2016

The Endophytes of Pediomelum Esculentum: A Unique Case in Legume Evolution

Tyrel Ryan Deutscher
South Dakota State University

THE ENDOPHYTES OF *PEDIOMELUM ESCULENTUM*: A UNIQUE CASE IN LEGUME EVOLUTION

BY

TYREL RYAN DEUTSCHER

A thesis submitted in partial fulfillment of the requirements for the

Master of Science

Major in Biological Sciences

Specialization in Biology

South Dakota State University

2016

THE ENDOPHYTES OF *PEDIOMELUM ESCULENTUM*: A UNIQUE CASE IN LEGUME EVOLUTION

This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science in Biological Sciences degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

R. Neil Reese, Ph.D.
Thesis Advisor Date

Volker Brözel, Ph.D.
Head, Department of Biology and Microbiology Date

Dean, Graduate School Date

To Mom and Dad, for their never-ending love, support,
and understanding through my extended adolescence.

"We can only have *opinions* about things that belong to the world of the senses, tangible things. We can only have true *knowledge* of things that can be understood with our reason."
- *Jostein Gaarder,* Sophie's World

ACKNOWLEDGMENTS

This research was support by the USDA Tribal Colleges Research Grant, the SDSU Agricultural Experiment Station, the SDSU Functional Genomics Core, and the SDSU Oak Lake Field Research Station.

Thank you to my advisor, Dr. Neil Reese, for allowing me to make this project my own, introducing me to the world of native plants, and his thoughtful advice throughout this project.

Thanks to my committee members Dr. Volker Brözel and Dr. Senthill Subramanian, for excellent conversations, wise guidance, and allowing me to be an honorary member of their labs. Also thanks to Dr. Evren Celik Wiltse, for her time and effort in helping me obtain this degree.

Thank you to Gitanjali Nande-Kafle and Bibha Dahal, for being excellent lab mates, and always willing to help in a time of need. Thanks to Mike Hildreth, for his guidance on microscopes, fixation protocols, and microtomes. Thank you to Liping Gu, for her assistance in running the gas chromatograph. A special thanks to Leslie Rae Henry for showing me how to find wild turnips and providing the best homemade goods.

Thanks to Maria, who's annoying at times, but I still put up with it; she does the same for me. And finally, to Kevin and Cindy Deutscher, who not only are responsible for my being, but also for fostering the type of guy who wants to get a Master's degree. As to what type of guy that is, I'll let them clarify.

CONTENTS

ABSTRACT

THE ENDOPHYTES OF *PEDIOMELUM ESCULENTUM*: A UNIQUE CASE IN LEGUME EVOLUTION

TYREL DEUTSCHER

2016

Pediomelum esculentum (commonly prairie turnip) is a perennial legume of the Great Plains, consisting of a deep taproot and large edible tuber, and has served as a nutritious staple in Native American diets. The tuber is capable of storing up to 20 percent protein by weight. *P. esculentum* is a legume, but not a prominent nodule former; instead, it grows in nitrogen-limited soils and produces large amounts of protein. This suggests the involvement of biological nitrogen fixation. We have investigated the presence of diazotrophic endophytes in *P. esculentum*. Bacteria were isolated from wild plants on nitrogen free media, identified with their partial *16S rRNA* gene sequences, and screened for the presence of the nitrogen fixation gene *nifH*. Select isolates were applied as a co-inoculum to seedlings grown under gnotobiotic conditions in a growth chamber with no nitrogen source. Seedlings in both the inoculated and uninoculated group developed nodules and showed no signs of nitrogen stress. Bacteria isolated from the nodules and tubers of both groups were closely related to the same *Bacillus* bacterium isolated from seeds germinated under sterile conditions, according to partial *16S rRNA* sequences. Bright field and fluorescence imaging revealed bacteria present in the intercellular space of four-week-old tubers and in the sterile germinated seeds. Sectioning and imaging of the nodules show a central nodule vasculature and infected cells extending inwards to the main root vasculature. Nitrogen fixation in the plants was indirectly confirmed by acetylene reduction. Our results suggest *P. esculentum* has formed a unique symbiosis with a nitrogen fixing *Bacillus* bacterium that transmits vertically in the seeds and induces nodules.

1. Introduction

All organisms require a source of fixed nitrogen to thrive. Nitrogen is required for the synthesis of nucleic acids and proteins, two biomacromolecules that dictate all of an organism's vital processes. Nitrogen is integrated into a cell at 2 to 20 atoms for every 100 carbon atoms acquired [1]. Prior to integration, the atmospherically abundant dinitrogen (N_2) must be reduced to the biologically available form ammonium (NH_4). Abiotic fixation of N_2 by lightning and mineral-based reduction accounted for initial sources of bioavailable nitrogen on earth, allowing for evolution of early life forms. The limitation of bioavailable nitrogen likely became the driving force in the evolution of biological nitrogen fixation (BNF), a metabolic pathway capable of converting N_2 to bioavailable NH_4 via reduction-oxidation [2].

BNF is limited to select bacteria and archaea, and is not evident in eukaryotes. Nitrogen-fixing organisms or diazotrophs relieve the nitrogen stress in natural and agricultural ecosystems, and have been considered keystone species [2]. Until recent anthropogenic times, the input of bioavailable nitrogen into the biosphere was almost solely dependent upon diazotrophs. The development of the Haber-Bosch process has allowed for the application of modern fertilizer [1] to supply bioavailable nitrogen. While this man-made fertilizer has increased agricultural output, it has contributed to abrupt shifts of the nitrogen cycle and eutrophication of waterways [3]. Recent research efforts are exploring the broad applications of natural BNF that may reduce the global dependency on artificial fertilizers.

Despite being limited to only bacteria and archaea, diazotrophs have a diverse ecological distribution, and thrive in all terrestrial and marine biomes [4]. Of particular interest for agricultural application are the nitrogen-fixing bacteria that form a root nodule symbiosis (RNS) in conjunction with angiosperm host plants of the nitrogen-fixing clade (NFC). The bacteria contribute bioavailable NH_4 to the plant while receiving a carbon supply, among other benefits. This relationship is the most studied system of BNF and plant-microbe interaction due to its agricultural importance [2]. In earlier literature, this type of symbiotic partnership was thought to be limited to the legume family (Fabaceae) and their partner bacterial counterpart of the bacterial, Rhizobia. The

term Rhizobia is used to generalize all unicellular bacteria capable of forming nodules, taken from the generic names of the early discovered symbionts in the Alphaproteobacteria class: *Bradyrhizobium*, *Mesorhizobium*, and *Rhizobium* [5].

It is now understood that the RNS partnership is much more diverse. Bacteria of the Betaproteobacteria class can form nodules with legumes [6]. The NFC has been expanded to include non-legumes able to form nodules with filamentous bacteria of the *Frankia* genus and the non-legume *Parasponia* that forms nodules with Rhizobia [6,7]. Among all RNS partnerships, a few laws remain constant. The relationships are non-obligate, non-permanent, and lack vertical transmission from one host generation to the next [8].

1.1. Biological nitrogen fixation and nitrogenase

BNF reduces N_2 to NH_4 via nitrogenase. The common nitrogenase is composed of the NifH protein (γ2 homodimeric azoferredoxin) and NifD/K proteins (α2β2 heterotetrameric molybdoferredoxin) [8]. The largest component NifD/K reduces N_2, while NifH hydrolyzes ATP for electron transfer [9]. The enzyme consumes a significant amount of ATP—the reduction of a single N_2 molecule requires 16 ATP and 8 electrons. Nitrogen fixation can be represented by the following equation: $N_2 + 8\ e^- + 8\ H^+ + 16\ MgATP \rightarrow 2\ NH_3 + H_2 + 16\ MgADP + 16\ P_i$ [10].

Three known types of nitrogenase complexes differ based on cofactors used in the active metal site for reduction. Nitrogenase (Nif) is the predominant form, uses molybdenum as the cofactor, and is common in cyanobacteria and rhizobia. The other two enzyme complexes, referred to as alternative nitrogenase, utilize either Iron (Anf) or Vanadium (Vnf) as their cofactors [2,11].

Numerous genes are required for protein coding and regulation of BNF. Six genes have been identified as the minimal requirement for successful nitrogenase expression. The main structural genes are *nifH*, *nifD*, and *nifK*, and code for the previously described proteins of the same names. Other sequences required include the FeMo-cofactor biosynthesis genes *nifE*, *nifN*, and *nifB*. The alternative nitrogenases have been shown to require the addition of a *nifG* gene for encoding the NifG protein [12]. The *nifH* gene is generally well-conserved among all the nitrogenase variants. It has also

been shown to exhibit similarity with chlorophyllide reductases and *nifH*-like genes present in some methanogenic Archaea [13].

All three variants of nitrogenase are sensitive to low concentrations of oxygen (O_2), and as a consequence multiple adaptations have evolved to guard against O_2 damage. Anaerobic diazotrophs are likely to contain a lower number of nitrogenase-associated genes, while aerobic organisms require an increase in genes for the evolved mechanisms of O_2 coping [2].

Bacteria, such as *Azotobacter*, have increased aerobic respiration and thick extracellular polysaccharides to effectively reduce intracellular O_2 concentrations [14,15]. Filamentous cyanobacteria fix nitrogen inside their heterocysts, where there is limited photosynthetic activity that helps to lower the O_2 concentration [16,17]. Unicellular cyanobacteria operate on dark/light cycles that alternate nitrogenase activity with photosynthesis [18]. Other approaches to reducing O_2 concentrations involve O_2-scavenging globin molecules. In the symbiotic partnership between plants and diazotrophs, the plant provides leghemoglobin to effectively lower O_2 concentration in the nodule environment [19].

1.2. Taxonomic perspectives of legume and nodule evolution

1.2.1. The nitrogen fixing clade of angiosperms

Host plants capable of RNS are confined to the NFC within the Rosid 1 clade of angiosperms consisting of the orders Fabales, Cucurbitales, Fagales, and Rosales [20]. Nodulation is rare in all orders, except for Fabales, which houses the family with highest occurrence of nodulation, the Fabaceae (Leguminosae) [21]. Actinorhizal plants form nodules with the filamentous bacterial genus *Frankia*, and are distributed throughout Cucurbitales, Fagales, and Rosales. *Parasponia* (Ulmaceae), of the Cannabaceae family of Rosales, is the only non-legume nodulated by Rhizobia [22]

The classical RNS that occurs between legumes and Rhizobia has been extensively studied compared to other interactions, largely due to the plant family's agricultural relevance. The Fabaceae family is the third largest plant family with ~19,500 species [23], and exhibits a significantly higher rate of evolution during the past 60 million years compared to other angiosperms [24]. Some of the fastest evolving plant

clades are within the legume family [25-27]. Legumes are an ecological component of virtually every ecosystem, and are major players in nitrogen cycling and the bioavailability of nitrogen. The morphology of the family varies wildly, including large trees, shrubs, herbs, aquatics, and climbing vines. The phylogenetic relationship among major nodulating clades of the Fabaceae are represented in Figure 1. The order in which the Fabaceae subfamilies diverged is reflected in the number of their species which can form nodules: Caesalpinioideae (23%), Mimosoideae (90%), and Papilionoideae (97%) [7] [28]. Papilionoideae is the largest legume subfamily with 478 genera that contain 13,800 species [23].

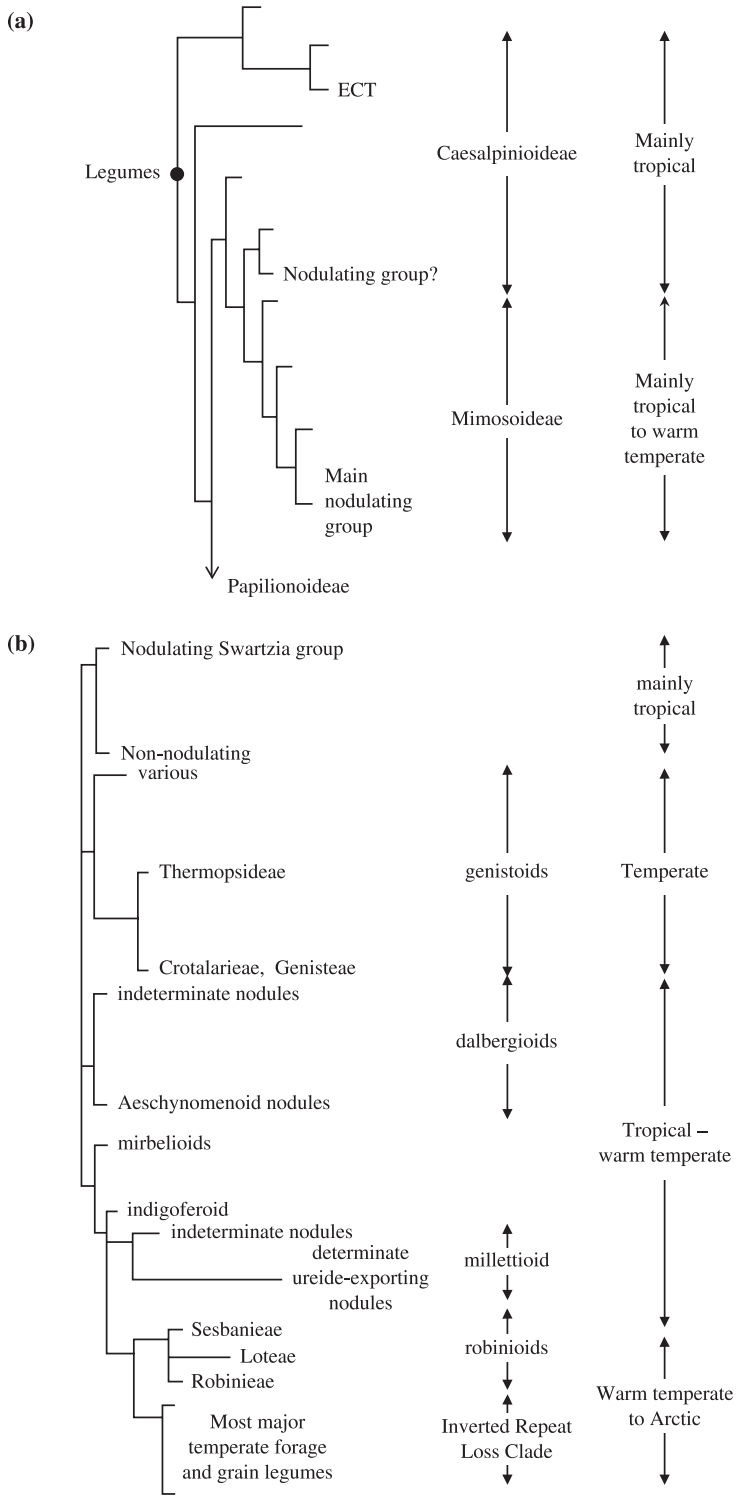

Figure 1. The major nodulating clades of Fabaceae in chronological order. (a) The subfamilies Caesalpinioidieae and Mimosoideae. (b) The Papilionoideae subfamily. Taken from Sprent 2007 [5].

1.2.2. Nodule morphology varies across lineages

Attempts to classify the Fabaceae based on nodule morphology and development have had some success but are relatively limited in their scope. Missing data on many species of the NFC create a lack of clarity in such taxonomical schemes [21]. Meristematic activity, route of infection, and the behavior in which the infection spreads are basic criteria for classifying nodules. Indeterminate nodules retain their meristems while determinate nodules grow to a distinct size. The two routes of bacterial entry into the plant occur through root hairs or intercellular spaces in the epidermis, "crack entry" Following infection, bacteria can spread through intercellular spaces or intracellularly through the advent of infection threads (ITs) formed by an invagination of the cell membrane and remodeling of the cell wall [29].

Indeterminate nodules have a wider distribution throughout the NFC lineages compared to determinate forms, and have longer lifespans. Therefore, indeterminate nodules have been considered more versatile [30]. The first subfamilies to diverge within the Fabaceae, Caesalpinioideae and Mimosoideae, form indeterminate nodules. The third divergent subfamily, Papilionoideae, is capable of forming both types, implying the indeterminate growth pattern may be ancestral [31].

Caesalpinioideae are infected through root hairs, and form indeterminate nodules where bacteria are maintained in ITs [5]. Mimosoideae form only indeterminate nodules, while the final diverging subfamily Papilionoideae is capable of both growth types. [31]. Mimosoideae nodules are very uniform throughout the family compared to Caesalpinioideae and especially Papilionoideae nodules, which exhibit a wider range of development and infection strategies. All Mimosoideae nodules are indeterminate with varying degrees of branching and infection initiated through root hairs followed by the development of ITs, which is common in the earlier branching Caesalpinioidieae [5]. Some species of the tribe Mimoseae in Mimosoideae have been reported to be nodulated by Betaproteobacteria [6].

The determinacy of nodules is not dependent upon either infection route or the development of ITs. The root hair infection route is an advanced, well-controlled process that occurs in an estimated 75% of nodulating legumes [30]. Crack entry is under limited host control and results in more promiscuous infection by diverse bacteria, which can

lead to the formation of nodules housing less efficient nitrogen fixers [32]. While Caesalpinioideae and Mimosoideae are both infected through root hairs, both modes of infection are common in the Papilionoideae subfamily, with some genera (i.e. *Sesbania, Arachis*) capable of both root hair and crack entry simultaneously [33].

