternative reason for this may be that the rapid increase in bacterial numbers applied pressure to the plant cell walls and as the bacteria grow and divide the exerted pressure re-shaped the plant cell walls.

The primordial leaf tissue of *P. edentula* was the only point at which a 'normal' bacterial shape could be seen. The older nodules were only filled with what appeared to be pleomorphic bacteria. This may be an indication that the shape of the bacterial endophytes in *P. edentula* played a role in the relationship shared between plant and bacteria. This role may be the excretion or absorption of beneficial substances that are shared with the plant.

The TEM image obtained from the young tissue of *P. edentula* showed an increase in cell wall thickness of the mesophyll cells that were in contact with the bacteria. This phenomenon was also found in *P. bacteriophila* and the reasoning given was that the thicker mesophyll cell walls may be a carbohydrate source for the bacteria (Lersten and Horner, 1967). Another possibility is that this may be a defence response from the plant cells brought about by the presence of the bacteria.

The decrease in plant cell activity within the mature nodule tissue of *P. edentula* agreed with the findings of Whitmoyer and Horner (1970) in that the benefits received by the plant are most likely when the leaf nodules are young. The absence of plant cell contents may indicate that the bacterial endophytes were not producing beneficial substances anymore and hence the plant cells in the vicinity need not be as active. As with the old nodule tissue of *P. lanceolata* an apparent increase of mucilage and decrease in bacterial numbers was observed within the mature nodule tissue of *P. edentula*. The whiter areas of mucilage found in the old nodule tissue of *P. edentula* strengthened the idea proposed by Whitmoyer and Horner (1970) that the bacteria begin to die off as the nodule ages.

The same result as that found by Lemaire *et al.* (2011) could be seen in *P. schumanniana* under the SEM, namely, rod-like non-flagellated bacteria. This result strengthens the fact that within *P. schumanniana* leaf nodules, rod-shaped non-flagellated bacteria can be found within the leaf nodule tissue. One difference that was noted between *P. schumanniana* and *P. edentula* under the SEM was that there seemed to be less mucilage-like

substance between the bacteria in *P. schumanniana*. This may be a result of the specific interactions that is shared between the bacteria and their plant host.

As indicated by the light microscope slides of the young nodule tissue of *P. schumanniana* the same was seen under the TEM in that the bacterial numbers increased drastically from primordial leaf tissue to young nodule tissue. This sudden increase in bacterial numbers suggests that the nodule forming bacteria are fast growing and that the intercellular spaces changed in order to create space for such large numbers. This result agreed with the theory suggested by Miller *et al*. (1983) specifically that the bacteria play a part in separating the plant cells. Again the distorted mesophyll cells within the bacterial mass raised the question of whether this was a result due to the plant cell increasing its surface area for the bacteria or the bacteria applying pressure to the cell wall as they multiplied.

Within the mature nodule tissue of *P. schumanniana* the plant cell wall thickening was not evident in all of the plant cells situated within the mass of bacteria. This finding disputes previous suggestions as to why this phenomenon occurs. Lersten and Horner (1967) hypothesized that the cell wall thickening was a carbohydrate source for the bacteria. If this was the case then how do the bacteria within the mature nodules of *P. schumanniana* gain nutrients? The idea of it being a defence reaction from the plant cells is also disproved in that all of the plant cells would show the same response.

The outcome of the nodule-forming bacterial endophytes not being fewer in numbers within the old nodule tissue of *P. schumanniana* is surprising. In both *P. edentula* and *P. lanceolata* there appeared to be less bacteria within the older nodules. This was similar to that found in the study by Whitmoyer and Horner (1970) in *P. bacteriophila*. This result may be explained by the fact that *P. schumanniana* is deciduous. The bacterial endophytes may continue to provide beneficial traits until the leaves drop from the tree or they are still preparing to move out of the leaf tissue whilst the leaf is still attached to the tree. Once again this is an indication that each *Pavetta* spp. has a

different method of nodule formation and that the relationship shared with the bacterial endophytes is unique.

Overall bacterial nodule formation in the three *Pavetta* spp. has both similarities and differences. The similarities include an increase in bacterial numbers as the nodules grow and healthy active plant cells during nodule development. Some of the differences that could be identified were plant cell wall thickening being most prevalent in *P. edentula* and the absence of plant cell distortion in *P. lanceolata*. This study shows that leaf nodule development in the three *Pavetta* spp. does not follow a set of rules but is rather a process that is dictated by the relationship shared between plant and bacteria.

3.6) Conclusions

- No precocious stomatal openings were found within the three *Pavetta* spp. Therefore a possible alternate mode of entry may be employed by leaf nodule-forming bacterial endophytes in *Pavetta* spp.
- Nodule-forming bacterial endophytes are rod-like and non-flagellated in *P. edentula* and *P. schumanniana*.
- The shape of the bacterial endophytes may play a role in substance transfer between plant and bacteria in the nodules of *P. edentula* i.e. it may enhance the secretion or absorption of beneficial substances.
- The question of whether the plant cells are separated by the bacterial endophytes or if the plant prepares space for bacterial growth is not clear in this study.
- The distorted plant cells may be a result of pressure being applied from growing bacterial numbers or an increase in surface area for increased contact with the bacteria.
- The purpose of cell wall thickening in *Pavetta* spp. is unclear.
- Nodule-forming bacterial endophytes decrease in number as the nodule ages except in *P. schumanniana*.

- Overall nodule morphology in *P. lanceolata* is very different to that seen in *P. edentula* and *P. schumanniana*.
- Leaf nodules in *Pavetta* spp. form by the growth and multiplication of bacterial endophytes which in turn push against the upper and lower leaf epidermis.
- Leaf nodule development and morphology is specific to the species of bacterial endophyte and the species of host plant involved.

3.7) References

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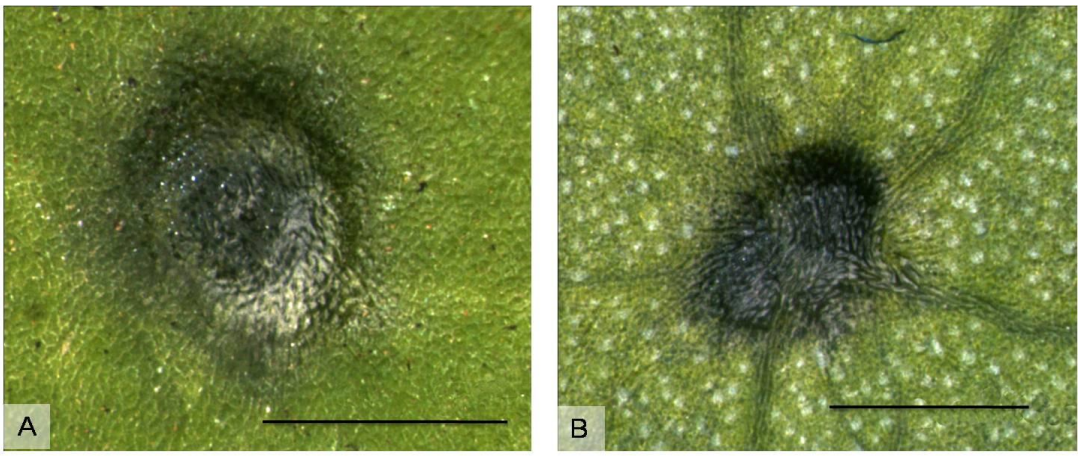

Figure 1: Stereo-microscope images of nodules on leaf surface of *P. lanceolata*. A – Adaxial surface, B – Abaxial surface. Scale bar = 0.5 mm .

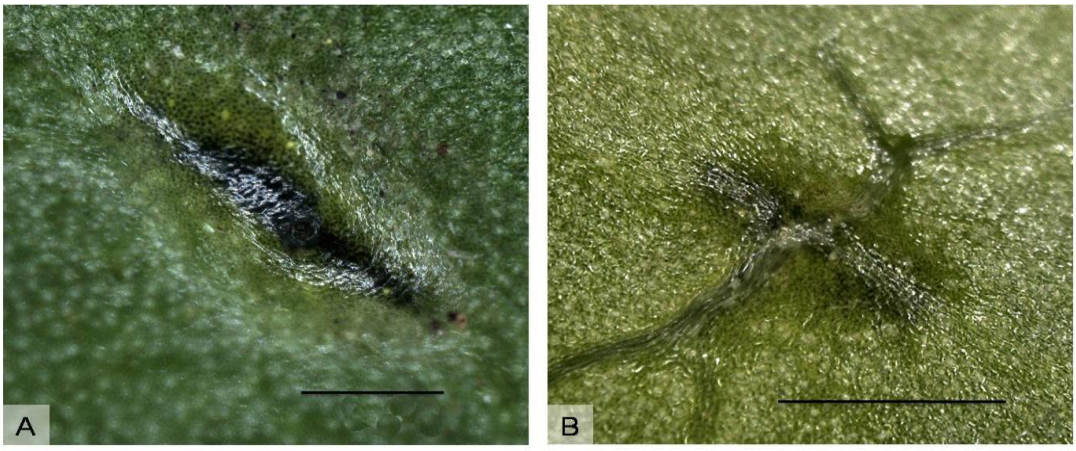

Figure 2: Stereo-microscope images of nodules on leaf surface of *P. edentula*. A- Adaxial surface B- Abaxial surface. Scale bar = 2 mm.

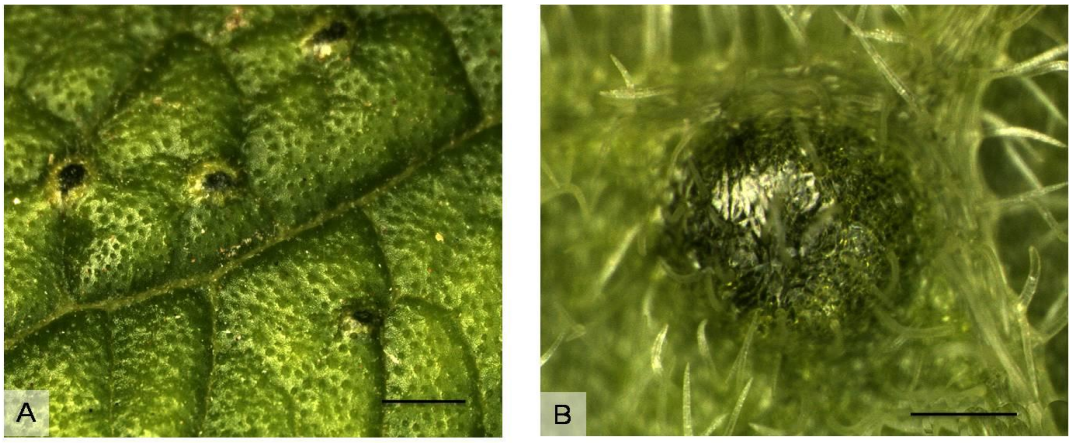

Figure 3: Stereo-microscope images of the leaf surface of *P. schumanniana*. A- Adaxial surface, bar= 1 mm. B- Abaxial surface, bar = 0.2 mm.

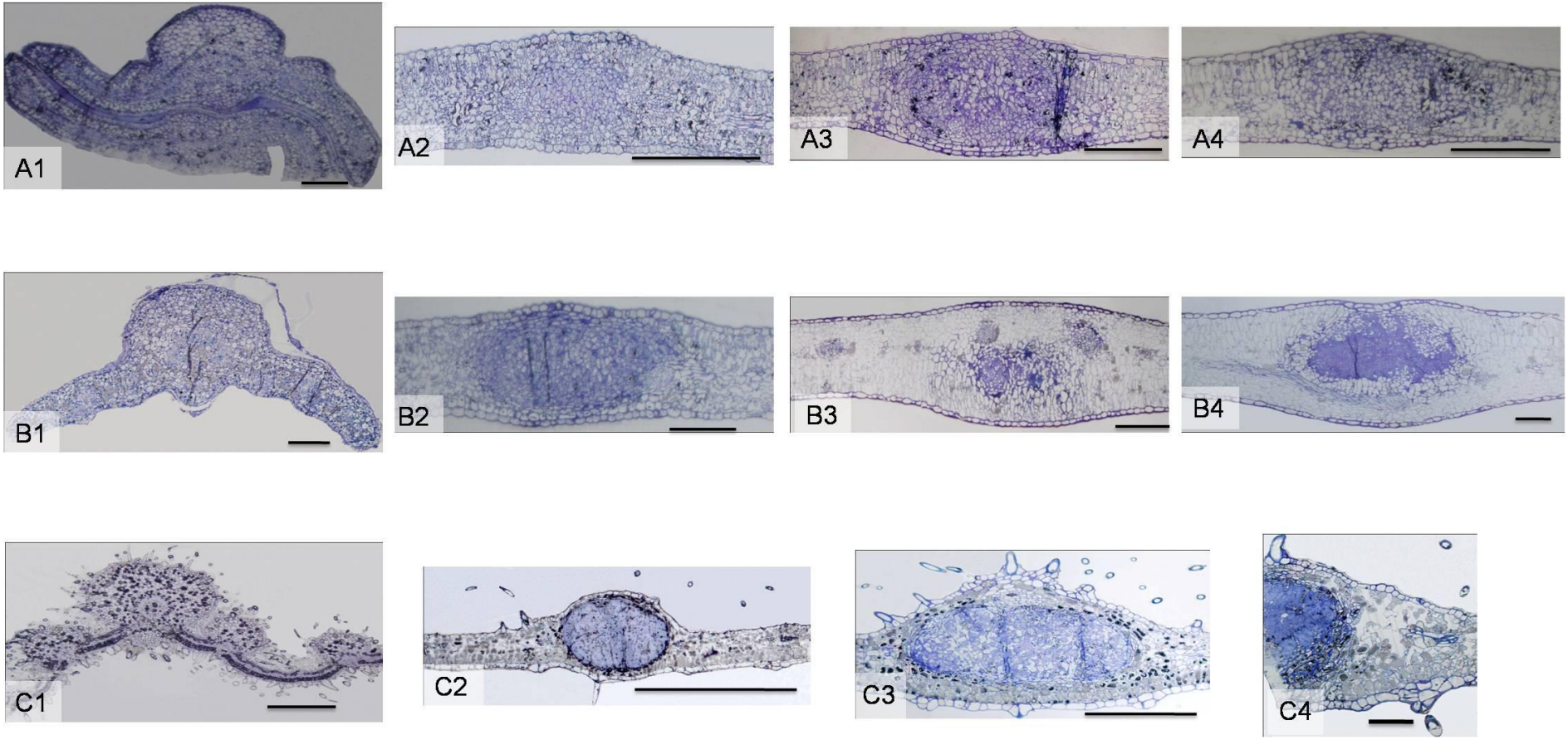

Figure 4: Cross section through leaf nodules of A – *P. lanceolata*, B – *P. edentula* and C – *P. schumanniana* during different leaf ages. 1- Leaf primordia, 2 – Young nodule tissue, 3 – Mature nodule tissue, 4 – Old nodule tissue. Bar = 0.2 mm.

***P. lanceolata*:**

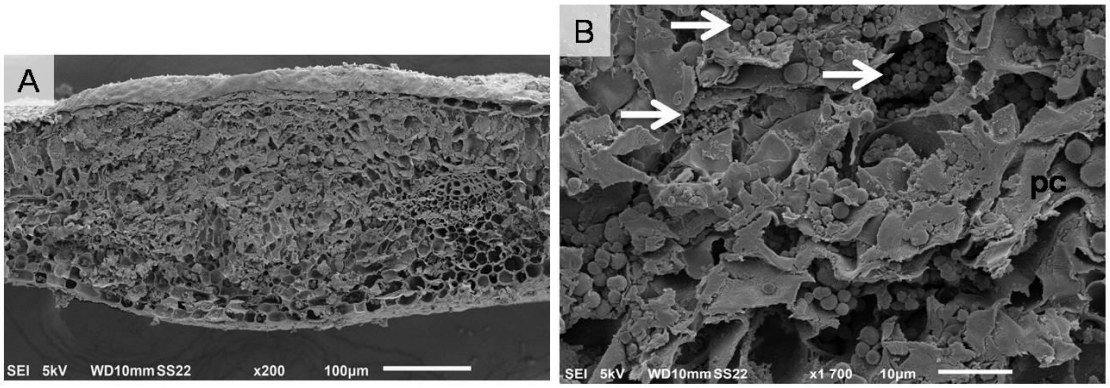

Figure 5: SEM micrograph of *P. lanceolata* nodule tissue. A – Nodule, B – Circular structures within nodule tissue indicated by arrows. pc = plant cell.

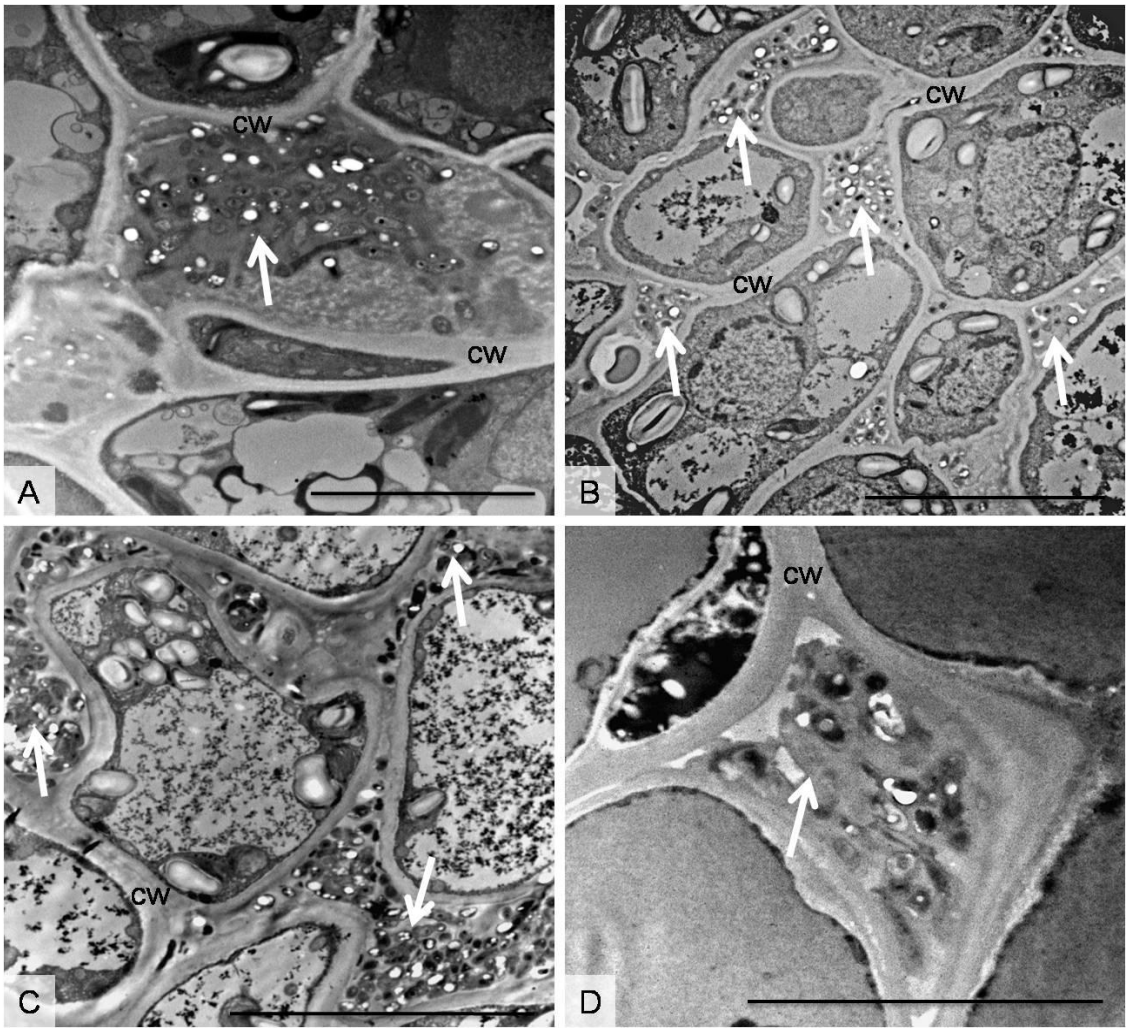

Figure 6: TEM micrographs of *P. lanceolata* nodule tissue. Bacteria indicated by arrows. A- Very young nodule tissue (bar = 5 µm), B – Young nodule tissue (bar = 5 µm), C – Mature nodule tissue (bar = 10 µm), D – Old nodule tissue (bar = 10 µm). cw = cell wall.