Indeterminate and determinate nodules appear to have evolved with and without the advent of ITs. The dalbergioid and genistoid clades of the Papilionoideae subfamily evolved nodules very early, around 55 MYA (refer to Figure 1) [34]. The determinate aeschynomenoid nodules formed by peanut (*Arachis*) of the dalbergioids and the indeterminate nodule of the genistoids develop without any ITs. Other nodules with origins around the same time period contain ITs, accordingly, the two different infection processes likely evolved in parallel [35]. It has been suggested that crack entry infection is a precursor to the more complex root hair infection [5,29,36]. However, the data concerning infection is lacking in many species to clearly determine which behavior is ancestral [21].

Determinate nodules have at least two independent origins within Papilionoideae. The earliest determinate is the aeschynomenoid nodule of the dalbergioid clade which evolved only a few MY after the emergence of indeterminate nodules. The other determinate nodule type, "desmodioid," appeared in more recent evolutionary times in the tribes Phaseoleae, Psoraleae, Desmodieae, Milletieae-1, and Loteae tribes [21,31]. The nonhomology and separate origins of these determinate nodules is reflected in the difference between their biochemical activities and development.

The Desmodieae, Phaseoleae, and Psoraleae tribes are typically shrubby and herbaceous, and live throughout a broad range of habitat. The species that have been investigated in this clade have determinate nodules with ITs that export ureides as the product of nitrogen fixation. The infected zone of the nodule has interspaced infected and uninfected cells [37]. The woody genus *Erythrina* of Phaseoleae has atypical nodules, exports low levels of ureides, and has very high levels of nitrate reductase compared to other studied legumes. *Erythrina* observations show both crack entry and root hair infection, and nodules associated with lateral roots, which is not typical of this tribe that forms nodules via root hairs [38].

1.3. Nodule development

1.3.1. Root hair infection

In most legumes, nodule development preferentially occurs just behind the root tip in a zone where root hairs are developing [39,40]. The process begins with the release of flavonoids from the plant which induce *nod* genes in the respective bacteria [41]. Expression of *nod* genes leads to the production of lipo-chitooligosaccharides (LCOs) or Nod factors [42,43]. LCOs in turn induce root hair deformation followed by division of cortical cells behind the root hair that become the nodule primordium [44].

In determinate nodules, cell division occurs first in the outer cortex, followed by the inner cortex and eventual marriage of the two dividing regions [44]. This nodule primordium continues to divide and differentiate while infection occurs. The cortex eventually stops dividing as the central primordium is infected with rhizobia, the cells cease dividing, and finally the peripheral cells differentiate to complete nodule development [45]. In indeterminate nodules, the first site of cell division is in the inner cortex opposite of a protoxylem pole [46]. A nodule meristem then forms from previously differentiated inner cortical cells adjacent to the nodule primordium. The meristem gives rise to the emerging nodule tissue, while the proximal end of the nodule, including the vasculature, is mainly derived from the endodermis and pericycle [47].

Bacteria infect the root hair first with the development of an IT formed by an inward invagination of the plant cell membrane and new cell wall synthesis [48]. Bacteria then advance from the root hair cells to the developing nodule primordium. ITs vary from highly advanced to primitive. In many legumes that have been studied, an IT that penetrates the cells of the nodule primordium will release bacteria into a droplet-like structure (symbiosome or bacteroid) formed through an endocytosis process that involves pinching off of the host cell membrane [44,48].

The ITs branch throughout the infected tissue with infected and uninfected cells interspersed. This interspacing likely plays a role in nodule functioning, specifically in the case of ureide exporting nodules [35,49]. These features are found in both indeterminate and determinate nodules, however the latter loses meristamic activity after a short while [35]. Once a host cell is penetrated via ITs, it ceases mitotic division but undergoes endoreduplication, resulting in large polyploid cells capable of tendering large

sums of bacteria. *Lupinus*, which does not exhibit infection threads, also undergoes endoreduplication in infected cells [50].

ITs are common in all studied Caesalpinioideae nodules. Many species in this subfamily form ITs that do not release bacteria into symbiosomes, instead nitrogen fixation takes place in modified ITs called fixation threads [37]. In *Chamaecrista*, the tree species forms fixation threads while the herbaceous species will fully release symbiosomes into the cell [51]. Two Caesalpinioideae genera, infected via root hair, have been shown to develop ITs without nodules [7,28]. These unique features led to the hypothesis that ITs served as the initial defense response to a pathogenic infection and ultimately evolved into the development of symbiosomes.

1.3.2. "Crack Entry" infection

The ability of legumes to nodulate is reduced in mature tissue due to the lack of freshly developing root hairs present in the zone directly behind the root tip [39]. However, legume nodules can form nodules without root hairs. Crack entry or infection between epidermal cells is thought to occur in 25% of legumes, which can be expected because not all extant legumes can form root hairs, and those capable may only form root hairs under specific conditions [5,38]. Crack entry infection does not always lead to the formation of ITs as with root hair infection. Sprent [5] postulated the ancestral entry point of bacteria is through crack entry. As bacteria enter into the intercellular space, they may be surrounded by elements that would later compose the ITs [52]. Known progression of nodule development following crack entry infection is described in Figure 2.

Nodules that develop in the mature root zone are typically associated with emerging lateral roots [53,54]. The distinct aeschynomenoid nodules formed by species of the Dalbergioid clade of Papilionoideae are determinate and develop following a crack infection where a lateral root is emerging [32]. In studies on peanut (*Arachis*) aeschynomenoid nodules, bacteria enter between the epidermal cells and the nodule originates from divisions of the pericycle [55]. Bacteria spread throughout the intercellular space without forming ITs and are endocytosed by the host cells in the nodule primordium which further divide post infection [55-57]. Aeschynomenoid nodules are short-lived and small, typically less than 5 mm in diameter, and can be observed on old growth roots still capable of forming lateral roots; a type of behavior important for

long-lived trees associated with a large degree of promiscuity and uncontrolled infection by numerous bacterial genera [32]. Also, some Papilionoideae species can form ITs post crack entry, as in *Lonchocarpus* sp. of the Milletieae tribe [58].

Sesbania (also in the Papilionoideae subfamily) has been well studied for its ability to develop nodules on both the root and stem through both root hair and crack infection [59]. *Sesbania* forms determinate nodules, however the development shares commonalties between both indeterminate and determinate type nodules [60]. The entry point is often associated with the emergence of lateral roots, as in the development of aeschynomenoid nodules. Bacteria will enter cells as they spread through the apoplast. These infected cells then divide, lose meristematic activity, and begin to form an evenly infected central tissue [61]. Studies conducted on Genistoid legumes reveal infection occurs either through epidermal cracks or at the base of root hairs. A few host cells are infected, divide repeatedly, and give rise to a central infected tissue. However, some cells are capable of retaining meristematic activity [50,62].

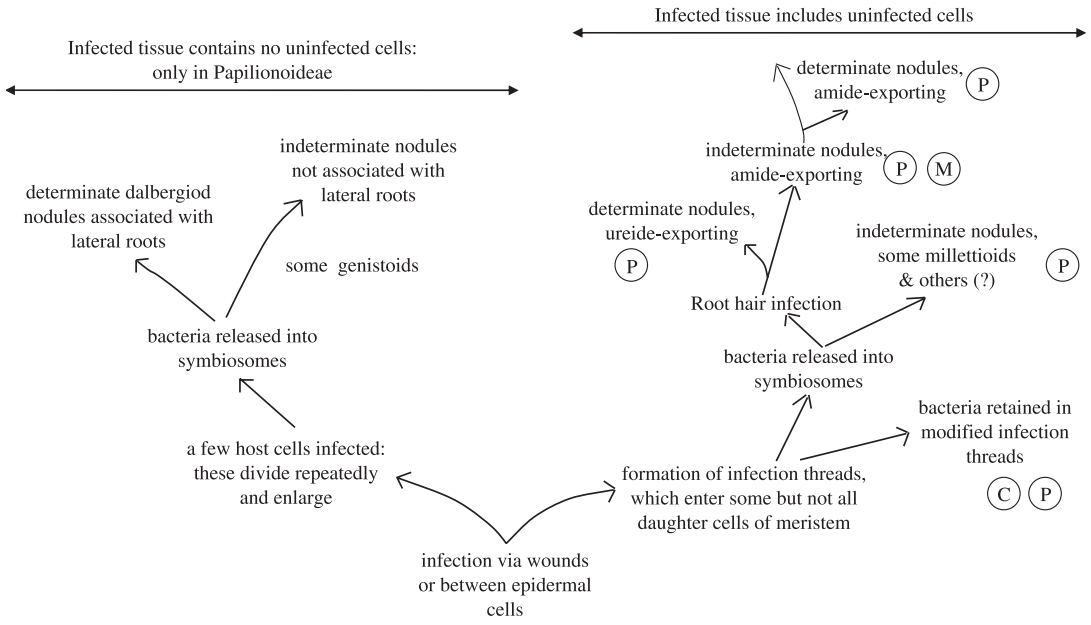

Figure 2. The possible routes of legume nodule development following crack entry. Circled letters indicate the subfamily in which the characteristic has been confirmed. From [5].

1.3.3. Non-legume nodules

Nodules in *Arachis*, *Sesbania*, *Parasponia*, and actinorhizal plants are formed in association with lateral roots but with some distinct differences. Legumes develop nodules from cortex cells; peanut from the main root cortex, and *Sesbania* from the cortical cells of the lateral root base. *Parasponia* and actinorhizal nodules are modified lateral roots that develop into nodules originating from the pericycle similar [44]. Lateral roots are initiated in the pericycle, and main root cortex divisions occur during the lateral root emergence [63,64] Actinorhizal and *Parasponia* nodules also uniquely form a central vasculature unlike legume nodules that have a peripheral nodule vasculature.

Actinorhizal nodules, infected by *Frankia*, can be infected through both crack entry and root hair infection, the latter most closely resembles *Parasponia* nodule development. During crack entry in the genus *Ceanothus*, the cortex divides to form the prenodule, but no cells are infected until the entral vasculature has fully differentiated, and then infection threads form [65]. Though invasion first occurs in the cortex, the nodule lobe primordium differentiates from the pericycle. Frankia forms ITs that will penetrate each individual cell from the intercellular space, with cell to cell spreading of the IT being rare [65]. In *Elaeagnus*, *Frankia* cells begin infection in pericycle cells that have fully differentiated to form the nodule lobe primordium. Similar to other actinorhizal plants, there is no cell to cell invasion in *Elaegnus* [66-68].

Nodule development in *Parasponia* more closely resembles *Frankia*-infected nodules rather than legume nodules, despite being infected by the Alphaproteobacteria *Bradyrhizobium*. Unique to Parasponia is the induction of multicellular root hairs by the bacteria which then enter the cortex via ITs [69]. Infection threads are not observed in the root hairs themselves or in the mature cortical cells, and the root hairs do not exhibit the classic curling as in legumes. Rhizobia reside in the apoplast during a prenodule formation, produced by random divisions of cells in the cortex, and are eventually entered by rhizobia using an infection thread [69]. As the prenodule and ITs are forming, the pericycle begins dividing to give rise to a nodule lobe primordium resembling a lateral root. The ITs of the dividing cortex penetrate into the nodule lobe primordium as the nodule expands and the infected zone of the cortex and primordium become

continuous [70]. In *Parasponia*, the rhizobia are never released from the infection threads [71].

1.4. Evolutionary origins of the root nodule symbiosis

There has been decades of discussion on whether the nitrogen fixing symbiotic root nodule evolved as a modified stem or leaf, or perhaps an entirely novel organ referred to as sui generis [44,46]. Other hypotheses for the origin of the nodule include the development from a carbon storage organ [72] or from modified stems [73]. The interaction between plants and microbes is likely to have developed from three different scenarios. The first is the ancient symbiotic interaction between plants and endomycorrhizae [74]; the second is a response mechanism against pathogenic bacteria [75]; and the third is from a wound response [76].

Structurally, nodules probably share the most in common with lateral roots. Compared to a lateral root, a legume nodule develops from cortical cells rather than from the pericycle cells like a lateral root, and have peripheral vasculature instead of a central vasculature. The non-legume nodules more closely resemble a lateral root, developing from the pericycle and exhibiting a central vasculature.

1.4.1. Predisposition hypothesis

The current view on the evolution of nodules is based on a predisposition hypothesis first described by Soltis et al. [20], and has been further investigated using phylogenetic modelling of the nitrogen fixing clade of angiosperms [21,77]. The predisposition hypothesis infers the evolution of nodules occurred independently across lineages that diverged from a common ancestor containing an underlying genetic trait that created a predisposition for nodulation to occur in future generations. This “deep homology” contains the regulatory circuits required for the most basic symbiotic interaction and is possibly present in all plants descending after this moment in time, whether they form nodules or not. Early rapid radiations in the legume family lineages [34] indicates that the major lineages were separated long before the independent evolution of nodules, owing to the diverse morphology, developmental patterns, biochemistry, and symbiotic partners across lineages [78].

Werner et al. [77] found quantitative evidence for a single origin of a deeply homologous predisposing trait for nodulation emerging around 100MYA when the NFC diverged from other angiosperms. Based on this phylogenetic model, there are 8 independent origins of nodulation and 10 losses. This model implemented a heterogeneous rate of evolution that predicted the fixing state of species as either non-precursor, precursor, fixer, or stable fixer. Stable fixers are plants unlikely to lose the symbiotic capacity. A state of stable fixing, common in Papilionoideae, has been attributed to duplication of the genome around 54MYA, making redundant copies of the genes required and decreasing chances of losing the ability [79-81].

The root nodule symbiosis is likely to have first evolved around 60 MYA in legumes, 30-40 MY after the predisposition trait appeared [21,82]. This lapse of time and conservation across lineages, suggests the predisposition mutation may have had a functional role in non-nodule-forming ancestors preceding a recruitment event that led to nodule emergence. The genetic basis for nodules may have been evolved through recruitment. For instance, a gene involved in root development could take on a new function involved in nodule development, and the gene would then be involved in both processes. Genome duplication may have contributed to recruitment also, in which a gene is copied and the new paralogue is utilized in a novel manner for nodule development [21]. If the predisposition trait was functional, contributing to fitness, it is likely to be present in extant non-nodule forming species that have nodulating lineages [21].

1.5. Endophytic bacteria

Plants have not evolved in isolation, but in intimate association with microorganisms such as fungi and bacteria abundant in the plant tissue, rhizosphere, and phyllosphere. Any microorganism that resides within plant tissues for a portion of its life cycle is considered an endophyte. An endophyte was first defined as an organism that can be isolated from surface sterilized tissue, and does not observably cause any harm to the plant [83]. Confirming a microorganism as an endophyte can often be problematic. The advent of modern molecular techniques has allowed for the detection of nucleic acids from unculturable organisms but techniques may introduce error while detecting non-endophyte nucleic acids that were not completely eliminated from the plant surface [84].

The current accepted criteria for identifying endophytes requires the isolation from surface-disinfected tissue and microscopic evidence of the "tagged" organism within the host plant tissue [85]. The criteria are not always met, and the term "putative" is often used.

Endophytes have been found in roots, tubers, nodules, shoots, leaves, seeds and ovules of plants [83,86]. Roots carry a higher load of endophytes compared to the shoot and leaf tissues [87]. The most common location of bacterial endophytes is within the intercellular space and xylem vessels [85,88]. Endophytic bacteria occur at a lower populations than rhizospheric bacteria [83], and the endophyte population is dependent upon soil type, season, plant genotype, plant age, and tissue type [89,90].

A compilation of all prokaryotic endophyte sequences was constructed in 2015 by Hardoim et al. [91]. Endophytes have been identified in 21 Bacteria phyla and two Archaea phyla. The majority of all the sequences fall into the following four bacterial phyla: Proteobacteria (54%), Actinobacteria (20%), Firmicutes (16%), and Bacteroidetes (6%). The highest occurring classes are Gammaproteobacteria with 26% and Alphaproteobacteria with 18%; the latter includes the symbiotic nitrogen fixing rhizobium genera. Betaproteobacteria accounts for 10% of endophytes described so far, and includes *Burkholdeira*, which has been identified has a root nodule symbiont [6] and has the broadest range of hosts and environments [92]. Within the Firmicute*s*, the genus *Bacillus* accounts for 15% of identified endophytes.

1.5.1. Effects of endophytes on the host plant

The level of the intimacy between plants and their endophytes can be categorized as obligate, opportunistic, or facultative [91]. Obligate endophytes include a number of mycorrhizal fungi that cannot complete their life cycle without association with plant tissue [93,94]. Opportunistic endophytes typically inhabit the rhizosphere or phyllosphere of the plant, and colonize the inner tissue sporadically when conditions permit [95]. The majority of endophytes are described as facultative, able to consume nutrients from the host plant, but their effect on the host plant is not understood [91]. It is under debate whether the plant selects these facultative bacteria, or the bacteria merely utilize the plant as a means of dispersal [96-99]. Many endophytes have no effect on the plant, and others are able to contribute to the plant by inhibiting pathogens and herbivorous arthropods

[91]. Endophytes cannot always be clearly defined as beneficial, harmful, or neutral. Their behavior can be dependent on the plant life stage and other environmental factors. *Fusarium verticillioides* can either benefit maize or act as a pathogen when either abiotic or biotic factors promote a change in the symbiotic balance [100,101].