P. edentula:

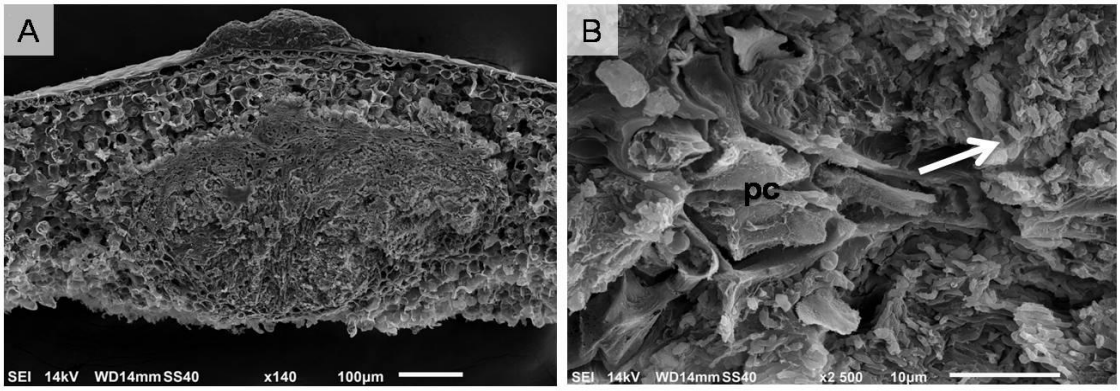

Figure 7: SEM micrographs of *P. edentula* nodule tissue. A – Nodule, B – Rod-shaped bacteria within nodule indicated by arrow. pc = plant cell.

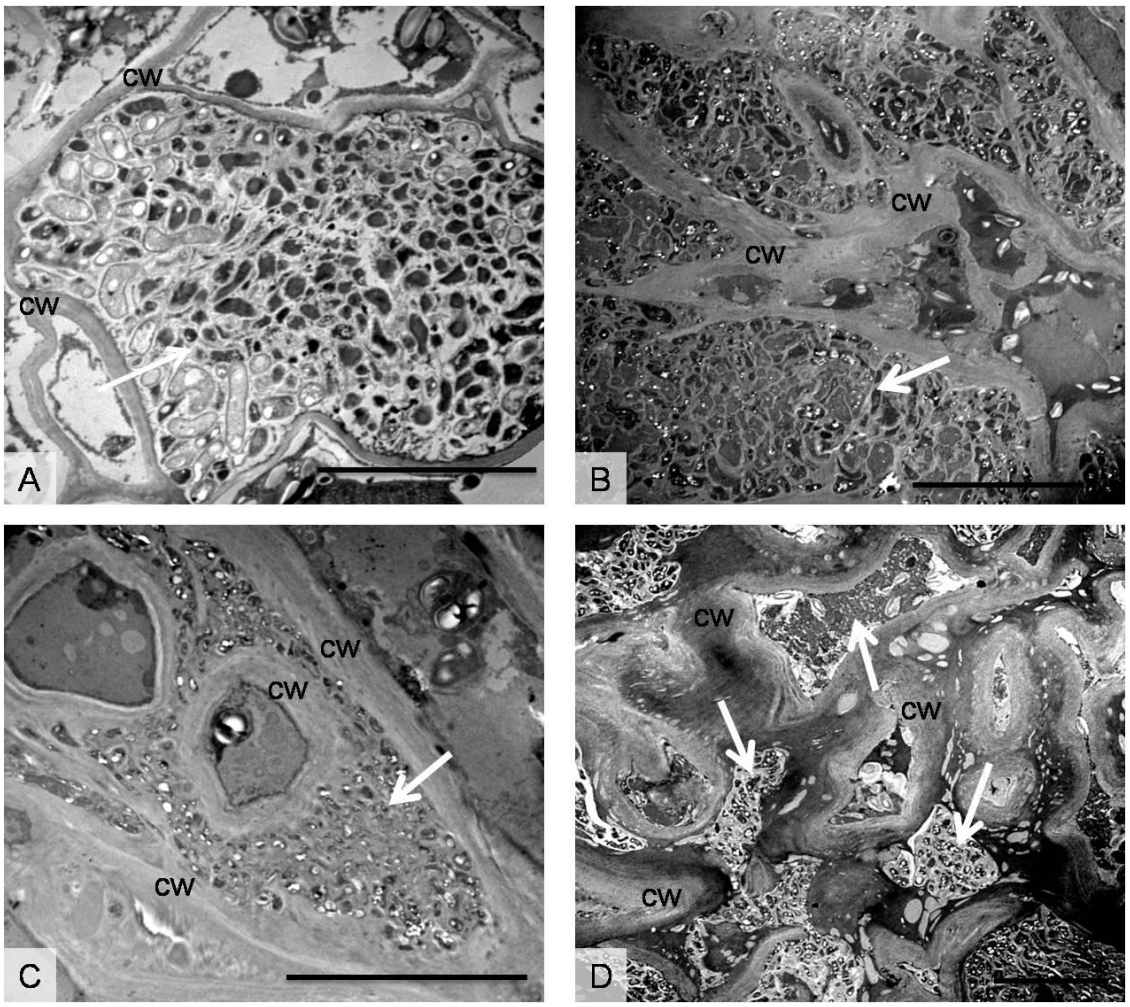

Figure 8: TEM micrographs of *P. edentula* nodule tissue. Bacteria indicated by arrows. A – Very young nodule tissue (bar = 5 µm), B – Young nodule tissue (bar = 10 µm), C – Mature nodule tissue (bar = 10 µm), D – Old nodule tissue (bar = 10 µm). cw= cell wall.

P. schumanniana:

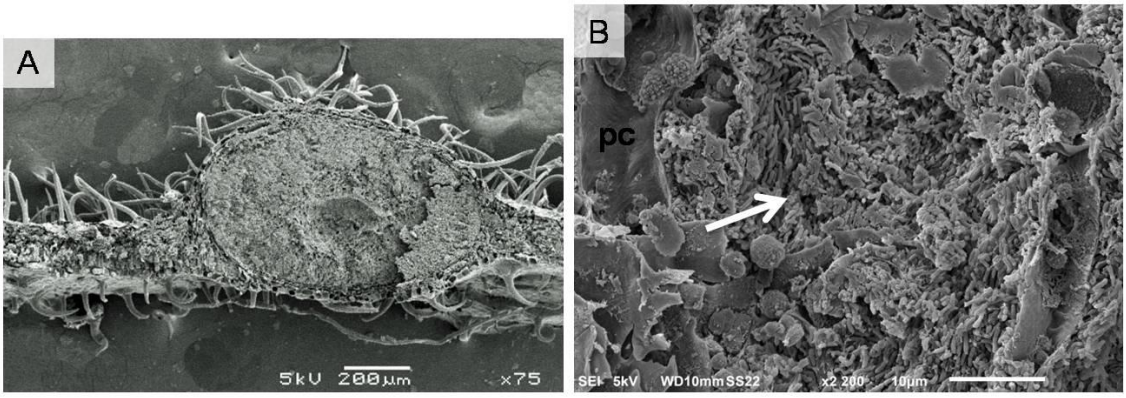

Figure 9: SEM micrographs of *P. schumanniana* nodule tissue. A – Nodule, B – Rod-shaped bacteria within nodule tissue indicated by arrows. pc = plant cell.

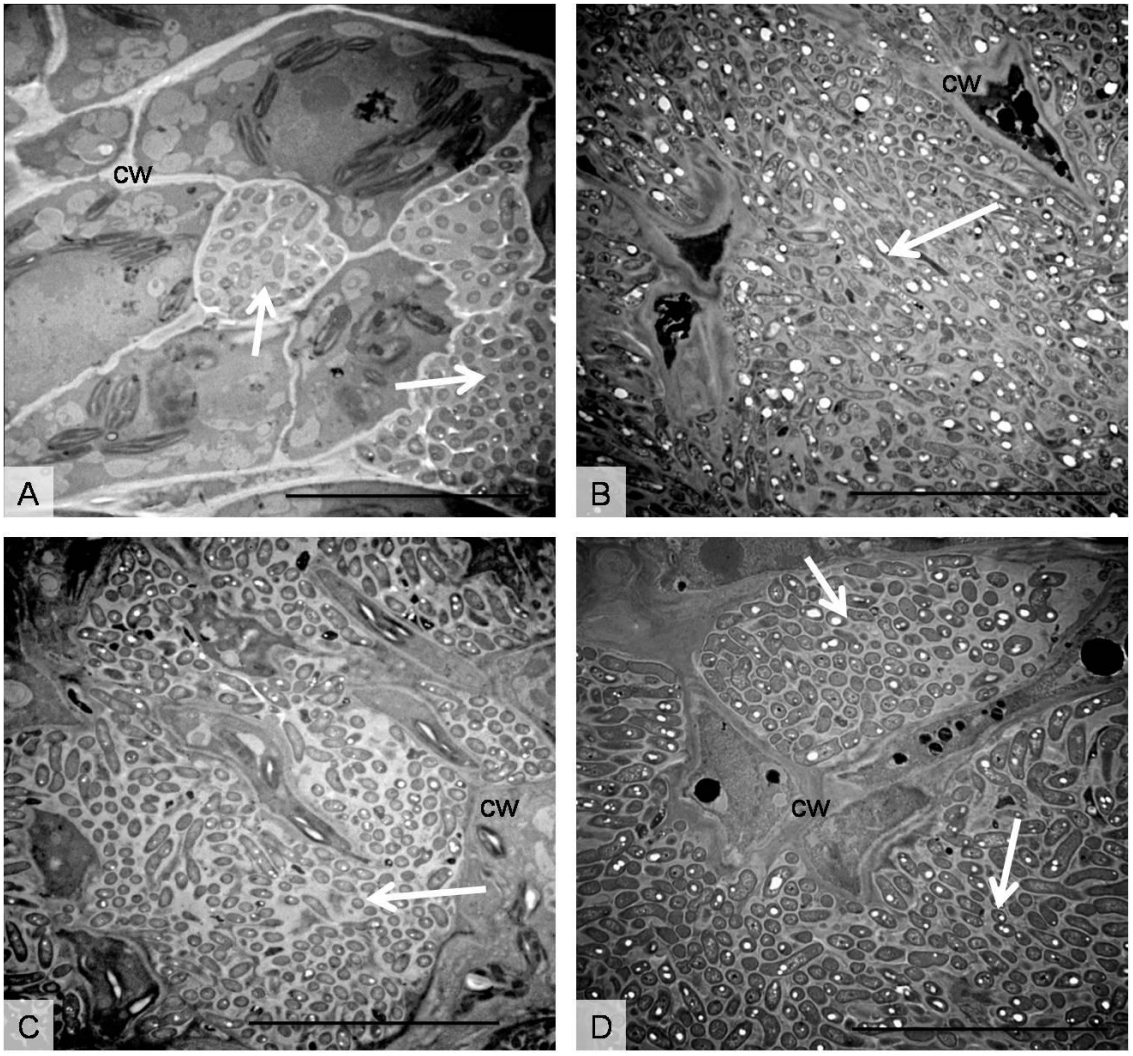

Figure 10: TEM micrographs of *P. schumanniana* nodule tissue. Bacteria indicated by arrows. A – Very young nodule tissue, B – Young nodule tissue, C – Mature nodule tissue, D – Old nodule tissue. Bar = 10 µm. cw = cell wall.

Chapter 4

Detection of leaf nodule bacterial endophytes in different tissue types of *Pavetta schumanniana and P. edentula*

Detection of leaf nodule bacterial endophytes in different tissue types of *Pavetta schumanniana and P. edentula*

4.1) Abstract

Pavetta is a plant genus in which various species harbour a symbiotic relationship with bacteria that form leaf nodules. The identity of these bacteria and how they initiate and establish these nodules has been of considerable interest to scientists over the years. The movement of these bacterial endophytes within the leaf and other areas of the plant are not well understood. The purpose of this study was initially to confirm the presence and location of the leaf nodule-forming bacterial endophytes within the deciduous *P. schumanniana*. A further objective was to detect these bacterial endophytes within different plant tissue types in both *P. schumanniana* and *P. edentula*. Previous studies had shown that the leaf nodules of *P. schumanniana* were colonised by a *Burkholderia* sp. and the same was shown for *P. edentula* in Chapter 2. *Burkholderia* specific primers were used to detect this bacterium within different aged leaf buds and stems of *P. schumanniana* in winter and the leaf buds and stems of *P. edentula*. Different aged leaf buds, stem, seed and flower samples collected from *P. schumanniana* in summer were also tested for the presence of a *Burkholderia* sp. A *Burkholderia* sp. was detected within the older leaf buds of *P. schumanniana* in both winter and summer as well as in the flowers. *Burkholderia* spp. could not be detected within the small leaf buds or seed of *P. schumanniana*, the leaf buds of *P. edentula* or any of the stem samples from both *Pavetta* spp. These results could be due to the limitation of the PCR method in that it may not have detected bacteria that were present at a low concentration. The presence of a *Burkholderia* sp. in the older leaf buds of *P. schumanniana* in winter indicate that the bacteria are maintained within these buds until conditions are optimal for the sprouting of new leaves. The different results obtained for the two *Pavetta* spp. suggests that the process of leaf nodule initiation may not be as simple as previously thought.

4.2) Introduction

Leaf nodules formed by bacterial endophytes have been of considerable interest to scientists over the years (Zimmerman, 1902; von Faber, 1912, Lersten and Horner, 1967; Miller, 1990; Van Oevelen *et al*., 2001; Lemaire *et al*., 2011a, b). Plant genera containing species exhibiting bacteria filled nodules on their leaves include *Psychotria* spp., *Pavetta* spp. and *Sericanthe* spp. (van Wyk *et al*., 1990; Van Oevelen *et al*., 2001). The genus *Pavetta* has approximately 353 leaf nodulated species (Miller, 1990) some of which are considered edible (Fox and Young, 1982) while others are poisonous to domestic ruminants (van Wyk *et al*., 1990; Coates Palgrave, 2002).

Pavetta schumanniana causes a disease known as 'gousiekte' (van Wyk *et al*., 1990; Coates Palgrave, 2002). Directly translated 'gousiekte' means "quick disease" which is an accurate description of the symptoms. A cow or sheep will drop dead from heart failure 28 to 56 days after eating the leaves of this plant without showing any other symptoms (van Wyk *et al*., 1990). Another interesting characteristic of *P. schumanniana* is that it is deciduous which leads to the question of how the leaf nodule–forming bacterial endophytes survive during winter.

The symbiotic relationship that is shared between a host plant and its leaf nodulating bacteria is considered to be a cyclic one (Miller, 1990). This in turn means that the nodule-forming bacterial endophytes are acquired through the process of inheritance (Miller, 1990). Von Faber (1912) attempted to study the movement of the nodule bacteria from parent to offspring in *Pavetta zimmermanniana*. In this study he was able to observe bacteria in various stages of flower development as well as in the seed between the embryo and endosperm. Von Faber (1912), despite all efforts, was never able to detect the bacterial endophytes in all of the reproductive stages of the host plant and thus was never able to prove that they were vertically transmitted. It was also noted by von Faber (1912) that the bacterial numbers within the flowers were very low. Even with the use of electron

microscopy, detection of the nodule-forming bacterial endophytes within the reproductive stages of a Rubiaceous host was reported to be difficult (Miller, 1990). Rosenblueth and Martinez-Romero (2006) mentioned that bacterial endophyte density is reliant on many factors such as the species of bacteria involved, the genotype of the host plant, the amount of initial inoculum, the environmental conditions and the developmental stage of the host plant.

Lersten and Horner (1967) used electron microscopy to study the development of bacterial leaf nodules in *Psychotria bacteriophila*. This study found that during leaf development the nodule forming bacteria could be found floating in an extracellular mucilage that surrounded the leaf primordia enclosed by stipules. The nodule bacteria were said to be maintained within a protein / carbohydrate-like mucilage within the enclosed leaf stipules and it is believed that it is at this stage that the bacteria 'inoculate' the new leaves (Lersten and Horner, 1967; Miller, 1990). The nodule forming bacteria are said to enter the leaf tissue through prematurely developed stomatal openings within the young leaves and hence form the leaf nodules within the leaf lamella (Lersten and Horner, 1967). As is the case with the leaf primordia, it is believed that the bacteria are maintained within a carbohydrate/protein mucilage within the flower buds during flower development (Miller, 1990).

The identity of the leaf nodule-forming bacterial endophyte has been an ongoing investigation with many theories (Horner and Lersten, 1972). Van Oevelen *et al*. (2002) were the first to identify the bacteria responsible for leaf nodule formation in *Psychotria kirkii* using 16S rRNA gene cloning and sequencing. The bacterium was found to belong to the genus *Burkholderia* and has since been classified as '*Candidatus* Burkholderia kirkii' sp. nov. (Van Oevelen *et al*., 2002). A further two *Candidatus* Burkholderia spp. have been identified from the nodule tissue of *Psychotria calva* and *Psychotria nigropunctata* (Van Oevelen *et al*., 2004). In 2011 (b), Lemaire *et al*. identified the nodule forming bacteria in *P. schumanniana* as belonging to the genus *Burkholderia* using the same technique as that adopted by Van Oevelen *et al*. (2002). This result has been confirmed by the author using

Denaturing Gradient Gel Electrophoresis (DGGE) in Chapter 2 and has also been shown to be the case in *P. edentula.*

The aim of this study is to determine the presence of *Burkholderia* spp. in leaf bud and stem samples collected from *P. schumanniana* in summer and winter as well as that from *P. edentula*. The seeds and flowers of *P. schumanniana* will also be tested for the presence of these bacteria.