The known effects of endophytes, which have been characterized in well-controlled environments for optimal host plant growth may not reflect the actual effect in a natural setting with complex interactions between multitudes of organisms [102]. As more data is collected with stronger high-throughput microbiome sequencing, a better understanding of the overall functioning of the plant microbiome can occur.

Some endophytes may be capable of increasing plant defense reactions, inducing a higher tolerance of pathogens. This induced systemic resistance in plants has been found to be stimulated by *Pseudomonas* and *Bacillus* genera [103,104]. This immune response in the host plant can be triggered through factors consisting of antimicrobial compounds, *N*-acylhomoserine lactones, salicylic acid, jasmonic acid, siderophores, acetoin, and lipopolysaccharides [104,105]. Aside from protection against biotic stress, endophytes also contribute to resistance of abiotic stresses, improving tolerance to drought, cold, salt, and nitrogen starvation [91].

Endophytes can also promote plant growth. Current understanding of plant growth promotion by endophytes is limited. They may contribute to increased host fitness through nitrogen fixation [106], production of phytohormones, siderophore production, production of antifungal/antibacterial products, or through increased availability of minerals [107,108]. There is evidence that phytohormone production by bacteria can stimulate morphological changes in the host plant [109-111]. Certain endophytes are capable of producing the plant hormones cytokinin, gibberellins, and indole-3-acetic acid that may play a role in plant growth promotion [109-114]. Other compounds produced by endophytes that may increase plant growth include adenine, adenine ribosides, acetoin, 2,3-butanediol and polyamines [115-121].

1.5.2. Associative and endophytic nitrogen fixers

Bacteria capable of fixing nitrogen in association with plants but outside of a nodule environment are referred to as associative nitrogen fixers [122]. They may

reside in the apoplast or colonize the surface of the root, however, they have not been shown to induce structural changes in the host plant. Alpha- and Betaproteobacteria have been confirmed as associative nitrogen fixers. Genera that are confirmed to be endophytes include *Azoarcus*, *Herbaspirillum*, and *Glucenobacter* [123].

The relationships between associative nitrogen fixers has been studied in rice, maize, and wheat, in hopes of transferring a nitrogen fixing symbiosis similar to that of the RNS. The extent to which associative nitrogen fixers contribute biologically available nitrogen to these plants has been shown to be variable depending upon host genotype and growth stage, bacterial strain, and environmental conditions [123].Mutants of *Azospirillum* spp. and *Azoarcus* spp. lacking the ability to fix nitrogen are still able to promote plant growth [124,125].

1.5.3. Endophyte colonization and transmission

The manner in which endophytes colonize plants can vary based on the plant species, plant tissue, plant genotype, microbial species, microbial genotype, and environmental factors. Bacterial endophytes most often originate from the rhizosphere rather than the phyllosphere, partly due to their attraction to certain root exudates and rhizodeposits [126,127]. Bacteria can enter between epidermal cells, at the sites of lateral root emergence, wounds, or intracellularly through root hair infection as during certain nodule formation [91]. Endophytes are able to migrate from the rhizosphere through the root cortex. The endodermis limits further colonization beyond the cortex [126,128]. Endophytes capable of bypassing the endodermis reach the xylem vasculature, and are then capable of systemic colonization [129]. From the xylem vessels, bacteria have been shown to travel to the reproductive organs of both angiosperms and gymnosperms, ultimately colonizing the seed [130-132].

Seeds can carry diverse endophytes that can be conserved independently of the soil environment [133,134]. The seed-carried endophytes are ensured by the host plant to be present for the next generation, and may not require any further infection. Some vertically transmitted bacteria have been shown to become epiphytes and colonize the surrounding host plant environment post-germination [96,135]. Maize transmission of

seed endophytes has been shown to be at least partly conserved from wild ancestors to modern varieties across evolutionary time-scales [119]. Pathogens have also been found to be carried in plant seeds [136,137].

1.6. *Pediomelum esculentum* background

Pediomelum esculentum, commonly called prairie turnip or Indian breadroot, is a perennial legume indigenous to the Great Plains and eastern foothills of the Rocky Mountains. Its range stretches from Oklahoma, north into Canada, and from Wisconsin to Western Montana. The plant prefers rocky well-drained soils where it puts down a deep taproot system. The plant breaks dormancy in the early spring from a large tuber that can grow over 5 cm in diameter. The tuber gives rise to a woody crown about 2 cm below ground from which the herbaceous top emerges to produce blue to purple papilionaceous flowers [138]. The plant completes its seasonal cycle in approximately two months, flowering and senescing by mid-July when the top breaks away from an abscission layer on the crown to spread seeds as a tumbleweed [139]. In mature *P. esculentum* the tuber is surrounded by a woody bark. The tuber is comprised of mostly xylem parenchyma where protein and starch are enclosed in proteinoplasts or amyloplasts, which are stored separately in adjacent cells (Figure 3) [138,140]. The tuber contains over of 7.5% protein and 70% starch, making it a significant source of protein and carbohydrates [141].

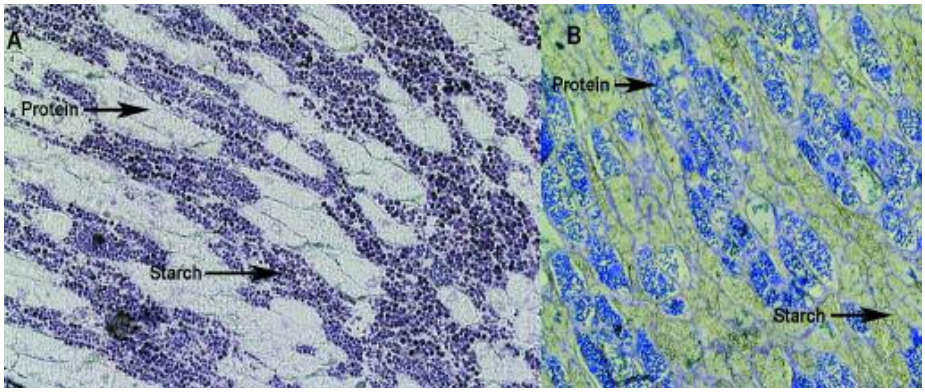

Figure 3. Cross section of *P. esculentum* tuber stained to show protein and starch. (a) Potassium iodide staining shows the starch as purple grains and (b) Coomassie Blue staining presents the protein as blue and starch as grayish green. Distinct proteinoplasts and amyloplasts can be in separate adjacent cells. Taken from [138].

In a 1939 study testing the cross-inoculation of rhizobia isolated from different legumes, *P. esculentum* was found to form nodules when inoculated with rhizobia isolates from *Caragana frutescens*, *Glycine max*, *Oxytropis lambertii*, *Phaseolus vulgaris*, *Stizolobium deeringianum*, and *Vicia villosa* [142]. In this study, no bacteria were isolated from nodules on *P. esculentum*, and no images of nodules on the root were published. In an earlier 1937 study of native legumes in Wisconsin, one strain from wild collected nodules was isolated. The isolate was described as being very short monotrichous rods. They were unable to test the isolates ability to nodulate *P. esculentum* due to a lack of seeds. The authors noted seeds were as difficult to come across as nodules on the roots [143].

P. esculentum is part of the Psoraleae tribe within the Phaseoloid clade of Papilionoideae. The Phaseoloid clade, further comprised of the Desmodeae and Phaseoleae tribes, is the only group in the Papilionoideae to form determinate, uriede exporting nodules. Nodule studies of the Phaseoloid clade have been limited to 42 of 128 genera and only tropical and sub-tropical species [29]. The Psoraleae tribe contains six genera with 29 species in the *Pediomelum* genus with three known to nodulate. Psoraleae began its divergence in North America around 5.8 MYA with *Pediomelum* arising around 4.8 MYA and *esculentum* diverging around 1.72 MYA. This rapid rate of diversification in *Pediomelum* was likely influenced by glacial cycles driven by climate shifts [144].

The aim of this work is to characterize the nitrogen fixing symbiotic partnerships maintained by *P. esculentum*. The lack of nodules observed on wild harvested plants, large stores of nitrogen, curious growth behaviors, and recent rapid evolution has prompted this investigation.

2. Materials and methods

2.1. Collection of endophytes

2.1.1. Initial collection from mature P. esculentum

Mature plants were collected from four different sources. Wild plants were harvested from the South Dakota State University Oak Lake Field Station in Eastern SD and south of Kyle, SD on the Pine Ridge Reservation. Cultivated plants were harvested

from the Oglala Lakota College Agriculture Research Extension grounds in Kyle, SD. The fourth source was obtained as rootstock from Prairie Moon Nursery (Winona, MN) and were propagated in 1.5" conetainers (Stuewe and Sons) in a greenhouse for 3 weeks prior to being harvested. Each plant was estimated to be at least three years old. All harvested plants were uprooted, wrapped in a moist paper towel, and placed on ice. Endophyte isolation was performed within 24 hours.

Prior to isolation, plants were washed of soil with a brush and detergent. They were then surface sterilized with 96% ethanol for 30s, rinsed in sterile water for 30s, 10% bleach for 5 min, 96% ethanol for 30s, and finally rinsed four times with sterile water [145]. Plants were transferred to a laminar flow hood and separated into three portions: shoot, edible tuber, and inedible tuber coat including the taproot. Thin sections, approx. 1 cm x 5 cm, were cut from each portion and placed onto nitrate-free MS media [146] (Caisson Labs) containing 5 g/l of both glucose and sucrose and 1.5% (w/v) noble agar. Sterilized roots were rolled on MS plates prior to sectioning to check for inadequate sterilization and epiphyte growth; samples with epiphyte growth were discarded. Plates were incubated under 2% oxygen inside a chamber equipped with an automatic gas oxygen sensor at 28 degrees Celsius for 21 days [147]. Bacterial growth extending from the plant tissue was selected and streaked onto the same media and incubated as before. Morphologically distinct colonies were selected and incubated under the previous conditions on a nitrogen free medium (NFM) containing the following (g/l): K_2HPO_4, 0.2; KH_2PO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; $FeSO_4 \cdot 7H_2O$, 0.1; $Na_2MoO_4 \cdot 2H_2O$, 0.005; NaCl, 0.2; Glucose, 5; Sucrose, 5.

2.1.2. Inoculation and re-isolation of endophytes

P. esculentum seeds were acid scarified with concentrated sulfuric acid for 30 min, rinsed, and surface-sterilized as previously described. Seeds were planted in 1.5" conetainers (stuewe.com), previously washed with a 10% bleach solution and containing a 2:1 (v/v) autoclaved mixture of vermiculite:perlite. Plants were watered with nitrogen free plant nutrient solution (N- PNS) (Table 1). The growth chamber was operated on a cycle consisting of 16 h of light at 25°C and 40% relative humidity followed by 8 hours of dark at 20°C and 40% relative humidity. Seedlings were broken into three treatment

groups: co-inoculated with 9 *Rhizobium* isolates, inoculated with one *Burkholderia* isolate, and non-inoculated. For inoculation, each isolate was individually grown in liquid MS media supplemented with 0.4 g/l of yeast extract and 5 g/l of both glucose and sucrose. Cultures were collected by centrifugation and re-suspended in N- PNS to OD_{600} = 0.08. The 9 *Rhizobia* suspensions were mixed in equal parts. The seedlings were flooded with the respective suspensions at 7 days post germination [148]. Bacteria were isolated from the nodules and tubers after 4 weeks as previously described.

Table 1. Nitrogen Free Plant Nutrient Solution (N-PNS).

Macronutrients	Concentration (µM)
$MgSO_4 \bullet 7H_2O$	496
$CaCl_2 \bullet 2H_2O$	2270
$K_2HPO_4 \bullet 3H_2O$	149
K_2SO_4	1262
$FeCl_3 \bullet 6H_2O$	38.2
Micronutrients	
H_3BO_3	2.3
$MnSO_4 \bullet H_2O$	0.455
$ZnSO_4 \bullet 7H_2O$	0.6
$CuSO_4 \bullet 5H_2O$	0.15
$NaMoO_4 \bullet 2H_2O$	0.1
$CoCl_2 \bullet 6H_2O$	0.01
$NiSO_4$	0.006

2.1.3. Isolation from seeds

P. esculentum seeds were acid scarified with concentrated sulfuric acid for 30 min, rinsed, and surface-sterilized as previously described. Seeds were allowed to germinate on water agar plates (1.5% w/v) for 5 days. The germinated seeds were again surface sterilized in a laminar flow hood as previously described, sliced with a sterile scalpel, and laid onto MS plates. Incubation and culture of endophytes followed the same procedures from the mature plants.

2.2. PCR and phylogenetic analysis

2.2.1. PCR of 16S rRNA

Genomic DNA was extracted from each isolate using the Quick-gDNA MicroPrep kit (Zymo Research). Partial *16S rRNA* was amplified using the universal bacterial primer pair, 27F/518R (Table 2). The amplicon was approximately 491bp, spanning the variable regions V1 to V3. PCR was carried out in 30 ul reactions containing 0.5 U of Taq DNA polymerase (New England Biolabs), 1x PCR buffer, 2 mM MgCl2, 200 μM dNTPS, 0.2 μM of each primer, and approximately 10 ng of template DNA. Reactions consisted of initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation (30s at 95°C), annealing (30s at 58°C), extension (60s at 72°C), and followed by a final extension for 72°C for 7 min. The products were resolved on a 1.2% agarose gel.

2.2.2. PCR of nifH

Isolates were screened for the presence of *nifH* using the primer pair PolF/PolR (Table 2). 30 μl PCR reactions consisted of 0.5 U of Taq DNA polymerase (New England Biolabs), 1x PCR buffer, 2 mM MgCl2, 200 μM dNTPS, 0.2 μM of each primer, and approximately 10 ng of template DNA. The following touchdown program was used for amplification: initial denaturation for 5 min at 95°C, followed by 8 cycles of 95°C for 30 s, 63°C for 25 s (decreasing 1 degree every cycle) and 72°C for 1 min, followed by 22 cycles of 95°C for 30 s, 55°C for 25 s, 72°C for 1 min, and a final extension of 72°C for 7 min. The 362bp product was resolved on a 1.2% agarose gel.

2.2.3. GC-clamp PCR of nifH and denaturing gradient gel electrophoresis

DNA was purified from three separated portions of *P. esculentum*: the taproot, the shoot, and the edible tuber. DNA was purified using the PowerPlant Pro DNA Isolation kit (MO BIO Laboratories) and *nifH* was amplified following the method previously described, except in 50 μl volumes and using a GC-clamp modified PolF primer (Table 2). The GC clamp was adapted from [149]. The entire 50 μl product was resolved on a 1.2% agarose gel and the approximately 400bp band was extracted from the gel using Zymoclean Gel DNA Recovery kit (Zymo Research). The extracted products were

loaded onto a 6% polyacrylamide denaturing gradient gel, where 100% is equivalent to 7 M urea and 40% (v/v) formamide. The Dcode system (BioRad) was used to perform DGGE in 1X TAE at 70 V and 60°C for 16 hours [149]. DGGE Gels were stained with SybrGold (Invitrogen).

Table 2. Primers used in this work.

PRIMER PAIR	TARGET GENE/ PRODUCT LENGTH (BP)	SEQUENCE (5'-3')	REF
27F/518R	Universal *16S rRNA*/491	GAGTTTGATCMTGGCTCAG/ATTACCGCGGCTGCTGG	[150,151]
POLF/POL	*nifH*/362	TGCGAYCCSAARGCBGACTC/ATSGCCATCATYTCRCCGGA	[152]
GC CLAMP POLF	*nifH*/400	CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCGCT GCGAYCCSAARGCBGACTC	

2.2.4. Phylogenetic analysis of 16S rRNA

Sequences were quality checked and trimmed with 4peaks (http://www.nucleobytes.com) and closest relatives identified using the SILVA Incremental Aligner [153]. Alignment of the isolate and reference sequences was performed using ClustalW [154]. The evolutionary history was inferred by using the maximum likelihood method based on the Tamura-Nei model [155]. The trees with the highest log likelihood are given in the results. 1000 bootstrap replicates were completed with the percentage of trees in which the associated taxa clustered together shown below the branches. Initial trees for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6 [156].