4.3) Materials and Methods

4.3.1) Sample preparation

Stem, young leaf bud (small) and older leaf bud (large) samples of *P. schumanniana* were collected in winter from a farm near Hoedspruit, Northern Province. The trees were bare and only stem and leaf buds were collected. Seeds, young leaf buds and older leaf buds of *P. schumanniana* were collected in summer from a tree growing in the Onderstepoort toxicology gardens, Gauteng. Flower samples were collected in summer from a *P. schumanniana* tree growing in the Nelspruit National Botanical Gardens, Mpumalanga. Samples of *P. edentula* were collected in summer from an open plot located on a hill in Barberton. For this chapter only the leaf buds and stems of *P. edentula* were of interest as the tree is evergreen. All samples collected were placed into plastic bags and were processed within 24 hours of collection.

Surface sterilisation of the stem, bud, seed and flower samples was done according to the protocol used by Garbeva *et al*. (2001) except that the final rinse steps were done in double distilled water. The last double distilled water solution for each of the samples was used to test if the surface sterilisation of the sample was successful by plating 100 µl of the solution onto Tryptone Soya Agar (TSA) plates. The TSA plates were incubated at 28 ˚C and examined after 24 and 48 hours for bacterial growth (Hallmann *et al*., 1997). The stem, young leaf buds, older leaf buds, seed and flower samples from *P. schumanniana* as well as the stem and leaf bud samples from *P.*

edentula were aseptically cut into smaller pieces (1 mm^2) using a sterile scalpel before being used for inoculation of the broth solutions.

4.3.2) Inoculation of enrichment media

Three types of incubation solutions were used in this experiment, namely, Congo Red Broth (CRB), Tryptone Soya Broth (TSB) and Sodium phosphate (NAP) buffer. The CRB was prepared as follows; Mannitol, 10 g, K_2HPO_4, 0.5 g, $MgSO_4 \bullet 7H_2O$, 0.2 g, NaCl, 0.1 g, Yeast extract, 0.5 g, distilled water to 1 litre and 25 µg ml^{-1} filter sterilized Congo red dye (Somasegaran and Hoben, 1994). The TSB was prepared according to the Manufacturer's instructions. All solutions contained 100 µg ml^{-1} cycloheximide (Garbeva *et al*., 2001).

Separate sets of 10 ml of each of the above three solutions were inoculated separately with either 3 cm stem, 2 leaf buds, 2 seeds or clumps of 20 - 25 flowers. The solutions were then incubated at 28 ˚C with shaking for up to 72 hours (Garbeva *et al*., 2001). Aliquots of 1 ml were taken from each sample every 24 hours. These aliquots were pelleted and kept at –20 ˚C until being processed further.

4.3.3) Evaluation of *Burkholderia* specific primers

Perin *et al*. (2006) developed a set of primers to detect *Burkholderia* spp. form the roots of maize and sugarcane. The development of the primers was accomplished by aligning *Ralstonia* spp. and *Burkholderia* spp. 16S rRNA gene regions that were available from the National Centre for Biotechnology Information (NCBI) database. This information was then used to determine areas of the gene that were specific to *Burkholderia* spp. At 16S rRNA gene regions corresponding to positions 85 to 104 in *B. unamae* the 20-mer forward primer GB-F (5' AGT AAT ACA TCG GAA CRT GT 3') was designed. At positions 1091 to 1110 corresponding to the 16S rRNA gene region of *B. unamae* the 19-mer reverse primer GBN2-R (5' GCT CTT GCG TAG CAA CTA G 3') was designed (Perin *et al*., 2006).

Cultures of authentic *Burkholderia* spp. were obtained from the Department of Microbiology and Plant Pathology, University of Pretoria and DNA was extracted using the Genomic DNA™ extraction kit (Zymo Research) according to the manufacturer's instructions. To confirm isolation of DNA the samples were run on a 1% agarose gel. To evaluate the primers GB-F (5' AGT AAT ACA TCG GAA CRT GT 3') and GBN2-R (5' GCT CTT GCG TAG CAA CTA G 3') (Perin *et al*., 2006) (Inqaba biotechnologies) the authentic *Burkholderia* spp. were used as positive controls and a *Bradyrhizobium* sp. culture previously isolated from leaf nodules was used as a negative control. A mixed DNA sample known to contain *Candidatus* Burkholderia schumannianae DNA was also included. The contents of each PCR reaction and the cycling conditions used were the same as described by Perin *et al*. (2006). A negative water control was included. The PCR products were run on a 1 % agarose gel.

4.3.4) *Burkholderia* specific primers used on Pavetta samples

DNA extraction was carried out on the pellets selected from each sample type using the Genomic DNA™ extraction kit (Zymo Research) according to the manufacturer's instructions. After DNA extraction each sample was run on a 1 % agarose gel to ensure that DNA was present. Once DNA extraction had been confirmed the samples were used for a PCR using the *Burkholderia* specific primers GB-F and GBN2-R (Perin *et al*., 2006) (Inqaba biotechnologies). The controls as previously described were included in each PCR run. The resulting PCR products were run on a 1 % agarose gel. All 1 % agarose gels were run by first mixing 5 µl of sample with 1 µl gel red loading dye (Biotium) before loading into the gel. A 1 kb DNA marker (Fermentas) was included in every gel. The gels were run at 80 V for 30 minutes in 1 x TAE buffer before being viewed under UV light.

4.3.5) Sequencing

PCR samples that showed a band of approximately 1, 025 bp in size on the 1 % agarose gels were cleaned. The cleaning procedure was carried out

using 2 U FastAP Thermosensitive Alkaline Phosphatase (Fermentas), 10 U Exonuclease I (Fermentas) and 20 µl of PCR product. The samples were mixed and incubated at 37 ˚C for 15 minutes after which they were incubated at 85 ˚C for 15 minutes so as to stop the reaction. The cleaned PCR products were then used as a template in the sequencing PCR reaction.

Those samples that were positive for the presence of *Burkholderia* spp. were sequenced using the forward primer GB-F (5' AGT AAT ACA TCG GAA CRT GT 3') (Perin *et al*., 2006) (Inquaba biotechnologies). Each 12 µl sequencing reaction contained 0.5 µl Big Dye Terminator v3.1 reaction premix (Applied Biosystems), 2.5 µl Big Dye sequencing buffer (Applied Biosystems), 3 pmol primer GB-F, 4.7 µl nuclease free water (Qiagen) and 4 µl cleaned PCR product. Sequencing PCR conditions included denaturation at 96 ˚C for 5 seconds, 25 cycles of denaturation at 96 ˚C for 10 seconds, primer binding at 55 ˚C for 5 seconds and elongation at 60 ˚C for 4 minutes. The resulting PCR products were sequenced with an ABI Prism DNA Automated Sequencer (Perkin-Elmer). The sequences were viewed and edited using BioEdit Sequence Alignment Editor v 7.0.9.0 (Hall, 1999). The edited sequences were then used for BLAST analysis (Altschul *et al*., 1990) against the National Centre for Biotechnology Information (NCBI) database to identify the most similar 16S rRNA gene sequences.

One sequence from each of the *P. schumanniana* plant tissue samples, including the leaf nodule tissue, was used for phylogenetic analysis. The closest match sequences from the BLAST analysis (Altschul *et al*., 1990) as well as two related type strains from the All – Species Living Tree (Yarza *et al*., 2010) were obtained from the NCBI database. All of the sequences were aligned using MAFFT (Version 6) online alignment tool (Katoh *et al*., 2002) after which they were trimmed with BioEdit Sequence Alignment Editor v 7.0.9.0 (Hall, 1999). The jModelTest software v. 0. 1. 1. (Posada, 2008) was used to select the best fit model and a Maximum Likelihood tree (Felsenstein, 1981) with a 1000 bootstrap replicates was constructed using PhyML 3.0 (Guidon *et al*., 2010).

4.4) Results

4.4.1) Evaluation of *Burkholderia* specific primers

It was evident from the electrophoresis gel (Fig. 1) that DNA was present in the two authentic *Burkholderia* spp. samples as well as in the mixed DNA sample containing *Candidatus* Burkholderia schumannianae DNA. The electrophoresis gel (Fig. 2) indicated that a gene approximately 1000 bp in size was amplified in both *Burkholderia* samples as well as in the mixed DNA sample containing *Candidatus* Burkholderia schumannianae DNA. No amplification was seen in the negative water control or in the *Bradyrhizobium* sp. sample. The BLAST results for both sequences from the authentic *Burkholderia* cultures were confirmed. The sequence from the mixed DNA sample closely matched the sequence of the *Candidatus* Burkholderia schumannianae identified by Lemaire *et al.* (2011b).

4.4.2) *P.schumanniana* winter leaf bud and stem samples

The *Burkholderia* specific primers were able to detect the *Burkholderia* sp. in the positive control and no bands were present in either of the negative controls (Fig. 3). In the older leaf bud samples for each of the enrichment solutions, a *Burkholderia* sp. could be detected. Unfortunately, not all of the older bud samples that showed bands yielded a sequence. Those sequences that were obtained closely matched the 16S rRNA gene sequence of *Candidatus* Burkholderia schumannianae (Lemaire *et al*., 2011b).

4.4.3) *P. schumanniana* summer leaf bud and stem samples

The electrophoresis gel for the young leaf buds, older leaf buds and stem samples collected from *P. schumanniana* in summer (Fig. 4) indicated that a *Burkholderia* sp. could be detected in the old leaf buds of *P. schumanniana* but not in the young leaf buds or stems. As before the PCR controls yielded the expected results. The BLAST results from the old leaf bud samples

closely matched the previously identified *Candidatus* Burkholderia schumannianae (Lemaire *et al*., 2011b).