2.3. Microscopic observations

Freshly harvested tubers and nodules were hand sectioned with a double-sided razor blade and stained with the green fluorescent nucleic acid stain SYTO13

(Invitrogen) according to Haynes et al. (2004) except without fixation. Hand cut sections were transferred to 1µl/ml of SYTO13 in DH_2O for 15 minutes. Tubers and nodules were imaged with or without a propidium iodide counter stain, which stains nucleic acids and cell walls. Following SYTO13 staining, sections were transferred to 5µl/ml of propidium iodide in DH_2O for ten minutes and then washed in DH_2O. Confocal images were collected with an Olympus Fluoview FV1200 laser scanning confocal system interfaced with an inverted IX81 microscope. SYTO13 was excited with a 488nm laser and emission acquired through a variable barrier filter from wavelengths 500nm to 545nm. Propidium iodide was excited at 559nm with emission collected between 600nm and 645nm. Interference from autofluorescence was tested on unstained sections. Single optical sections or a z-series of optical sections were collected. Maximum-intensity z-stacks and 3D projections were generated using the FIJI processing package of ImageJ software [157].

Endophytes were also visualized using tetrazolium chloride (TTC). Surface sterilized whole roots were soaked for 2-3 days in a filter sterilized 0.05 M potassium phosphate solution (pH 7.0) containing 1.5g/L TTC and 625 mg/L malic acid. [158]. Tubers and nodules were then sectioned by hand using a double-edged razor blade. Select TTC soaked sections were further stained with SYTO13 as previously described and imaged with an epiflourescence Olympus BX53 microscope equipped with an X-Cite 120LEDmini light source (Excelitas Technologies) and a band pass 515-550nm filter.

2.4. Nitrogen treatments and acetylene reduction assays

Seeds were acid scarified and surface sterilized as previously described. Seeds were then planted in 1.5” conetainers (stuewe.com), containing a 2:1 (v/v) autoclaved mixture of vermiculite:perlite. The plants were divided into three groups given different variations of N-PNS (Table 1). The treatment groups consisted of N-PNS, N-PNS with 5 mM KNO_3, or N-PNS with 5mM $(NH_4)_2(SO_4)$. Whole plants were harvested after 4 weeks, rinsed and placed in a 40ml test tube with moist filter paper. The method for acetylene reduction was adapted from [159]. The tube was sealed with a Suba-Seal rubber stopper (Sigma-Aldrich). One percent of the headspace was exchanged with acetylene. Following 10 days of incubation, the concentration of ethylene gas was

determined using a gas chromatograph (Agilent Technologies 7890A) equipped with a flame ionization detector and an Agilent CP7348 column. Plants without the addition of acetylene and containers with only acetylene served as controls for ethylene production by the plant and the spontaneous conversion of acetylene to ethylene or contamination.

2.5. Ureide concentration analysis

Total ureides were determined with the method proposed by Goos et al [160]. After the acetylene reduction assay, whole plants were dried, weighed, and ground for the ureide assay.

2.6. Statistical analysis

Statistical analysis was performed in R using the stats, Rmisc, and agricolae packages.

3. Results

3.1. Isolation of endophytes

A total of 38 unique isolates were obtained from mature harvested *P. esculentum* (Appendix: Table A1). No nodules were observed on the mature plants prior to isolation (Figure 4c). All of the isolates exhibited the ability to grow on nitrogen-free media. Unique isolates were defined as having unique morphology among the other colonies formed from the same tissue of the given plant. Bacteria were isolated from three tissue types (shoot, tuber, and peel) from three separate plants harvested at each site for a total of 12 plants. Many of the initial plates were discarded without successful isolation due to an overgrowth of fungi. Isolation was most successful from plants obtained as rootstock and grown for a period of time in the greenhouse. These plates had considerably less fungal growth than plates inoculated with tissue from wild and outdoor cultivated plants. Qualitatively, there was no difference in the diversity or abundance of the isolated bacteria at the genus or class level between plants, tissue type, or location (Appendix: Figures A7-A10). Figure 5 shows products resulting from *16S rRNA* PCR with the 27F/518R primer pair. Results of the *16S rRNA* phylogenetic analysis is presented in

Figure 6, and includes isolates from the inoculation experiment as well those found in the sterile seeds.

Isolates were screened for *nifH* using the primer set PolF/PolR (Table 2). 14 of 38 isolates presented a band corresponding to the correct length (362 bp) as tested with a known positive control (Figure 7). Sequencing of the PCR products did not yield any sequences related to previously identified *nifH* sequences in the GenBank. Comparison of these sequences with those of the *nifH* database constructed by Gaby and Buckley [161] placed all sequences in phylogenetic cluster IV of the *nifH* gene tree, a branch of paralogous *nifH* genes.

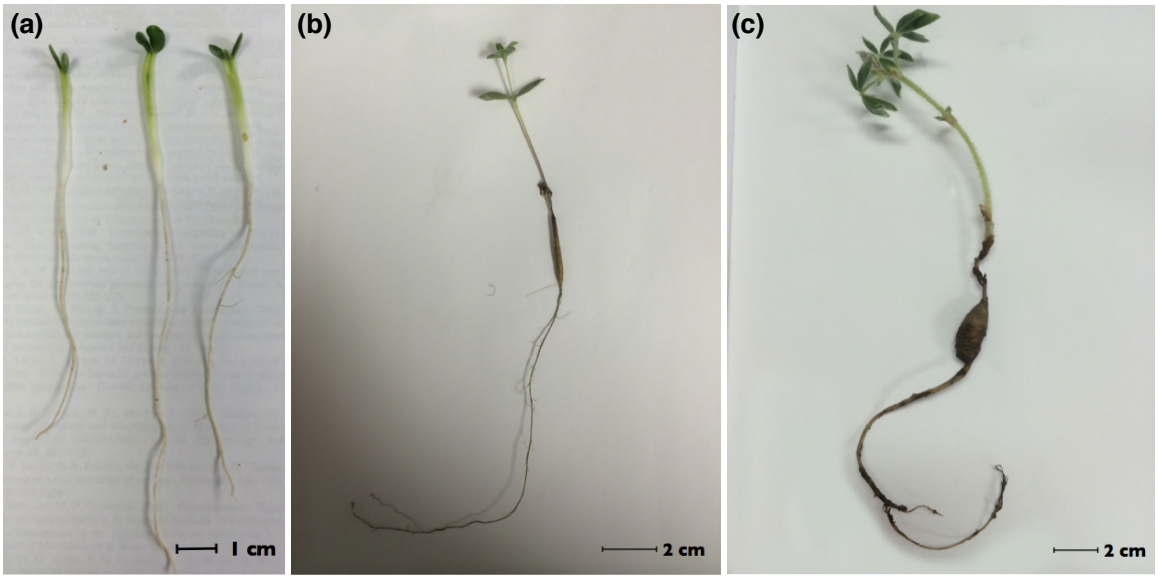

Figure 4. Development of *P. esculentum*. (a) Seedlings two weeks post germination. (b) 8 weeks post germination. (c) Mature plant estimated to be 3 years old.

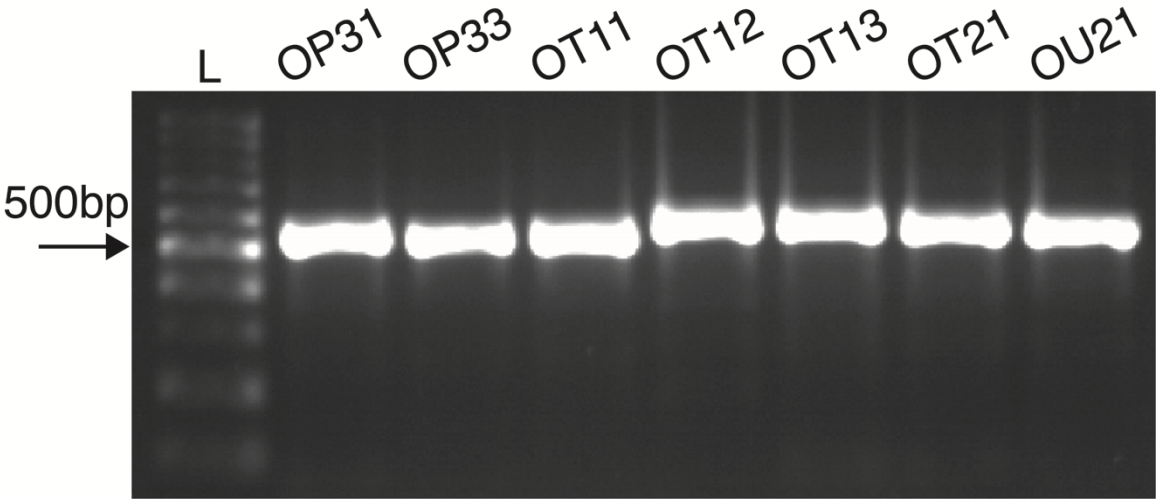

Figure 5. Representative example of partial *16S rRNA* PCR amplification from isolates. The primer pair 27F/518R produced approximately 500bp bands. Resolved on a 1.2% agarose gel. Shown with 100bp ladder and isolate ID.

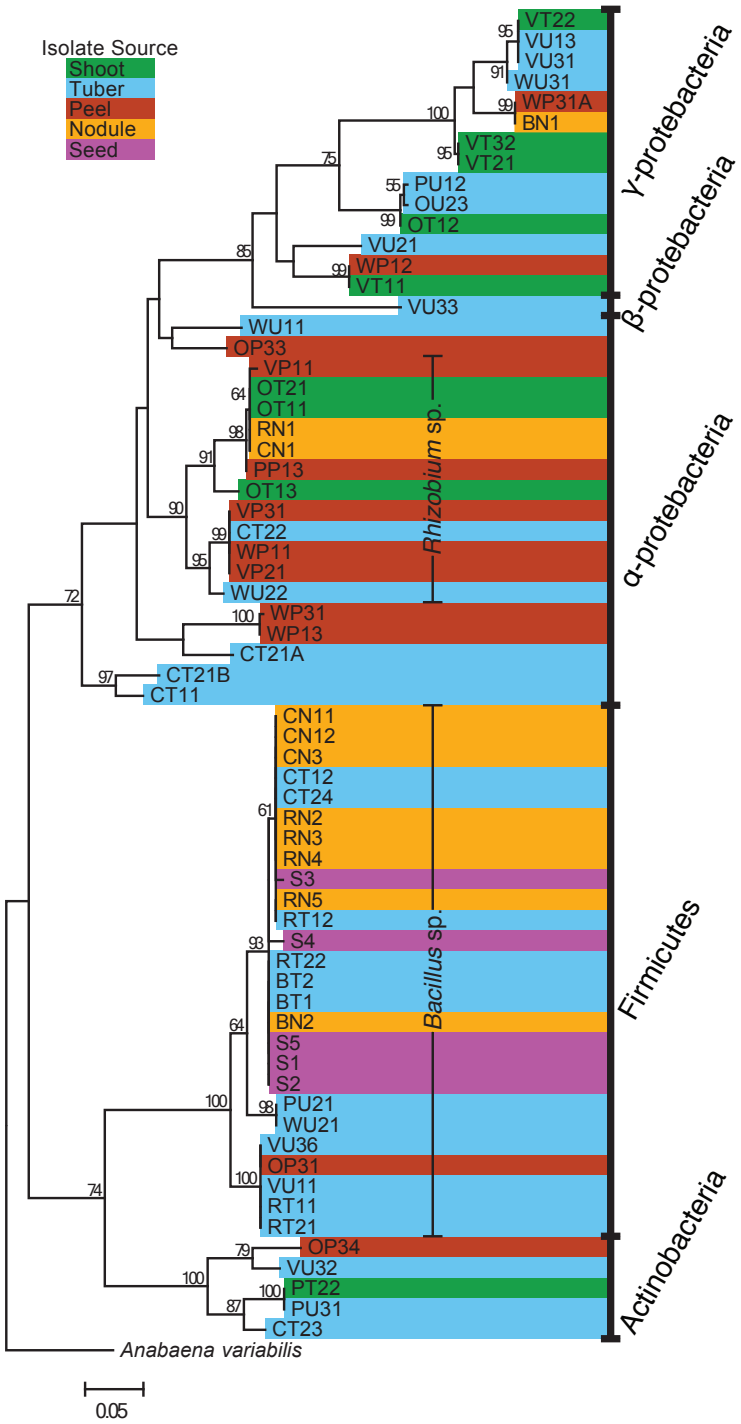

Figure 4

Figure 6. Phylogenetic tree based on *16S rRNA* of all isolated bacteria. Color indicates plant tissue from which the isolate was extracted. The maximum-likelihood tree is based on partial *16S rRNA* sequences of approximately 500bp including variable regions V1-V3. Bootstrap values shown are based on 1000 repetitions. Scale bar indicates number of substitutions per site.

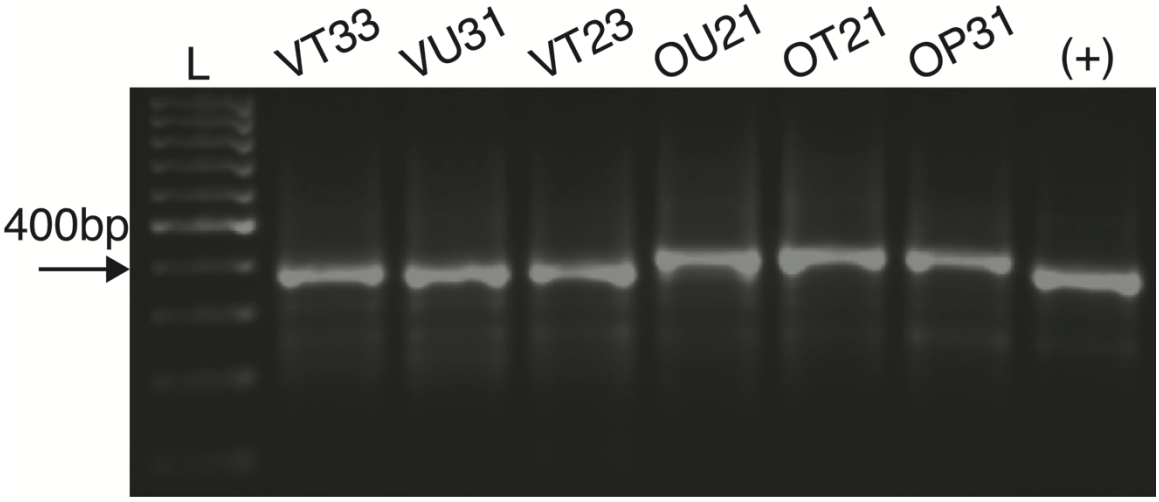

Figure 7. Representative example of partial *nifH* PCR amplification from isolates. The primer pair polF/polR produced approximately 360bp bands. Resolved on a 1.2% agarose gel. Shown with 100bp ladder and isolate ID.

3.2. Inoculation and subsequent re-isolation

Plants grown from seed were inoculated with either a co-mixture of 9 *Rhizobia* isolates, a single *Burkholderia* isolate, or no bacteria. The 9 Rhizobia isolates included the following isolate IDs: OT11, OT13, OT21, PP13, VP11, VP21, VP31, WP11, and WU22. The *Burkholderia* isolate applied was VU33 (Appendix: Table A1).

Plants in all three treatment groups were found to form nodules with no significant difference in the number of nodules formed (Figure 8). There was no discernable difference between plants or signs of nitrogen starvation in any of the groups. Isolation of bacteria from the tuber and nodules of these plants on NFM and subsequent identification with partial *16S rRNA* analysis (Figure 6, Appendix: Table A2) only recovered a single Rhizobium present in the Rhizobia inoculum applied. Three isolates from the non-inoculated control plants were closely related to the Rhizobium previously identified from the mature plants. Other isolates included a Gammaproteobacteria (*Pantoea* sp.) isolated from a nodule found in the root of a mature plant, three Alphaproteobacteria (2 *Sphingobium* sp., 1 *Methylobacterium* sp.) not previously isolated, and a single Actinobacteria (*Mycobacterium* sp.) not previously isolated.

The majority of isolates (14/24) from the three treatment groups were most closely related to *Bacillus nealsonii* or *Bacillus circulans*. The same *Bacillus* spp. were found in both the nodules and tubers of inoculated and non-inoculated plants. The only isolates obtained from seeds were also closely related to these *Bacillus* spp. (Appendix: Table A3) Complete sequencing of the *16S rRNA* and phylogenetic analysis of a seed isolate (S2) showed the bacterium was deeply rooted among *Bacillus* but within an ill-defined clade with low bootstrap support (Figure 9).

Six of the *Bacillus* spp. isolated from the inoculation experiment produced the expected 361bp *nifH* PCR amplicon with the PolF/PolR primer pair. However, the sequences of these PCR products did not match to any previously identified *nifH* products in the NCBI GenBank. None of the isolates from seeds produced the expected *nifH* product.