4.4.4) *P. schumanniana* seed and flower samples

The *Burkholderia* specific primers were able to detect the *Burkholderia* DNA in the positive control but no bands were evident in any of the seed samples as indicated by the electrophoresis gel (Fig. 5). A *Burkholderia* sp. was detected in both CRB flower samples, one TSB flower sample and one NAP buffer flower sample was amplified (Fig. 6). The controls were the same as that recorded in the previous PCR reactions. The BLAST results for the flower samples showing amplification of a *Burkholderia* sp. closely matched to the 16S rRNA gene region of *Candidatus* Burkholderia schumannianae (Lemiare *et al*., 2011b).

4.4.5) *P. edentula* leaf bud and stem samples

DNA was present in all of the *P. edentula* samples. However, no amplification occurred within the *P. edentula* leaf bud and stem samples even though the positive control did show amplification of the *Burkholderia* sp. (Fig. 7). The negative controls were the same as that seen previously with the other samples.

4.4.6) Phylogenetic analysis

The sequences identified from the various tissues of *P. schumanniana* clustered in a well supported group (bootstrap value of 99 %) with the sequence of *Candidatus* Burkholderia schumannianae as can be seen in Figure 8. Due to the relatively short sequences used for this analysis the sequences could not be differentiated from the *Burkholderia* sp. sequence identified from *P. edentula* by Lemaire *et al*. (2011a).

4.5) Discussion

The *Burkholderia* specific primers, GB-F and GBN2-R, were able to amplify the 16S rRNA gene region of authentic *Burkholderia* cultures and the sample of mixed DNA containing *Candidatus* Burkholderia schumannianae DNA. Additionally the primers did not amplify the 16S rRNA gene region of a *Bradyrhizobium* sp. This result indicated that these *Burkholderia* specific primers developed by Perin *et al.* (2006) could be used to detect the nodule-forming bacterial endophyte within the various *P. schumanniana* and *P. edentula* samples.

The *Burkholderia* specific primers were able to detect a *Burkholderia* sp. within the older leaf buds collected from *P. schumanniana* during winter. This outcome indicates that during winter the leaf nodule-forming bacterial endophytes most likely reside within the leaf buds. The feint bands within the large leaf bud samples do, however, also indicate that during winter these bacterial endophytes are not present in high numbers. The absence of a band in the young leaf bud samples may be explained by a low concentration of *Burkholderia* within these leaf buds. The same may apply to the young leaf buds collected from *P. schumanniana* in summer. As von Faber (1912) noted, the bacterial numbers were very low within the flowers he examined, hence this might be the case for the young leaf buds. The absence of a *Burkholderia* sp. within the stem tissue of both summer and winter samples of *P. schumanniana* cannot be as easily explained due to a lack of published research on the stem tissue of nodulated *Pavetta* species. The stem tissue may, therefore, not harbour a *Burkholderia* sp. or the number of bacteria within the stem tissue was too low for their DNA to be detected by the primers.

The results from the leaf bud and stem samples collected from *P. schumanniana* in summer were similar to that found in the winter samples. The only samples showing the presence of a *Burkholderia* sp. were that of the older leaf buds. One noticeable difference, however, was that the bands

were brighter than that found in the winter samples even though the amount of sample used to inoculate each enrichment solution was the same. This result suggests that the number of bacteria was higher in the older leaf buds during summer. Whitmoyer and Horner (1970) found that in the early stages of nodule development in *Psychotria bacteriophila* there were high levels of RNA within the bacteria indicating a very active biological network. This may explain the difference between the summer and winter older leaf bud samples in that the bacteria become active and begin to multiply as leaf development is initiated in summer.

The *Burkholderia* specific primers were not able to detect *Burkholderia* spp. within the seed samples of *P. schumanniana*. This is surprising as bacterial endophytes have been identified within the seeds of various *Pavetta* spp. (von Faber, 1912; Miller, 1990). This result may be due once again to the fact that when the nodule forming bacteria are low in number the *Burkholderia* specific primers may not detect them. One reason for a low concentration of *Burkholderia* within the seeds may be that the seed samples were collected too early in summer. It is possible that if given more time to develop and mature on the tree the bacterial numbers may have been higher within the seed. The seeds of *P. schumanniana* may be found on the trees from February to June (Boon, 2010) and the samples for this experiment were collected in late February. According to Rosenblueth and Martinez-Romero (2006) bacterial endophyte population density may be affected by the environmental conditions as well as the developmental stage of the host plant. This may be the reason for the absence of a *Burkholderia* sp. within the seed samples of *P. schumanniana* in that the seeds were not fully developed and the environmental conditions may not have favoured bacterial growth at the time of sample collection.

A *Burkholderia* sp. was detected within the flower samples collected from *P. schumanniana* in summer. This outcome is not surprising as von Faber (1912) was able to find bacterial endophytes within the flowers of *Pavetta zimmermanniana*. The presence of the nodule-forming bacterial endophytes within the flower samples suggests that the possibility of vertical transmission

of the bacteria is plausible (Miller, 1990). It is surprising, however, that the *Burkholderia* specific primers were able to detect the bacteria within the flowers and not the seeds. An explanation for this outcome other than the time at which the seeds were sampled may be due to the amount of sample used to inoculate each of the enrichment solutions. Only two seeds were used to inoculate each enrichment broth whereas a clump of 20 – 25 flowers was used for inoculation of each enrichment solution. As a result of this the number of nodule-forming bacterial endophytes within each flower solution was most likely higher than that of the seed solutions from the point of inoculation.

Phylogenetic analysis of the 16S rRNA gene sequences obtained from the different tissues of *P. schumanniana* using *Burkholderia* specific primers indicated that these *Burkholderia* spp. were closely related to those identified in *P. schumanniana* and *P. edentula* by Lemiare *et al*. (2011b). The flower and winter leaf bud samples of *P. schumanniana* were collected from trees growing in the northern region of South Africa. The summer large leaf bud and leaf nodule samples were collected from a *P. schumanniana* tree growing in Gauteng. The geographic location of the *P. schumanniana* trees that were sampled may account for the small differences seen between these sequences in the Maximum Likelihood tree.

The absence of a *Burkholderia* sp. within the leaf bud samples of *P. edentula* was surprising in that the leaf buds of this plant species were large in comparison to those found on *P. schumanniana*. This result suggests that the process of nodule formation within the leaf primordia of *P. edentula* may be different to that of *P. schumanniana*. One such possibility is that the nodule-forming bacterial endophytes in *P. edentula* do not reach high numbers until after the leaves have emerged from the stipules. This result can also be explained by the concept of bacterial endophyte density within plants being affected by plant host genotype and inoculum density (Rosenblueth and Martinez-Romero, 2006). The absence of a *Burkholderia* sp. in the stem samples of *P. edentula* once more indicates that the bacteria are either at low concentrations or not present at all within the stem tissue.

4.6) Conclusions

- The *Burkholderia* specific primers GB-F and GBN2-R (Perin *et al*., 2006) are useful in detecting high concentrations of the nodule-forming bacterial endophytes within the host plant.
- During winter the leaf nodule-forming bacterial endophytes most likely reside within the dormant leaf buds that develop on *P. schumanniana*.
- *Burkholderia* sp. can only be detected in the older leaf buds of *P. schumanniana* in summer. This result may be due to the limitations of a PCR based approach indicating that a more specific technique that is able to detect the bacterial endophytes at low concentrations is required.
- The limitation of a PCR based approach in detecting the bacterial endophytes is shown by the seed samples from *P. schumanniana* as it is known that the bacteria are present within the seed of *Pavetta* spp. The time period at which samples are collected is also emphasized with this result.
- The detection of a *Burkholderia* sp. in the flowers of *P. schumanniana* indicates that this bacterium is most likely vertically transmitted.
- The *Burkholderia* sp. identified from certain plant tissues of *P. schumanniana* are closely related to the nodule forming-bacterial endophytes previously described by Lemaire *et al*. (2011a, b).
- The leaf nodule-forming bacterial endophytes within the leaf buds of *P. edentula* may follow a different approach to nodule initiation compared to that of *P. schumanniana*.
- The leaf nodule bacterial endophytes may not be present in the stem tissue of their leaf nodulated plant hosts or may be at very low concentrations within this tissue.

4.7) References

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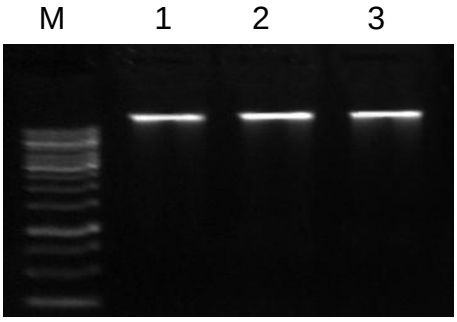

M: DNA marker (1 kb)
Lane 1: *Burkholderia* sp. culture 1
Lane 2: *Burkholderia* sp. culture 2
Lane 3: Mixed DNA sample

Figure 1: DNA extracted from authentic *Burkholderia* spp. cultures (*Burkholderia* sp. culture 1 and *Burkholderia* sp. culture 2) and a mixed DNA sample containing nodule-forming bacteria DNA.

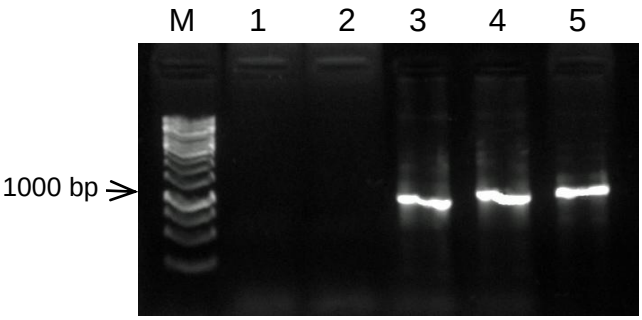

Figure 2: PCR with *Burkholderia* specific primers on authentic *Burkholderia* spp. cultures (*Burkholderia* sp. culture 1 and *Burkholderia* sp. culture 2) and a mixed DNA sample containing nodule-forming bacteria DNA.