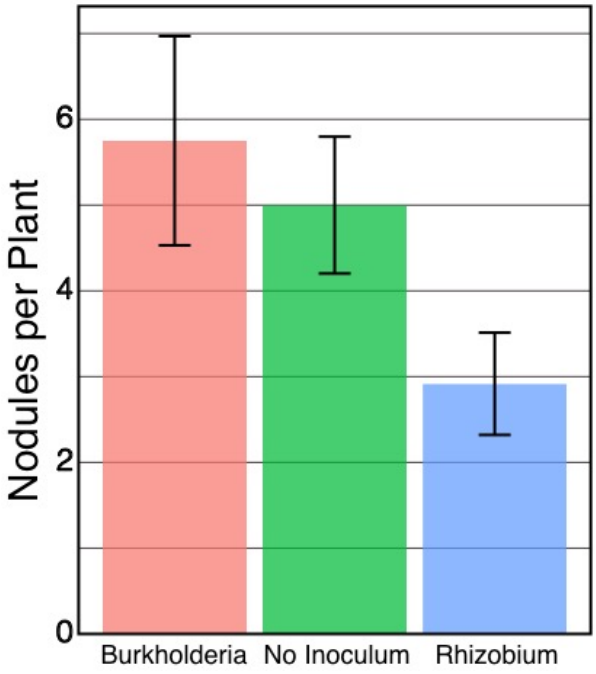

Figure 8. Number of nodules observed per plant for each inoculum treatment. An ANOVA test indicated no significance between means.

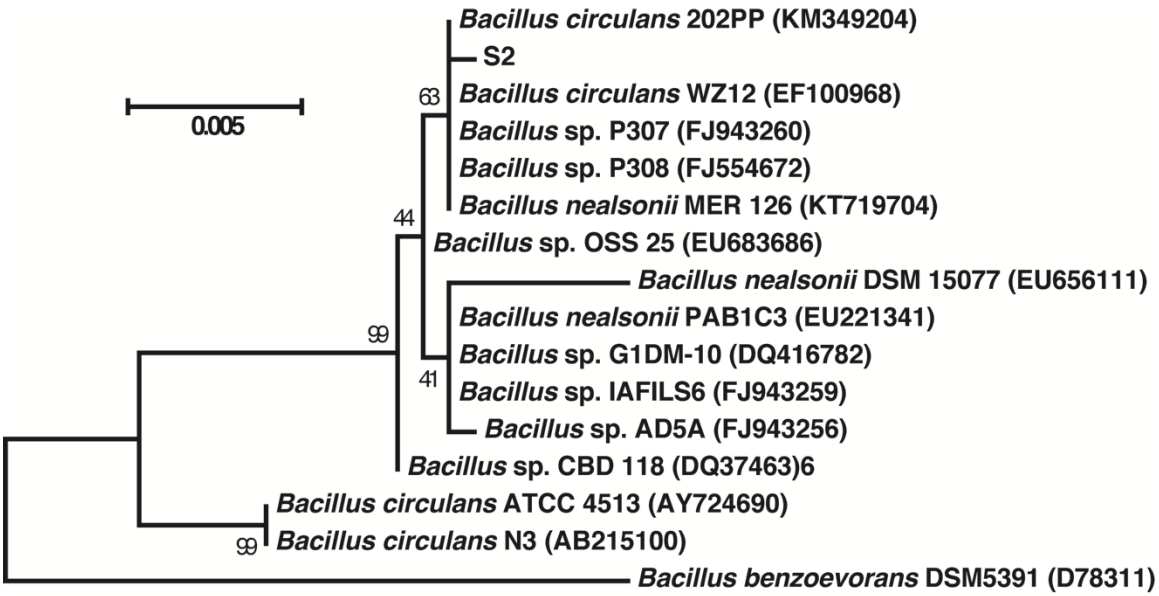

Figure 9. Phylogenetic tree based on whole *16S rRNA* of the S2 *Bacillus* spp. isolate. The maximum-likelihood tree is based on complete *16S rRNA* sequences. Reference sequences were obtained from SILVAngs database for reference. Accessions numbers in parenthesis. Bootstrap values shown are based on 1000 repetitions. Scale bar indicates number of substitutions per site.

3.3. Denaturing gradient gel electrophoresis of *nifH*

The diversity of *nifH* amplified from DNA extracted from the tissue of three mature plants was assessed with DGGE. The partial *nifH* was amplified using a forward primer modified with a GC-clamp (Figure 10). The DGGE profiles (Figure 11) of the root exhibited a single predominant product with no diversity. The shoot had a different profile with slight diversity.

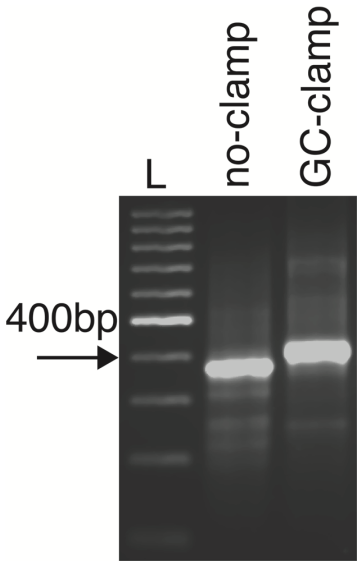

Figure 10. Representative example of partial *nifH* PCR amplicons obtained using GC-clamp primers. The primer pairs polF/polR and GC-polF/polR produced approximately 360bp and 400bp products, respectively. Resolved on a 1.2% agarose gel. 100 bp ladder.

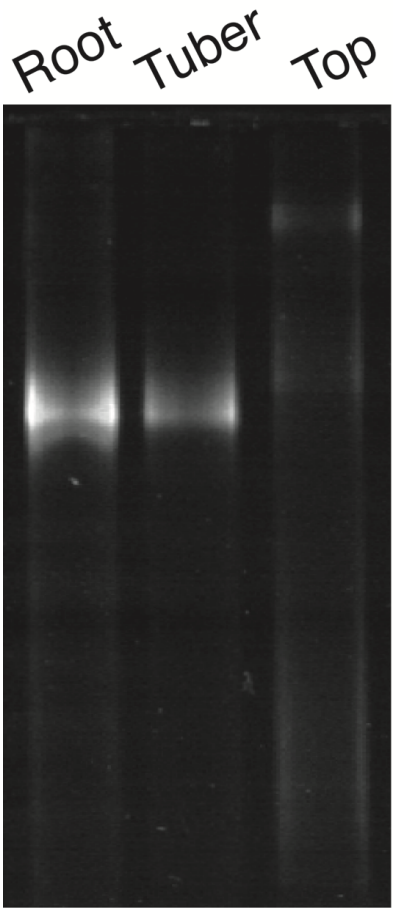

Figure 11. DGGE profile of *nifH* sequences across three tissue types of a mature plant. Partial *nifH* sequences were amplified using a GC-clamp forward primer and resolved with a 35-65% density gradient. Profiles of the root and tuber show the same limited diversity while the shoot profile exhibits a different slightly broader diversity.

3.4. Nitrogenase activity and ureide content in *P. esculentum*

Plants were given either ammonia or nitrate as a sole nitrogen source or given no nitrogen. Figure 12 compares the mass, ureide content, and nitrogenase activity as measured by acetylene reduction between the three groups. There was no difference in mass between the groups. The plants given ammonia showed diminished nitrogen fixation (2.23 μmol of C_2H_4 per g of total plant dry weight per hour) while those given nitrate fixed significantly more ($p < 0\ .05$) and reduced 5.13 μM of C_2H_4 per g of total plant dry weight per hour. Plants not given a nitrogen source showed no significant difference in fixation rate from those given nitrogen. Total ureide content was assessed as an indicator of nitrogen fixation. All three treatment groups significantly differed in their

ureide concentration. Plants given ammonia had the highest concentration (1524.63 mg/kg) followed by those given nitrate (796.82 mg/kg), and those with no nitrogen source (328.04 mg/kg).

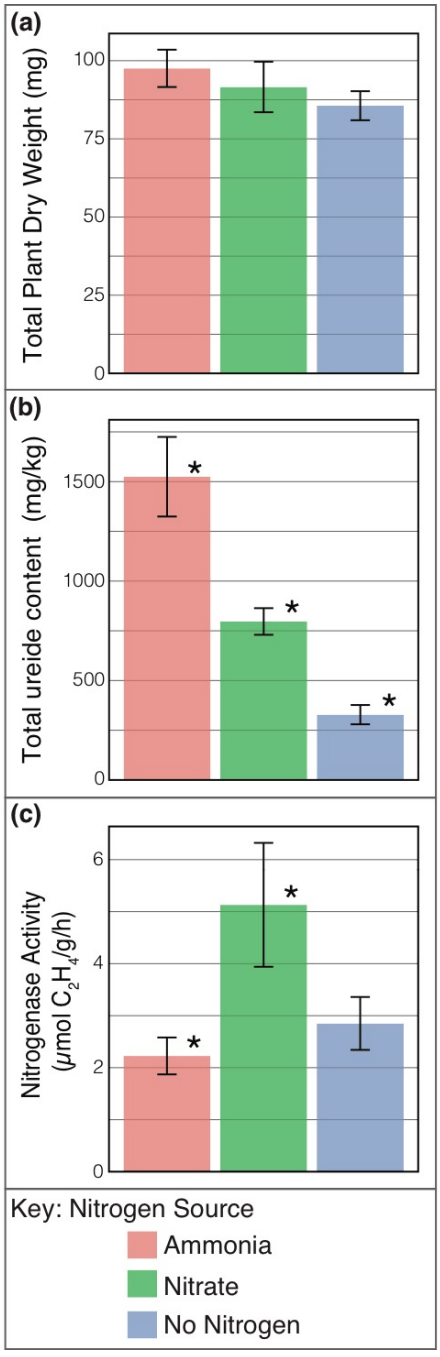

Figure 12

Figure 12. Mass, ureide content, and nitrogenase activity of plants supplemented with ammonia, nitrate, or no nitrogen. (A) Total plant dry mass measured after acetylene reduction assay. (B) Total ureide content measured per total plant dry mass. (C) Nitrogenase activity as measured by the acetylene reduction assay. Rates are based on total plant dry mass. * indicates a significantly different mean as determined with ANOVA ($p < 0.05$).

3.5. Imaging of nodules and endophytes

Inoculated and uninoculated plants grown in gnotobiotic conditions were imaged after 4 weeks of growth. Bacteria were visualized with SYTO13 and TTC within the intercellular space of the tuber of all plants. Surface sterilized seeds were imaged 5 days post germination in a petri dish and also contained bacteria within the intercellular space of the sectioned developing root (Figure 13). Prolific bacteria were also observed on the outer epidermis of the root of the developing root (Figure 14). SYTO13 staining also exhibiting staining patterns that show bacteria inside the xylem parenchyma cells of the tuber (Figure 15). A 3D rendering of the confocal slices in the z plane show that SYTO13 was localized inside of the cell. The staining was observed in rays of cells spreading from the central vascular bundle and extending out towards the outer cortex. Rays of cells adjacent to those with SYTO13 staining did not stain.

Plants were observed with both determinate nodules and intermediate structures between that of a nodule and lateral root (Figure 16). Both of these were typically associated with a lateral root. Sectioning and subsequent SYTO13 staining of the determinate nodules reveals interspersed infected and uninfected cells. No infection threads were observed. A central vasculature can be seen, as well as infected cells around the main root vascular bundle opposite the nodule infected zone (Figure 17). The bacteria appear as individual cells, and no bacteroids were observed (Figure 18). Staining of the intermediate nodule structures showed tightly packed infected cells and bacteria residing in the intercellular space (Figure 19).

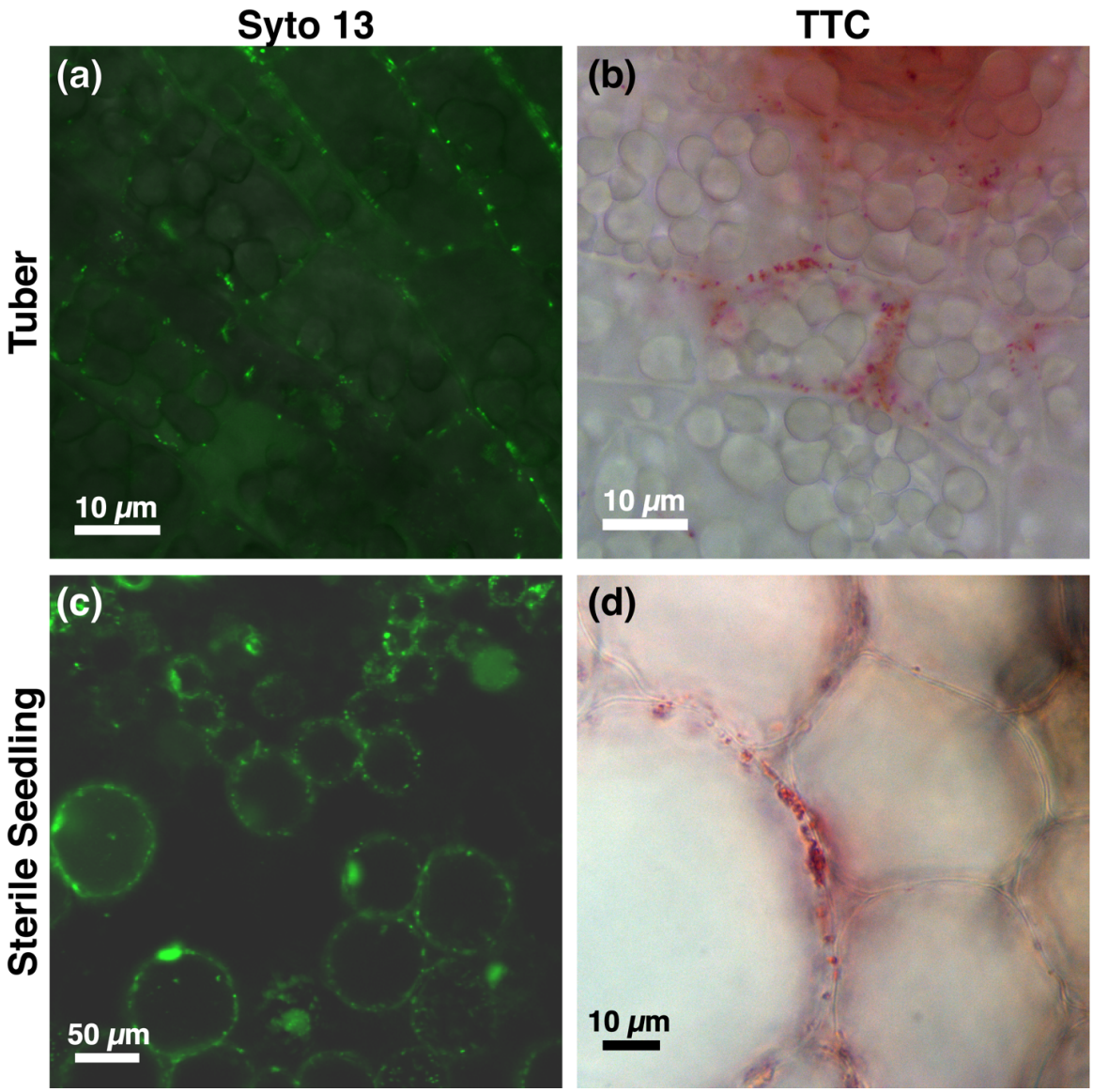

Figure 13. Imaging of bacterial cells in the intercellular space of tubers and sterile seedlings. SYTO13 is used to stain the nucleic acids (green) and TTC (red) used to indicate cellular respiration. A cross section of a 4-week old tuber stained with SYTO13 (a) and TTC (b) shows bacteria are residing within the intercellular spaces of the xylem parenchyma. A cross section of a seedling germinated under sterile conditions reveals bacteria in the intercellular space of the developing root shoot. 5 separate observations were made both the seedlings and tubers.

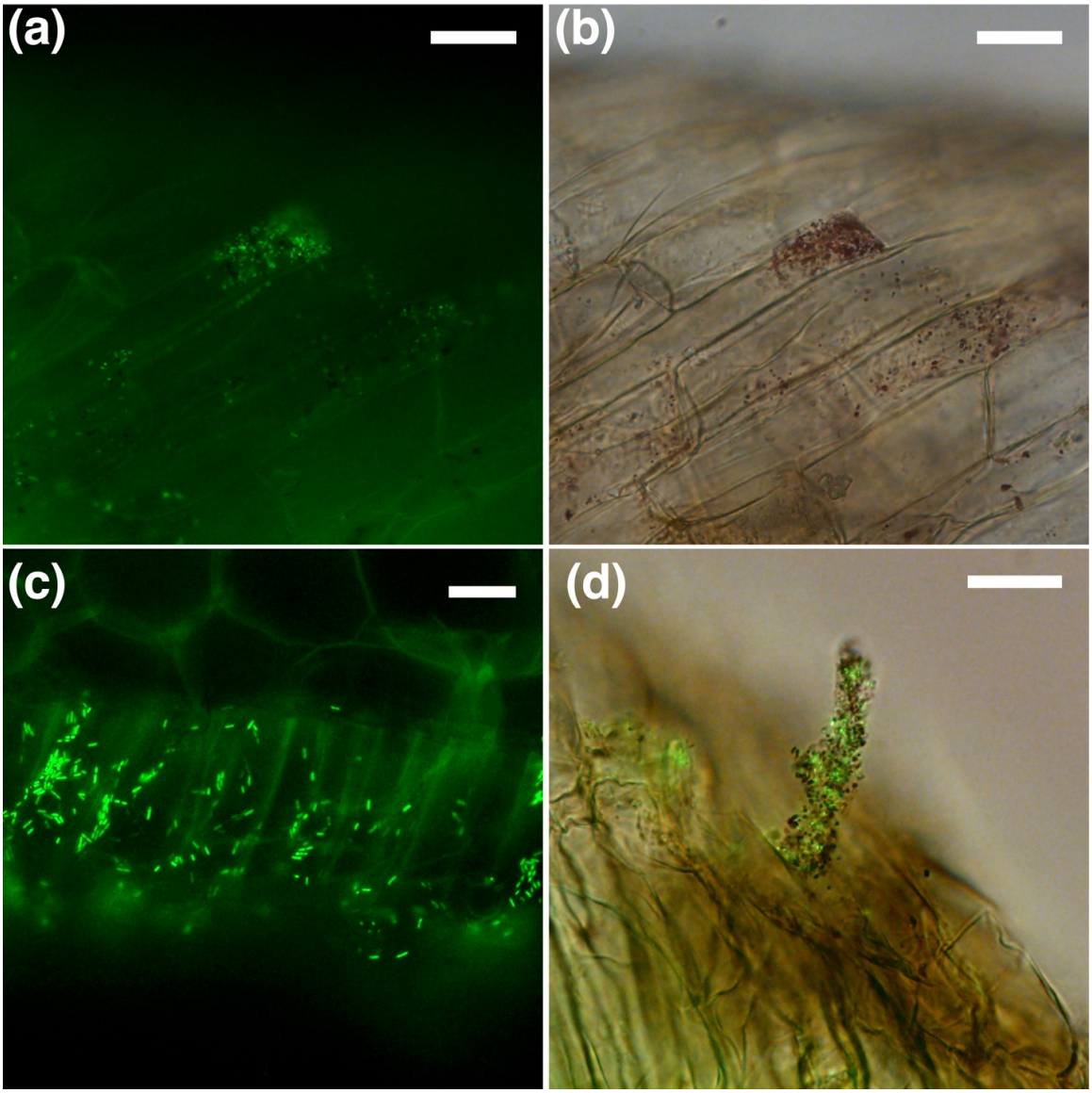

Figure 14. Bacteria of the root epidermis in seedlings 5 days post germination. SYTO13 is used to stain the nucleic acids (green) and TTC (red) used to indicate cellular respiration. Epiflourescent (a) and brightfield (b) microscopy (b) show that the SYTO13 and TTC are co-localized where bacteria are present on the epidermis. Confocal scan of a root cross section (c) shows bacteria attached to the epidermis of the root. Overlay of an epifluorescence and brightfield of a root hair like structure is seen to be infected with bacteria (d). All scale bars = 20 μM.

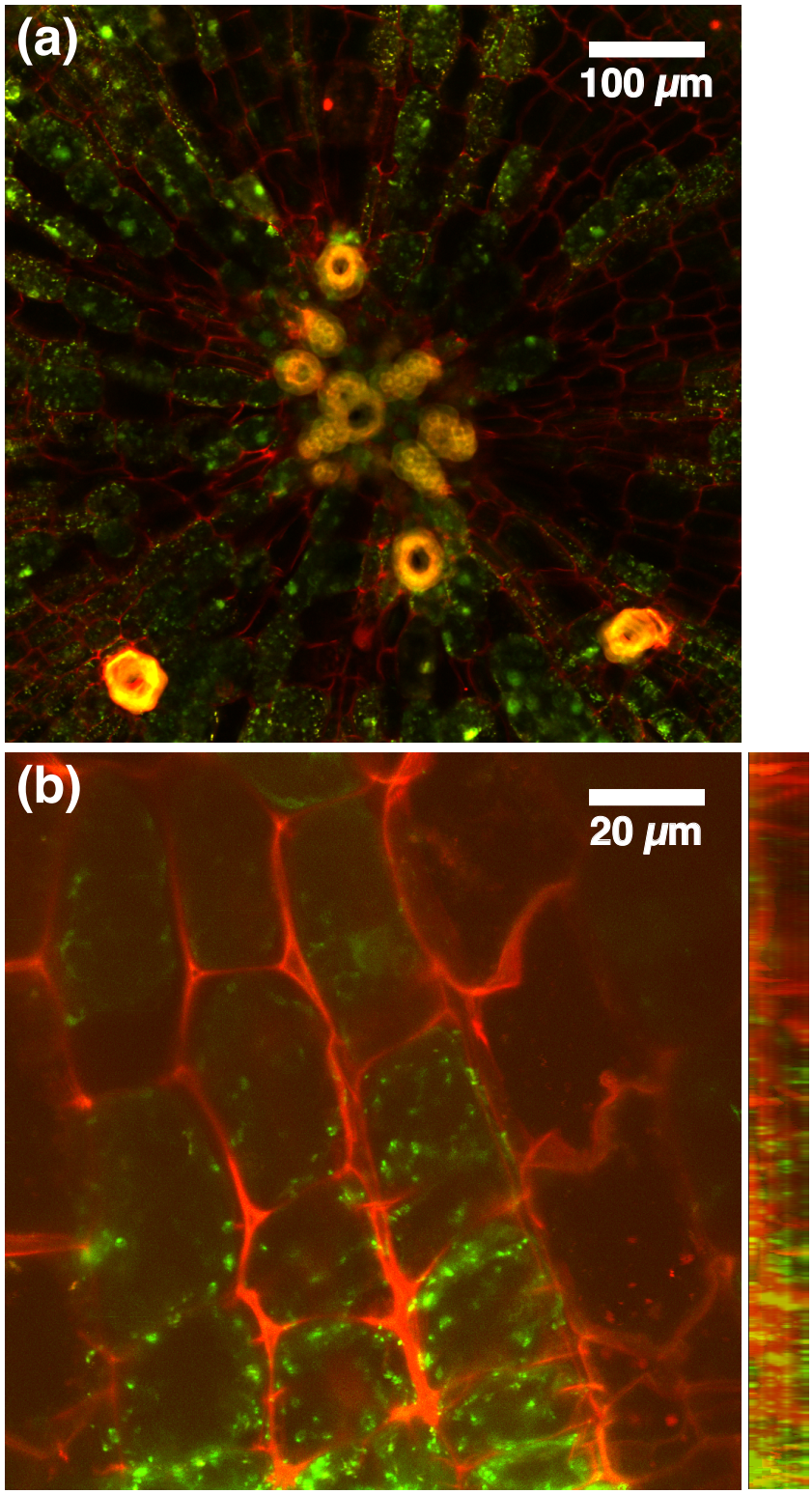

Figure 15. Laser scanning confocal imaging of a tuber cortex stained with SYTO13 and propridium iodide. (a) Rays of infected cells adjacent to uninfected cells. (b) Z-stack of 7 optical sections taken every 1.5 μm. Maximum intensity z projection. 1 of 9 tubers observed.

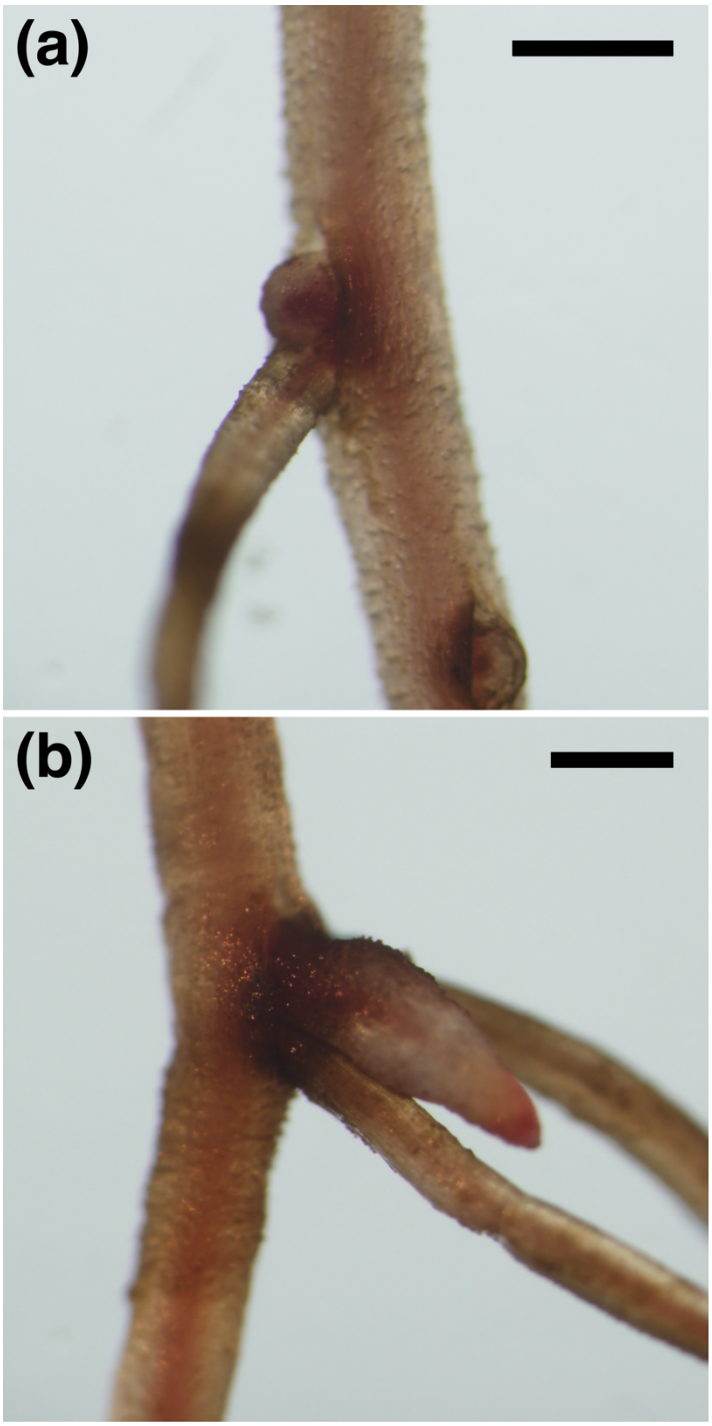

Figure 16. Nodule development in *P. esculentum*. Roots were soaked in TTC solution prior to sectioning. (a) A typical nodule emerged from a lateral root junction. (b) An intermediate nodule structure resembling a lateral root. Scale bars are 1 mm.

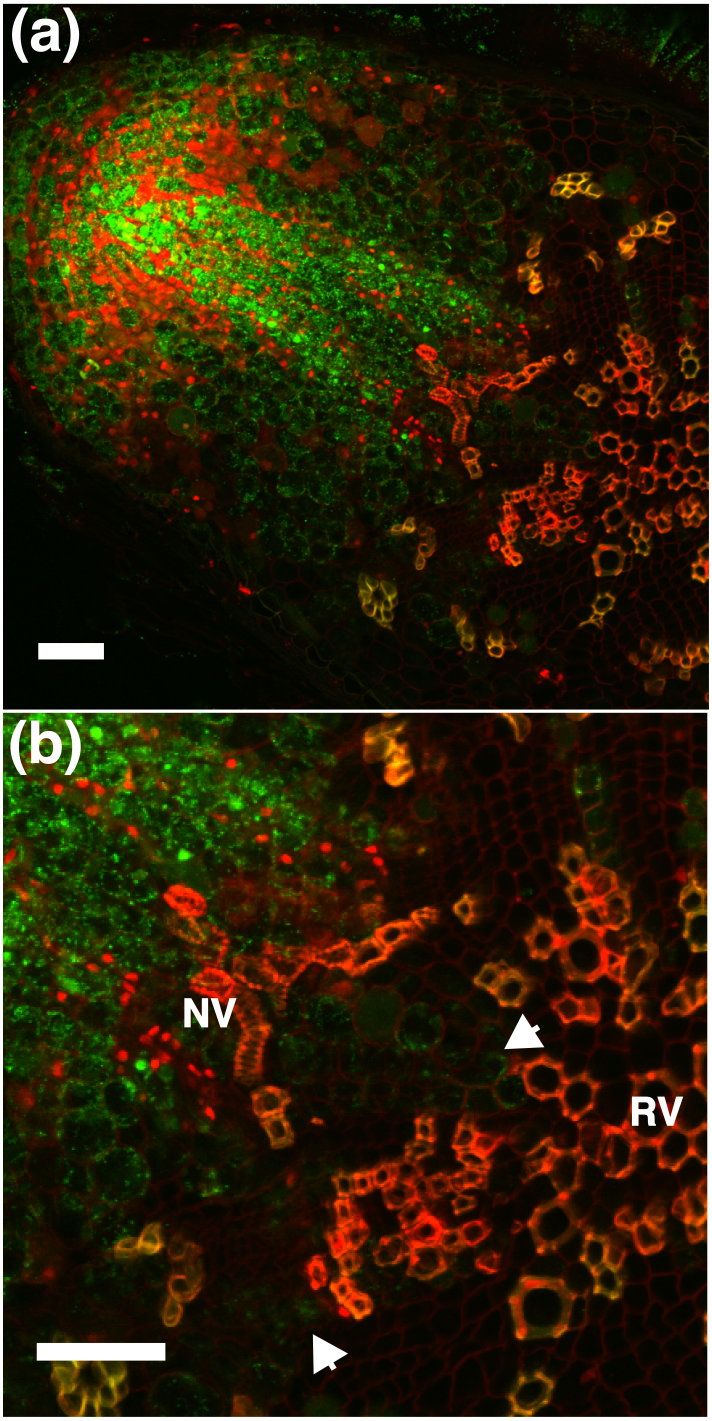

Figure 17. Laser scanning confocal image of a longitudinally sectioned nodule. Nucleic acids (green) stained with SYTO13 show the location of bacteria and cell walls (red) are stained with propidium idiodide. (a) Whole section shows extent of the infection in the nodule. (b) Infected plant cells (arrows) are between the central nodule vasculature (NV) and the main root vasculature (RV). Scale bars are 50 μm. 1 of 5 nodules investigated.

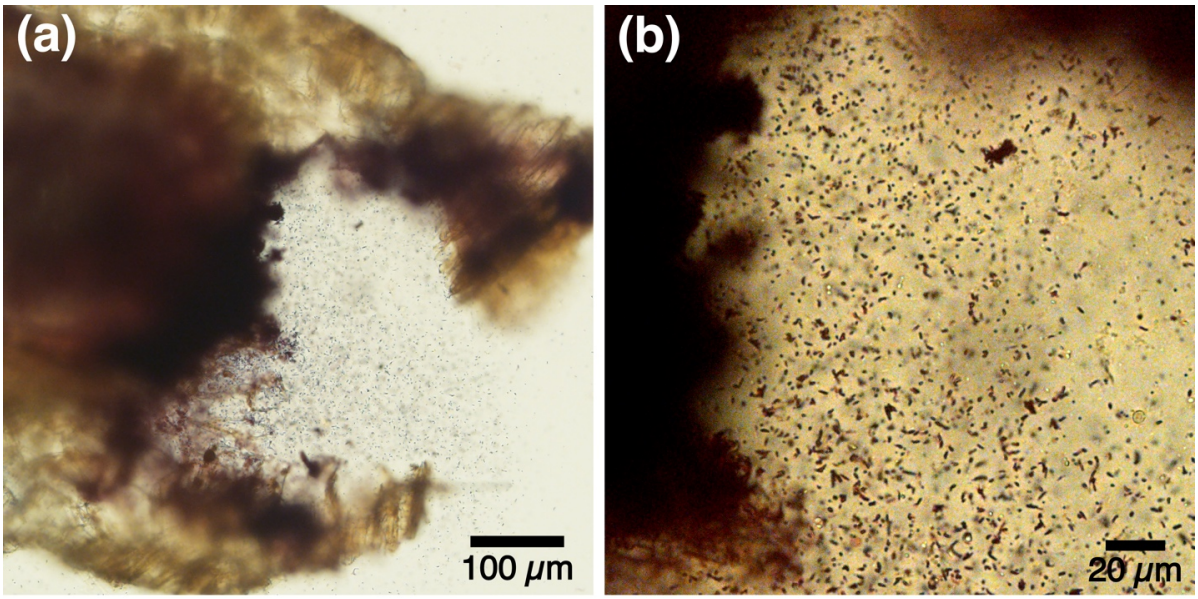

Figure 18. TTC stained nodule broken open to show bacterial cells.