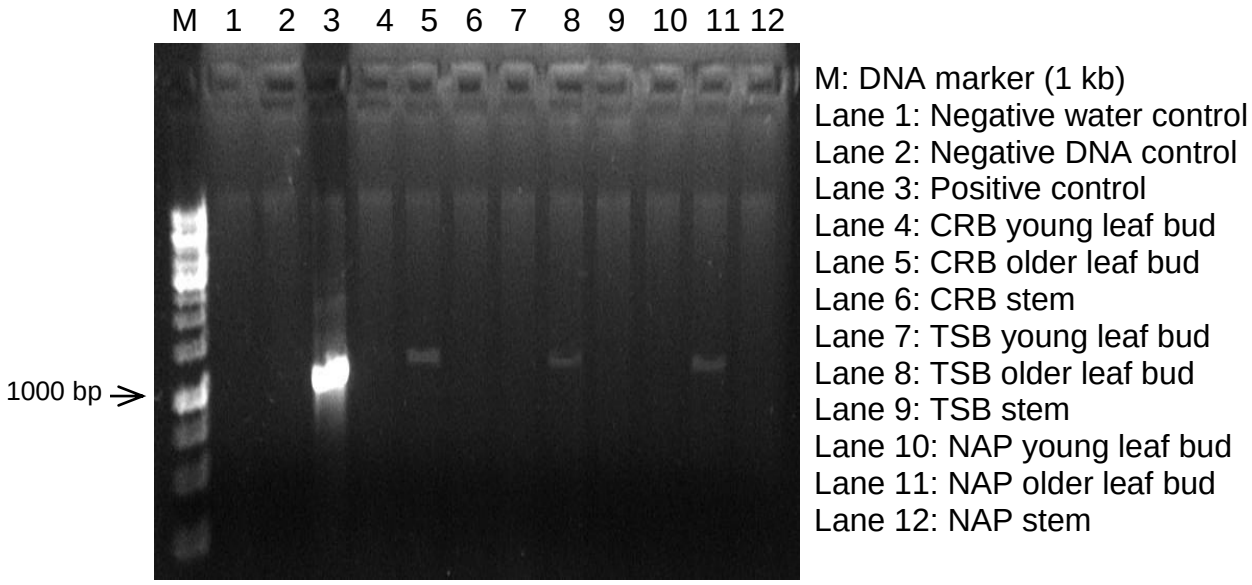

Figure 3: PCR using *Burkholderia* specific primers on samples (young leaf buds, older leaf buds and stem) collected from *P. schumanniana* in winter in the respective enrichment solutions (CRB, TSB and NAP buffer).

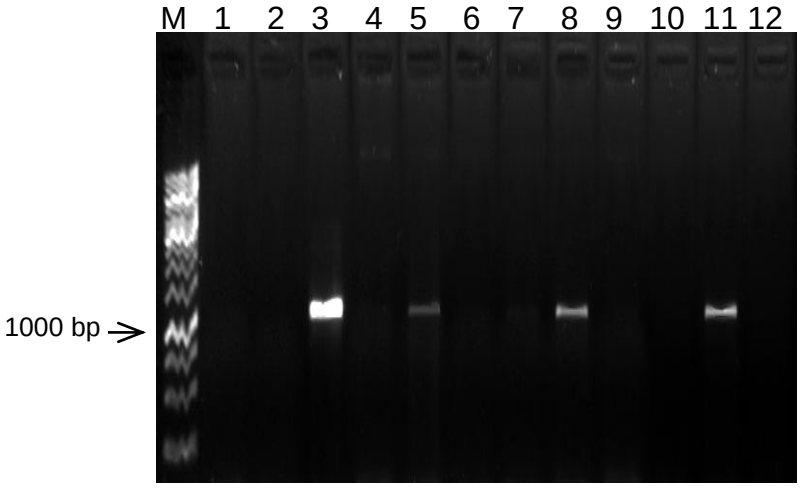

Figure 4: PCR using *Burkholderia* specific primers on samples (young leaf buds, older leaf buds and stem) collected from *P. schumanniana* in summer in the respective enrichment solutions (CRB, TSB and NAP buffer).

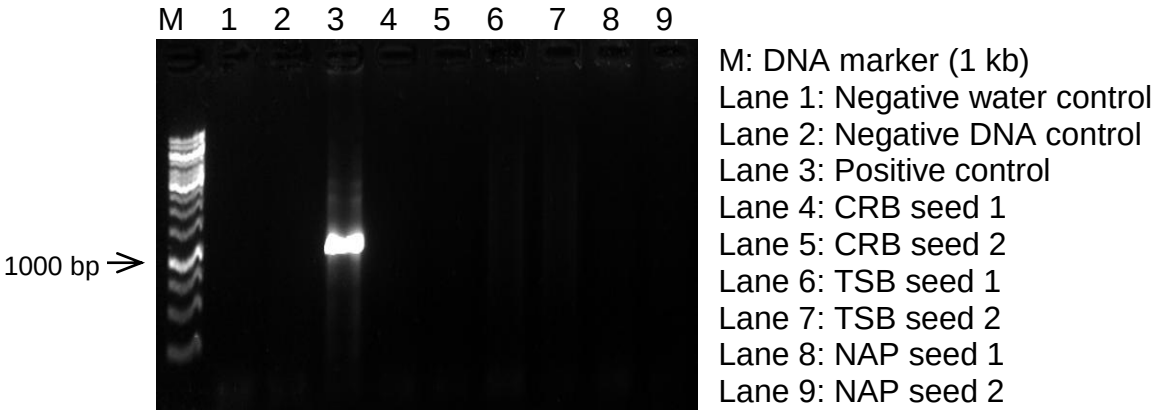

Figure 5: PCR using *Burkholderia* specific primers on seed samples collected from *P. schumanniana* incubated in the respective enrichment solutions (CRB, TSB and NAP buffer).

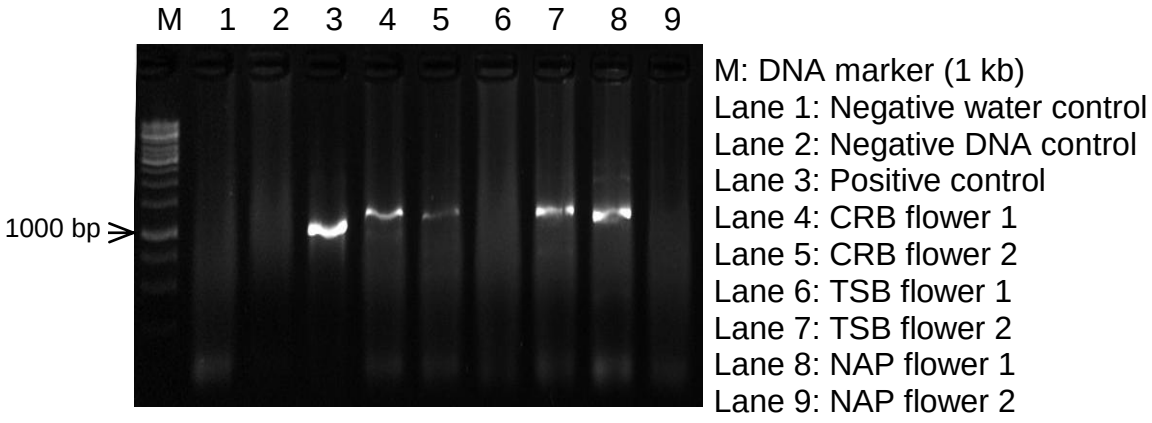

Figure 6: PCR using *Burkholderia* specific primers on flower samples collected from *P. schumanniana* in summer incubated in the respective enrichment solutions (CRB, TSB and NAP buffer).

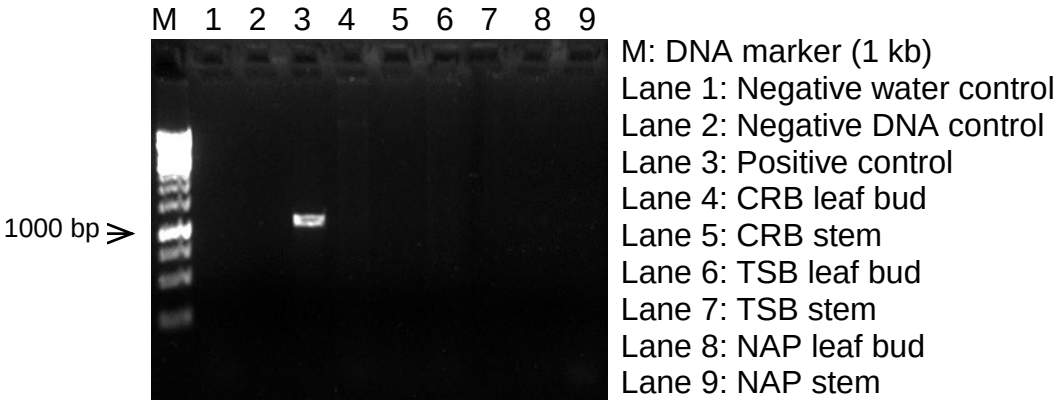

Figure 7: PCR using *Burkholderia* specific primers on leaf bud and stem samples collected from *P. edentula* in the respective enrichment solutions (CRB, TSB and NAP buffer).

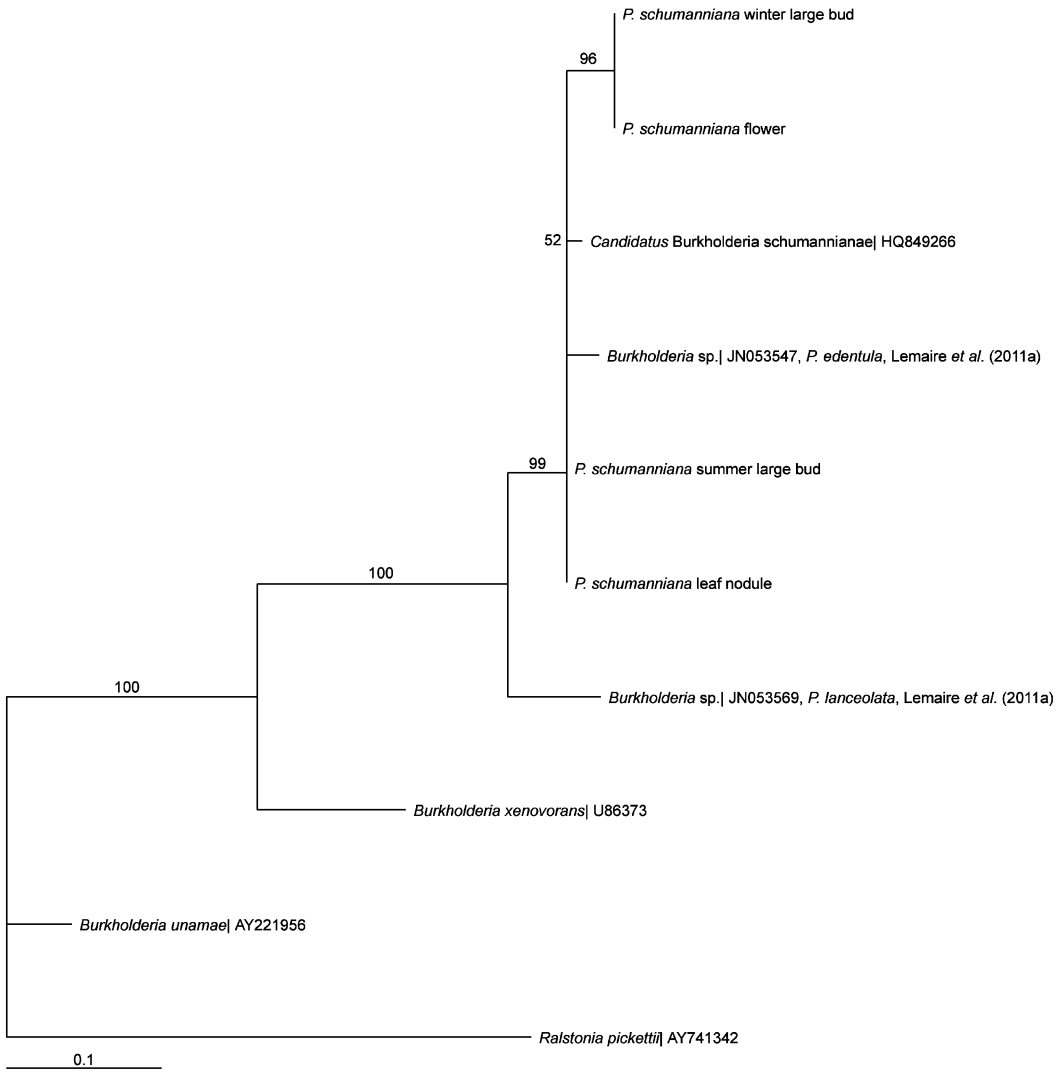

Figure 8: Maximum Likelihood tree of 16S rRNA gene sequences from different plant tissues of *P. schumanniana*. The sequence length was 448 bp. Bootstrap support was indicated as a percentage of 1000 replicates. Values under 50 % were not included. *Ralstonia picketti* was used as the outgroup.

Chapter 5

Summary and Conclusions

Summary and Conclusions

Within the Rubiaceae there are certain plant species with distinctive leaf nodules that is a result of a symbiotic relationship with bacterial endophytes. Three *Pavetta* spp. indigenous to South Africa that exhibit this trait are *P. lanceolata*, *P. edentula* and *P. schumanniana*. Recent studies on selected *Psychotria* spp. and *Pavetta* spp. have shown these nodule-forming bacterial endophytes to belong to the genus *Burkholderia*. These bacteria have, however, never been cultured and are, therefore, classified under the provisional status of *Candidatus.*

With the use of electron microscopy, leaf nodule morphology has been studied in selected *Psychotria* spp. The findings of this research included the observation of stomatal openings within the leaf primordia, large masses of bacterial endophytes within mature nodules and unusually thickened plant cell walls suspended within the bacterial mass. The process of nodule formation and the associated morphological characteristics have not been studied as thoroughly in *Pavetta* spp.

The leaf nodule-forming bacterial endophytes have been studied within the leaf tissue to a large extent but to a lesser extent in the flowers and seeds of many leaf nodulated plant hosts. The presence of these nodule-forming bacteria has as yet not been documented in the stem tissue. In addition to this the location of the leaf nodule-forming bacterial endophytes during the winter months in deciduous hosts is unknown. The use of molecular techniques to test for the presence of the nodule-forming bacteria have now become available. This is due to the identification of the bacteria that form these nodules as belonging to the genus *Burkholderia*. A molecular based approach may also contribute to answering the question of how the bacterial endophytes are distributed within the plant and transmitted from parent to offspring.

With the use of culturing and Denaturing Gradient Gel Electrophoresis (DGGE) this study identified bacterial endophyte genera within the leaves of *P. lanceolata*, *P. edentula* and *P. schumanniana* that have been reported to occur in other plants. Despite this, the bacterial endophyte population consisted of different bacterial genera between each *Pavetta* sp. In *P. edentula* and *P. schumanniana* the nodule-forming bacterial endophytes were identified as belonging to the genus *Burkholderia* using DGGE. More specifically these bacterial endophytes were found to be similar to the previously identified *Candidatus* Burkholderia schumannianae. The bacterial endophytes identified in the nodule tissue of *P. lanceolata* specimens growing in pots did not group with the genus *Burkholderia* but rather in the family Bradyrhizobiaceae. These nodule-forming bacterial endophytes were also cultured form the nodule tissue of these *P. lanceolata* plants. Samples of *P. lanceolata* that were collected from the National Botanical Gardens were tested for the presence of a *Burkholderia* species within the nodule tissue and the result was positive. This result may indicate that there is a link between the species of nodule-forming bacterial endophyte and the environment that the host plant is growing in.

The leaf nodules of *P. lanceolata*, *P. edentula* and *P. schumanniana* were studied using light and electron microscopy in order to determine the characteristics associated with leaf nodule development in the *Pavetta* spp. No stomatal openings were observed within the primordial leaf tissue of any of the *Pavetta* spp. This suggests that the bacterial endophytes may use an alternative mode of entry into the young leaf tissue. Within the older nodules of *P. edentula* and *P. schumanniana* the bacterial endophytes were rod-like in shape and did not posses flagella. Within the leaf nodules of *P. lanceolata* the bacterial endophytes were never seen in large clusters as was the case with *P. edentula* and *P. schumanniana*. Overall within the three *Pavetta* spp. nodule formation appeared to be mainly driven by the growth and multiplication of the bacterial endophytes.

The presence of a *Burkholderia* sp. was detected with the use of *Burkholderia* specific primers in the older leaf buds of *P. schumanniana*

sampled in winter. This indicates that the nodule-forming bacterial endophytes most probably reside within the leaf buds during the winter period. A *Burkholderia* sp. was also detected in the older leaf buds and flowers collected from *P. schumanniana* in summer but not in the young leaf buds or stem tissue. *Burkholderia* spp. were also found to be absent from the leaf bud and stem samples collected from *P. edentula*. The absence of *Burkholderia* spp. from the small leaf buds, stem and seed samples may be due to the bacteria being at very low concentrations and hence undetectable by the primers.

The conclusions of this study are that:

- The bacterial endophytes within the nodules of *P. edentula* and *P. schumanniana* were identified as *Burkholderia* spp.
- The nodule tissue of *P. lanceolata* was found to be colonized by different bacterial species depending on where the host specimen was growing.
- The relationship between plant and its nodule-forming bacterial partner may not be as strict as initially thought.
- Leaf nodule development in the *Pavetta* spp. is a result of growth and multiplication of the nodule-forming bacterial endophytes which expand the nodule as the leaf ages.
- Characteristics associated with leaf nodule development in the *Pavetta* spp. are specific to the plant species and its bacterial partner.
- The leaf nodule-forming bacterial endophytes most likely overwinter within the leaf buds of deciduous hosts.
- The detection of a *Burkholderia* sp. in the flowers of *P. schumanniana* indicates that the nodule-forming bacterial endophytes are most likely vertically transmitted.
- The absence of a *Burkholderia* sp. in the *P. edentula* leaf buds may be due to an alternative method used for the inoculation of new leaves with nodule-forming bacterial endophytes.

The results of this study will facilitate further research in this specialised relationship shared between plants and endophytic bacteria and will add to the existing knowledge of the *Pavetta* spp. It has been theorised that the nodule-forming bacterial endophytes within the leaves of *P. schumanniana* and *P. harborii* may play a role in 'gousiekte'. The identification of the nodule-forming bacterial endophytes may help to further the etiological studies of this disease. The finding of different bacterial species within the leaf nodules of *P. lanceolata* growing under different conditions raises the question of whether similar results can be found in other *Pavetta* spp. The use of *Burkholderia* specific primers will also make nodule-forming bacterial endophyte identification easier within both leaf tissue and other plant tissues.