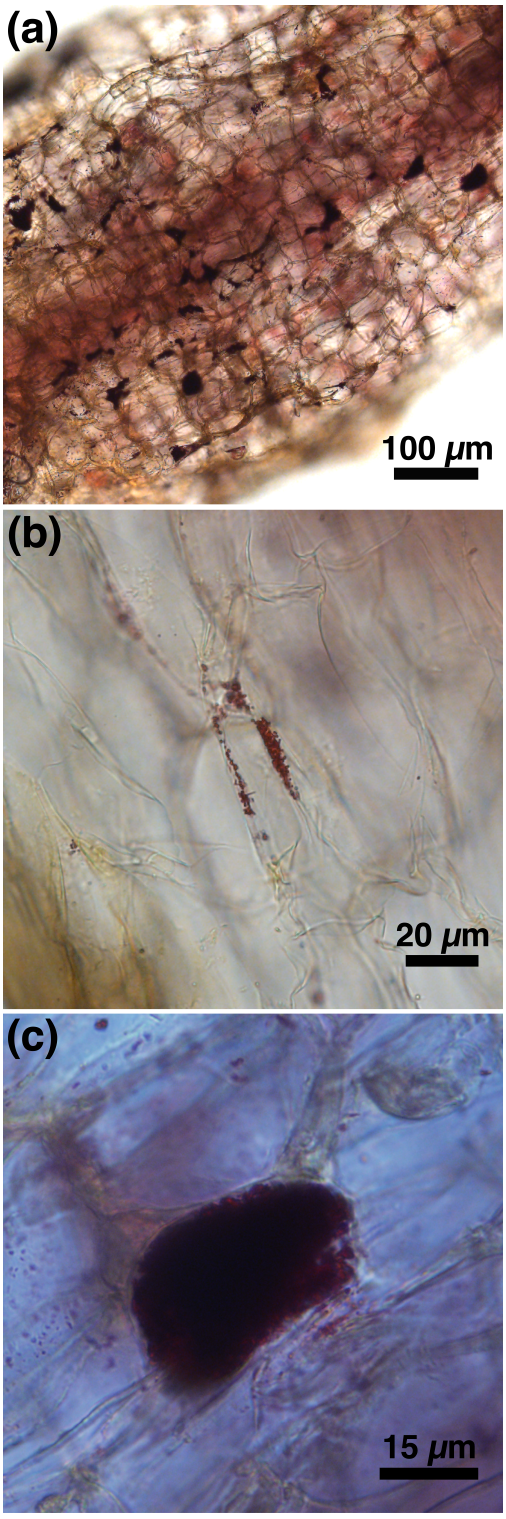

Figure 19. TTC staining of an intermediate nodule structure. (a) A transverse section showing multiple cells packed with the red colored bacteria (b) Bacterial cells within the apoplastic space. (c) The host cell tightly packed with bacterial cells. Representative of 5 nodules sectioned.

4. Discussion

Despite legume RNS being the most well-studied form of a nitrogen fixing symbiosis in nature, the extant legumes of this large plant family are relatively understudied. RNS, once thought to be limited to only legumes and Rhizobia, has since been discovered to be more diverse in its morphology and symbiotic partners. The non-legume *Parasponia* can form a RNS with Rhizobia, actinorhizal plants are capable of forming nodules with filamentous bacteria of the genera *Frankia*, and certain legumes of the Mimosoideae subfamily maintain the symbiotic partnership with Betaproteobacteria [6,162,163].

The complex process of nodule development varies wildly, and the order in which the diverse traits evolved remains unclear. The evolutionary track is in discrepancy based upon root nodule structure development, mode of infection, and mode of housing bacteria. Research to date suggests that determinate nodules may have evolved from indeterminate nodules and that crack entry without the advent of infection threads represents the most primitive form of infection [5]. Furthermore, the process likely evolved from a promiscuous host plant susceptible to infection by multiple bacterial genera with little control over the process, to a highly controlled infection process where the host plant is capable of recruiting and selecting specific favored bacterial species [164]. The underlying predisposition genes must have a single origin, while the full evolution of the RNS has multiple origins across and within plant families of the nitrogen fixing clade [20,77].

The information on the RNS of *Pediomelum esculentum* is minimal – only early reports confirming its ability to nodulate could be found [142,143]. *P. esculentum* is a perennial of the Psoralea tribe of the legume subfamily Papilionoideae. This tribe belongs to a clade known as the phaseoloid group; all of the studied plants form determinate, ureide-exporting nodules only [29]. *P. esculentum* is able to store large amounts of the nitrogen in the proteinoplasts housed in cells of the xylem parenchyma of the tuber [138]. This excessive storage of nitrogen in a plant that grows in relatively nitrogen limited soils has prompted our investigation into the symbiotic mechanisms.

4.1. Isolation of endophytes

Mature *P. esculentum* plants used for the initial isolation did not present with any nodules prior to isolation of endophytes. The nodules may have been severed from the root upon harvesting or were overlooked, as nodules later observed to develop on plants grown from seed were approximately 1mm in diameter, and were difficult to identify properly without the use of a stereomicroscope. The isolation of endophytes from all tissue types of these mature plants revealed a microbiome in agreement with previously identified endophytes [91]. The Rhizobia isolates and a single *Burkholderia* isolate from this initial isolation were used to inoculate seedlings under gnotobiotic conditions, wherein only the introduced bacteria should have been present. Isolates from the nodules and tubers of the seedlings revealed a different microbiome than what was applied.

The majority of isolates (14/24) from the seedlings were closely related to either *Bacillus nealsonii* or *B. circulans.* The *Bacillus* spp. were present in the nodules and tubers of both inoculated and uninoculated plants, as well as the only bacteria isolated from seeds of 3 different sources. The presence of these *Bacillus* spp. in all of these tissue implies the endophyte is passed vertically from one generation to the next and, upon germination, begins a systemic colonization process where it spreads through the plant.

Endophytes, including *Bacillus*, can be passed vertically in seeds [133], and *Bacillus* can systemically infect plants, promoting plant growth and increasing pathogenic resistance [165]. Seed-carried endophytes can either infect the plant by colonizing the exterior and subsequent re-entry post-germination or by maintaining a constant interior infection [166]. This presence during germination gives them an advantage over other bacteria from the external environment, granting immediate access to space and nutrients from the host plant [96,166]. However this relationship is not necessarily static; rhizospheric bacteria can colonize the plant and create shifts in the microbiome dependent upon soil type, season, weather, and even the plant genome [83,90]. *Bacillus* spp. were isolated from the mature plants, but were different than the *Bacillus* spp. isolated from the seedlings and seeds (See phylogenetic tree, Figure 6). This could be attributed to the culture-dependent conditions that would favor the fastest growing species present.

It is unclear whether *Bacillus* is responsible for fixing nitrogen in the nodules, though it is clearly present. If Rhizobia were present simultaneously as *Bacillus* in the nodules, it should have appeared as a predominant isolate, as the previously isolated Rhizobia from the mature plants exhibited faster growth rates on solid NFM than the *Bacillus* isolates. All *Bacillus* isolates were able to grow on nitrogen-free media. None of the isolates throughout this entire work showed the ability to reduce acetylene with the implemented method, despite they can all grow on N-free media and *nifH* gene products were amplified from many isolates (Appendix: Tables A1:A3).

The *Bacillus* genus is well known as a plant endophyte. *Bacillus* is one beneficial genera capable of creating induced systemic resistance in the host plant, brought on by triggering an immune response that boosts resistance to pathogens [165,167]. *B. thuringiensis* is able to produce parasporal crystal proteins that act as an insecticide [168]. *B. subtilis* is capable of enhancing the nitrogen fixation of *Rhizobium* in *Lens esculenta* in field studies [169]. *B. bacillus* and *B. subtilis* have been observed to reside within cells of the host plant [170,171]. *B. subtilis* has been shown to induce root hair deformation in the legume *Robinia pseudoacacia* and subsequently infect the host via infection threads. Though no nodules were described by the authors, *B. subtilis* was released into root cortical cells as bacteroids with the full development of a peribacteriod membrane and as free-living individual cells [171].

The inability to reduce acetylene may be due to a number of factors. Multiple studies have reported bacteria *nifH* positive bacteria capable of growing on NFM but unable to reduce acetylene [172,173]. *Streptomyces thermoautotrophicus* has been shown to have a functional nitrogenase that does not reduce acetylene [174]. A single amino acid mutation in the nitrogenase can result in the inability to reduce acetylene while maintaining nitrogen fixation capabilities [175]. Furthermore, experimental conditions may not have created the proper balance of acetylene and nitrogen, saturating the enzyme with acetylene and restricting nitrogen fixation required for growth. The acetylene reduction assay may not be an unconditional test for nitrogen fixation.

4.1.1. NifH *amplification and acetylene reduction in isolates*

NifH, the most highly conserved gene associated with nitrogen fixation, was amplified using the primer pair POLF/POLR. Many of the isolates produced a PCR

product corresponding to the correct length when compared to a known nitrogen fixer (Figure 7). Upon phylogenetic analysis of these products, all of the sequences fell into cluster IV of the nitrogenase gene tree, the portion of the tree housing genes paralougous to *nifH*. [161]. This could be due to a number of reasons. The degenerate *nifH* primers can have a phylogenetic bias and create the opportunity for false negatives [9]. The primers could also amplify the incorrect product; however, such a product is not likely to be the expected target length.

4.2. Nodule structure and endophyte imaging

The nodules formed on *P. esculentum* were associated with lateral roots (Figure 16); occasional nodules without a lateral root association and emerging directly from the root tuber were also observed. Neither infection threads nor bacteroids were observed. The nodules appear to be either determinate in fate or develop into an intermediate structure resembling a lateral root. Others have reported instances of such intermediate structures. One observation found that exposure to high temperature and subsequent return to lower temperatures can change the nodule apex to a root-like apex [176]. Auxin inhibitors can cause the formation of an intermediate structures [177,178] and a mutant of *Rhizobium meliloti* can induce them on alfalfa [179].

The central vasculature of the nodules is typical of the non-legume Parasponia, but has not been observed in legumes which develop a peripheral vasculature [44]. The nodules lack infection threads despite interspersed infected and uninfected cells, a feature associated with infection thread formation. The bacteria may be free to move through the intercellular spaces of the nodule and select cells for infection through an unknown process.

Furthermore, the presence of infected cells behind the central vasculature of the nodule and adjacent to the main root vasculature (Figure 17) indicates the infection could arise from a bacterium that is systemically infecting the plant and travelling throughout the vasculature. In studied legumes that form determinate nodules, whether the infection progresses through root hairs or epidermal cracks with or without infection threads, the infection progresses through the cortex where the nodule primordium forms from infected cells that divide to a certain extent and eventually stop. The presence of infected

cells outside of the infection zone has not been observed to the extent of the authors knowledge.

4.3. Lateral root association

It is unclear whether the nodules of *P. esculentum* develop from the main root cortical cells as in legumes or from the pericycle of the mainroot as *Parasponia* [44]. No root hairs were observed on *P. esculentum*, limiting possible modes of infection to either "crack entry" or perhaps from the inner vasculature. The lack of ITs as well as infected host cells adjacent to the main root vasculature support this hypothesis. Bacteria residing in the main root vasculature could conveniently induce nodules associated with lateral roots. The plant would not need to recruit bacteria through the external release of flavonoids nor would the bacteria be required to induce root hair formation through the expression of nod factors.

The cortical cells associated with emerging lateral roots of white clover have been shown to accumulate the flavonoid formononetin and a DHF derivative (7,4'-dihydroxyflavone) normally induced by Rhizobia during root hair infection. These cortical cells can be "hijacked" by the bacteria and induced to form a nodule [180]. The process bypasses the need for the production of LCOs or nod factors by the bacteria. Furthermore, the genes *ENOD40* and *ENOD12A* are also activated during lateral root and nodule initiation [181,182].

A systemically colonized endophyte present within the vasculature would have a distinct advantage in accessing these cortical cells versus a bacterium attempting to infect from the rhizosphere. Based on the results of this work, the *Bacillus* spp. of *P. esculentum* may take advantage of potentially the most primitive pathway for nodule initiation. The bacteria may also be the controlling partner that found an evolutionary loophole, allowing the bacteria to outcompete the classical Rhizobia that must infect from the exterior of the plant. Since the pathway is ancestral to the more complex nodule development schemes in other determinate forming extant plants of the Papilionoideae subfamily, it would not necessarily require a gain or loss of function. More advanced mechanisms may be simultaneously present, and could explain the limited isolation of Rhizobia from a few nodules.

4.4. Bacteria in the tuber and seedlings

Bacteria appeared to be colonizing the outside epidermis of sterilely germinated seeds within days after germination, and also residing in the apoplast of the developing root tissue (Figures 13 and 14). These visualized bacteria are presumptively *Bacillus*, as it was the only bacterial genera to be isolated from seeds. The *Bacillus* may begin proliferating immediately upon germination, move into the intercellular space of the cortex, and ultimately reach the xylem vessels where it is free to spread throughout the plant.

In the tuber, bacteria were observed in the intercellular space with both SYTO13 and TTC (Figure 13). Staining patterns of SYTO13 (Figure 15) also suggests the presence of bacteria intracellularly. The intracellular staining pattern was not observed with TTC, which could result in a reduced cellular uptake of the TTC that might improve with longer incubation times. Imaging of endophytes show the co-localization of both the TTC and SYTO13 for extracellular bacteria (Figure 14, Appendix: Figure A1). The presence of bacteria in the apoplast of the tuber resembles the relationship between sugar beet and *Burkholderia*, where the tuber houses large sums of the endophyte within the intercellular space and xylem vessels [183]. The infected cells, observed with SYTO13, are oriented in rays originating near the central vasculature of the tuber and extending out towards the epidermis. This staining pattern could not be replicated with TTC, perhaps due to the host cell permeability of TTC. The rays of infected cells and uninfected cells could be seen adjacent to each other, similar to the organization of protein and starch storage cells of the tuber [138]. This implies the bacteria are associated with either cells holding starch or cells holding protein. They are most likely associated with those storing starch as a carbon source, then the fixed nitrogen could be easily transported as ureides to the adjacent cells for storage.

4.5. Acetylene reduction and ureide assays

The plants given nitrate as a nitrogen source fixed significantly more atmospheric nitrogen than those given ammonia, but did not fix more nitrogen than plants without a nitrogen source (Figure 12). Despite fixing the most nitrogen, the nitrate-treated plants

contained fewer ureides than plants treated with ammonia, while the plants given no nitrogen had the lowest concentration of ureides.

Ureides are known exported products of nitrogen fixation from the nodules of legumes within the Phaseoloid clade of the Papilionoideae subfamily. As a member of this group, *P. esculentum* is expected to export ureides [29]. Ureides, as allantoin and allantoate, are exported from the nodule for transport through the xylem, and is more carbon-economical than the export of amides by other nodules [184]. Reports suggest ureides passing through the xylem may also serve as temporary nitrogen storage [185-187]. Ureides may diffuse out of the xylem vessels and into the xylem parenchyma, then migrate symplastically to the phloem parenchyma, and then ultimately released into the phloem [188].

P. esculentum has a well-developed thick xylem parenchyma that would be well adapted to diffusing and storing large amounts of ureides passing through the xylem. The plants given ammonia had the highest concentration of ureides, perhaps because they favored a ureide transport pathway also used for nitrogen export from the nodules. The ammonia would be converted into ureides upon absorption and prior to diffusing into the xylem. This process might serve as an intermediate step prior to the incorporation into amino acids via the glutamine synthetase – glutamate synthase pathway.

The plants given nitrate as a nitrogen source had a higher concentration of ureides than those with no nitrogen source (Figure 12). This increase in ureides may be a result of the higher fixation rates or due to increased NO_3. The literature is conflicting on whether the addition of nitrate reduces or increases nitrogenase activity in a RNS. Generally, nitrate at high concentrations will reduce fixation and nodulation; lower concentrations can enhance [189]. When viewed from the host plant controlling viewpoint, one would consider the addition of nitrate to cause the plant to cease bacterial symbiosis in favor of the more carbon-efficient nitrogen source. However, if viewed from the bacterial side of things, the addition of nitrate can increase nitrogenase efficiency in bacteria capable of nitrate respiration.

An investigation on different strains of *Bradyrhizobium* with varying nitrate reductase ability showed strains with highly efficient nitrate reductases were able to induce nodules in the presence of nitrate, while those without nitrate reductase did not

infect the host. The nitrate reducing bacteria exhibited increased nitrogenase activity at nitrate concentrations of 1-2 mM [190]. Other studies also report enhanced nitrogen fixation with the addition of low levels of nitrate [191-193]. These bacteria may have the ability to perform dissimilatory nitrate reduction to ammonia (DNRA).

DNRA is characterized by the production of excess ammonia beyond what is required for the bacterial cell. The first step, nitrate respiration, would produce ATP in the conversion of NO_3 to NO_2. The toxic nitrite is then used for production of ammonium [194]. DNRA would allow all bacteria to use nitrate as an electron acceptor instead of oxygen in the micro-aerobic environment of the nodule. DNRA may account for the increase in ureides when *P. esculentum* is given nitrate and the increase in nitrogen fixation rates. The NO_3 absorbed by the plant may be exported into the xylem as ureides after bacterial DNRA.

4.6. Denaturing gradient gel electrophoresis

The DGGE experiment showed only a single band profile for the bulk root and tuber tissue (Figure 11). These profiles agree with the potential for a single predominant bacteria species that is systemically present to be responsible for nitrogen fixation. Further investigation is needed to confirm the *nifH* sequence of the *Bacillus* spp. and that of *nifH* sequences extracted from bulk plant tissue.

5. Conclusions

This work suggests *P. esculentum* vertically transmits a nitrogen fixing endophyte capable of inducing nodules. The species is a candidate for further studies as a highly advanced or perhaps very practical RNS to evolve to date. The Psoralea tribe has a very high diversification rate. At the time *Pediomelum* diverged, about 4.8 MYA, the group underwent one of the most rapid diversification events known [144]. Such a rapid appearance of new species in this *Pediomelum* genus could account for the variation in the RNS. *P. esculentum* is an incredibly hardy plant well-adapted to drought, cold, and fire regimes. The plant is able to survive droughts and burns by remaining underground for entire seasons [195,196]. The seeds are dispersed by the wind over long distances, and can remain viable for years. It would be advantageous for such a plant that thrives in

nitrogen limiting soils to ensure the presence of its symbiotic counterpart via vertical transmission.

Given the slow growth rate of the *P. esculentum*, the plant should not require highly efficient nitrogen fixers, and this point is reflected in the low rates of acetylene reduction. The number of nodules formed on plants, inoculated or not, were the same. The same *Bacillus* spp. were the only isolate obtained from seeds, and the most common bacteria found in the nodules of plants. The DGGE profiles of *nifH* sequences amplified from root tissue indicated a single nitrogen fixing species is present.

References

1. Canfield DE, Glazer AN, Falkowski PG: **The Evolution and Future of Earth's Nitrogen Cycle**. *Science* 2010, **330**:192-196.
2. Boyd E, Peters JW: **New insights into the evolutionary history of biological nitrogen fixation**. *Frontiers in Microbiology* 2013, **4**.
3. Galloway JN, Townsend AR, Erisman JW, Bekunda M, Cai Z, Freney JR, Martinelli LA, Seitzinger SP, Sutton MA: **Transformation of the Nitrogen Cycle: Recent Trends, Questions, and Potential Solutions**. *Science* 2008, **320**:889-892.
4. Gallon JR: **The oxygen sensitivity of nitrogenase: a problem for biochemists and micro-organisms**. *Trends in Biochemical Sciences* 1981, **6**:19-23.
5. Sprent JI: **Evolving ideas of legume evolution and diversity: a taxonomic perspective on the occurrence of nodulation**. *New Phytol* 2007, **174**:11-25.
6. Moulin L, Munive A, Dreyfus B, Boivin-Masson C: **Nodulation of legumes by members of the beta-subclass of Proteobacteria**. *Nature* 2001, **411**:948-950.
7. Allen ON, Allen EK: **The Leguminosae. A source book of characteristics, uses and nodulation**. *The Leguminosae. A source book of characteristics, uses and nodulation.* 1981.
8. Kneip C, Lockhart P, Voss C, Maier UG: **Nitrogen fixation in eukaryotes--new models for symbiosis**. *BMC Evol Biol* 2007, **7**:55.
9. Zehr JP, Jenkins BD, Short SM, Steward GF: **Nitrogenase gene diversity and microbial community structure: a cross‐system comparison**. *Environmental Microbiology* 2003, **5**:539-554.
10. Dixon R, Kahn D: **Genetic regulation of biological nitrogen fixation**. *Nat Rev Micro* 2004, **2**:621-631.
11. Bishop P, Permakumar R: **Alternative nitrogen fixation systems**. In *Biological nitrogen fixation*. Edited by Stacey G, Burris RH, Evans DJ: Chapman & Hall; 1992:736-762.
12. Howard JB, Rees DC: **Structural Basis of Biological Nitrogen Fixation**. *Chem Rev* 1996, **96**:2965-2982.
13. Zehr JP, Turner PJ: **Nitrogen fixation: Nitrogenase genes and gene expression**. In *Methods in Microbiology*. Edited by: Academic Press; 2001:271-286. vol Volume 30.]
14. Dalton HF, Postgate JR: **Growth and physiology of Azotobacter chroococcum in continuous culture**. *J Gen Microbiol* 1969, **56**:307-319.
15. Yates MG: **Effect of non-haem iron proteins and cytochrome C from Azotobacter upon the activity and oxygen sensitivity of Azobacter nitrogenase**.
16. Almon H, Bohme H: **Components and activity of the photosynthetic electron transport system of intact heterocysts isolated from the blue-green alga Nostoc muscorum**. *Biochim Biophys Acta* 1980, **592**:113-120.

17. Donze M, Haveman J, Schiereck P: **Absence of photosystem 2 in heterocysts of the blue-green alga Anabaena**. *Biochim Biophys Acta* 1972, **256**:157-161.
18. Berman-Frank I, Lundgren P, Falkowski P: **Nitrogen fixation and photosynthetic oxygen evolution in cyanobacteria**. *Res Microbiol* 2003, **154**:157-164.
19. Appleby CA: **Leghemoglobin and Rhizobium Respiration**. *Annual Review of Plant Physiology* 1984, **35**:443-478.
20. Soltis DE, Soltis PS, Morgan DR, Swensen SM, Mullin BC, Dowd JM, Martin PG: **Chloroplast gene sequence data suggest a single origin of the predisposition for symbiotic nitrogen fixation in angiosperms**. *Proc Natl Acad Sci U S A* 1995, **92**:2647-2651.
21. Doyle JJ: **Phylogenetic Perspectives on the Origins of Nodulation**. *Molecular Plant-Microbe Interactions* 2011, **24**:1289-1295.
22. Trinick MJ, Hadobas PA: **Biology of theParasponia-Bradyrhizobium symbiosis**. *Plant and Soil* 1988, **110**:177-185.
23. Group LPW: **Legume phylogeny and classification in the 21st century: progress, prospects and lessons for other species-rich clades**. *Taxon* 2013, **62**:217-248.
24. Magallon S, Sanderson MJ: **Absolute diversification rates in angiosperm clades**. *Evolution* 2001, **55**:1762-1780.
25. Hughes C, Eastwood R: **Island radiation on a continental scale: exceptional rates of plant diversification after uplift of the Andes**. *Proc Natl Acad Sci U S A* 2006, **103**:10334-10339.
26. Richardson JE, Pennington RT, Pennington TD, Hollingsworth PM: **Rapid diversification of a species-rich genus of neotropical rain forest trees**. *Science* 2001, **293**:2242-2245.
27. Scherson RA, Vidal R, Sanderson MJ: **Phylogeny, biogeography, and rates of diversification of New World Astragalus (Leguminosae) with an emphasis on South American radiations**. *Am J Bot* 2008, **95**:1030-1039.
28. Bryan J, Berlyn G, Gordon J: **Toward a new concept of the evolution of symbiotic nitrogen fixation in the Leguminosae**. *Plant and Soil* 1996, **186**:151-159.
29. Sprent JI: *Legume Nodulation: A Global Perspective*. Ames, IA, U.S.A.: Wiley-Blackwell; 2009.
30. Sprent JI: **60Ma of legume nodulation. What's new? What's changing?** *J Exp Bot* 2008, **59**:1081-1084.
31. Doyle JJ, Doyle JL, Hortorium LHB: **Phylogenetic Perspectives on the Origins and Evolution of Nodulation in the Legumes and Allies**. In *Biological Fixation of Nitrogen for Ecology and Sustainable Agriculture*. Edited by Legocki A, Bothe H, Pühler A: Springer Berlin Heidelberg; 1997:307-312.
32. Sprent J: **Nitrogen fixation**. In *The Groundnut Crop: A scientific basis for improvement*. Edited by Smartt J: Springer Netherlands; 1994:255-280.
33. Capoen W, Oldroyd G, Goormachtig S, Holsters M: **Sesbania rostrata: a case study of natural variation in legume nodulation**. *New Phytologist* 2010, **186**:340-345.

34. Lavin M, Herendeen PS, Wojciechowski MF: **Evolutionary rates analysis of Leguminosae implicates a rapid diversification of lineages during the tertiary**. *Syst Biol* 2005, **54**:575-594.
35. Sprent JI, James EK: **Legume evolution: where do nodules and mycorrhizas fit in?** *Plant Physiol* 2007, **144**:575-581.
36. Madsen LH, Tirichine L, Jurkiewicz A, Sullivan JT, Heckmann AB, Bek AS, Ronson CW, James EK, Stougaard J: **The molecular network governing nodule organogenesis and infection in the model legume Lotus japonicus**. *Nature Communications* 2010, **1**:1-12.
37. Sprent JI: *Nodulation in Legumes*. Kew, UK: Royal Botanic Gardens; 2001.
38. Sprent JI, Parsons R: **Nitrogen fixation in legume and non-legume trees**. *Field Crops Research* 2000, **65**:183-196.
39. Bhuvaneswari TV, Turgeon BG, Bauer WD: **Early Events in the Infection of Soybean (Glycine max L. Merr) by Rhizobium japonicum: I. LOCALIZATION OF INFECTIBLE ROOT CELLS**. *Plant Physiology* 1980, **66**:1027-1031.
40. Bhuvaneswari TV, Bhagwat AA, Bauer WD: **Transient susceptibility of root cells in four common legumes to nodulation by rhizobia**. *Plant Physiol* 1981, **68**:1144-1149.
41. Phillips DA, Joseph CM, Maxwell CA: **Trigonelline and stachydrine released from alfalfa seeds activate NodD2 protein in Rhizobium meliloti**. *Plant Physiology* 1992, **99**:1526-1531.
42. Spaink HP, Carlson RW: **Regulation of plant morphogenesis by Lipo‐Chitin oligosaccharides**. *Critical Reviews in Plant Sciences* 1996, **15**:559-582.
43. Denarie J, Debelle F, Prome JC: **Rhizobium lipo-chitooligosaccharide nodulation factors: signaling molecules mediating recognition and morphogenesis**. *Annu Rev Biochem* 1996, **65**:503-535.
44. Hirsch AM, Larue TA, Doyle J: **Is the Legume Nodule a Modified Root or Stem or an Organ sui generis?** *Critical Reviews in Plant Sciences* 1997, **16**:361-392.
45. Patriarca EJ, Tate R, Fedorova E, Riccio A, Defez R, Iaccarino M: **Down-regulation of the Rhizobium ntr system in the determinate nodule of Phaseolus vulgaris identifies a specific developmental zone**. *MPMI-Molecular Plant Microbe Interactions* 1996, **9**:243-251.
46. Libbenga K, Harkes P: **Initial proliferation of cortical cells in the formation of root nodules in Pisum sativum L**. *Planta* 1973, **114**:17-28.
47. Bond L: **Origin and development morphology of root nodules of Pisum sativum**. *Botanical Gazette* 1948:411-434.
48. Verma D: **Signals in root nodule organogenesis and endocytosis of Rhizobium**. *The Plant Cell* 1992, **4**:373.
49. Sprent JI, Embrapa: **Root nodule anatomy, type of export product and evolutionary origin in some Leguminosae**. *Plant, Cell & Environment* 1980, **3**:35-43.
50. Gonzalez-Sama A, de la Pena TC, Kevei Z, Mergaert P, Lucas MM, de Felipe MR, Kondorosi E, Pueyo JJ: **Nuclear DNA endoreduplication and expression of**

the mitotic inhibitor Ccs52 associated to determinate and lupinoid nodule organogenesis. *Mol Plant Microbe Interact* 2006, **19**:173-180.
51. Naisbitt T, James EK, Sprent JI: **The evolutionary significance of the legume genus Chamaecrista, as determined by nodule structure**. *New Phytologist* 1992, **122**:487-492.
52. Brewin NJ: **Plant Cell Wall Remodelling in the Rhizobium–Legume Symbiosis**. *Critical Reviews in Plant Sciences* 2004, **23**:293-316.
53. Nutman PS: **THE INFLUENCE OF THE LEGUME IN ROOT-NODULE SYMBIOSIS**. *Biological Reviews* 1956, **31**:109-151.
54. Nutman PS: **Physiological Studies on Nodule Formation: I. The Relation between Nodulation and Lateral Root Formation in Red Clover**. *Annals of Botany* 1948, **12**:81-96.
55. Allen O, Allen EK: **Response of the peanut plant to inoculation with rhizobia, with special reference to morphological development of the nodules**. *Botanical Gazette* 1940:121-142.
56. Chandler MR: **Some observations on infection of Arachis hypogaea L. by Rhizobium**. *Journal of Experimental Botany* 1978, **29**:749-755.
57. Chandler MR, Date R, Roughley R: **Infection and root-nodule development in Stylosanthes species by Rhizobium**. *Journal of Experimental Botany* 1982, **33**:47-57.
58. Cordeiro L, Sprent JI, McInroy SG: **Some developmental and structural aspects of nodules of Lonchocarpus muelhbergianus**. *Naturalia* 1996, **21**:9-21.
59. Goormachtig S, Capoen W, Holsters M: **Rhizobium infection: lessons from the versatile nodulation behaviour of water-tolerant legumes**. *Trends in Plant Science* 2004, **9**:518-522.
60. Ndoye I, de Billy F, Vasse J, Dreyfus B, Truchet G: **Root nodulation of Sesbania rostrata**. *J Bacteriol* 1994, **176**:1060-1068.
61. Lavin M, Pennington RT, Klitgaard BB, Sprent JI, de Lima HC, Gasson PE: **The dalbergioid legumes (Fabaceae): delimitation of a pantropical monophyletic clade**. *Am J Bot* 2001, **88**:503-533.
62. Vega-Hernández MC, Pérez-Galdona R, Dazzo FB, Jarabo-Lorenzo A, Alfayate MC, León-Barrios M: **Novel infection process in the indeterminate root nodule symbiosis between Chamaecytisus proliferus (tagasaste) and Bradyrhizobium sp**. *New Phytologist* 2001, **150**:707-721.
63. Tschermak-Woess E, Doležal R: **Durch Seitenwurzelbildung induzierte und spontane Mitosen in den Dauergeweben der Wurzel**. *Österreichische botanische Zeitschrift* 1953, **100**:358-402.
64. McCully ME: **The development of lateral roots**. In *The Development and Function of Roots*. Edited by Torrey JGC, D.T.: Adademic Press; 1975:105-124.
65. Liu QB, A.M.: **The infection process and nodule initiation in the *Frankia-Ceanothus* root nodule symbiosis. A structural and histochemical study.** *Protoplasma* 1991:82-92.
66. Miller I, Baker D: **The initiation, development and structure of root nodules inElaeagnus angustifolia L.(Elaeagnaceae)**. *Protoplasma* 1985, **128**:107-119.

67. Racette S, Torrey JG: **Root nodule initiation in Gymnostoma (Casuarinaceae) and Shepherdia (Elaeagnaceae) induced by Frankia strain HFPGpI1**. *Canadian journal of botany* 1989, **67**:2873-2879.
68. Berry AMS, L.A.: **The infection process and nodule development**. In *The biology of Frankia and actinorhizal plants*. Edited by Schwintzer CRT, J.D.: Academic Press; 1990:61-81.
69. Lancelle SA, Torrey J: **Early development ofRhizobium-induced root nodules ofParasponia rigida. I. Infection and early nodule initiation**. *Protoplasma* 1984, **123**:26-37.
70. Lancelle SA, Torrey JG: **Early development of Rhizobium-induced root nodules of Parasponia rigida. II. Nodule morphogenesis and symbiotic development**. *Canadian journal of botany* 1985, **63**:25-35.
71. Price GD, Mohapatra SS, Gresshoff PM: **Structure of Nodules Formed by Rhizobium Strain ANU289 in the Nonlegume Parasponia and the Legume Siratro (Macroptilium atropurpureum)**. *Botanical Gazette* 1984, **145**:444-451.
72. Caetano-Anolles G, Joshi PA, Gresshoff PM: **Nodule Morphogenesis in the Absence of Rhizobium**. In *New Horizons in Nitrogen Fixation: Proceedings of the 9th International Congress on Nitrogen Fixation, Cancún, Mexico, December 6–12, 1992*. Edited by Palacios R, Mora J, Newton WE: Springer Netherlands; 1993:297-302.
73. Sprent JI: **Tansley Review No.15. Which Steps are Essential for the Formation of Functional Legume Nodules?** *The New Phytologist* 1989, **111**:129-153.
74. LaRue TA, Weeden NF: **The symbiosis genes of the host**. In *1st European Nitrogen Fixation Conference*; *Szeged*,Edited by Kiss GB, Endre G: Officina Press: 1994:147-151.
75. Verma DPS, Delauney AJ: **Root Nodule Symbiosis: Nodulins and Nodulin Genes**. In *Temporal and Spatial Regulation of Plant Genes*. Edited by Verma DPS, Goldberg RB: Springer Vienna; 1988:169-199.
76. Sprent JI: **Co-Evolution of Legume—Rhizobial Symbioses: Is It Essential for Either Partner?** In *Biological Fixation of Nitrogen for Ecology and Sustainable Agriculture*. Edited by Legocki A, Bothe H, Pühler A: Springer Berlin Heidelberg; 1997:313-316.
77. Werner GDA, Cornwell WK, Sprent JI, Kattge J, Kiers ET: **A single evolutionary innovation drives the deep evolution of symbiotic N2-fixation in angiosperms**. *Nat Commun* 2014, **5**.
78. Pawlowski K, Sprent JI: **Comparison between actinorhizal and legume symbiosis**. In *Nitrogen-fixing actinorhizal symbioses*. Edited by: Springer; 2007:261-288.
79. Young ND, Debellé F, Oldroyd GE, Geurts R, Cannon SB, Udvardi MK, Benedito VA, Mayer KF, Gouzy J, Schoof H: **The Medicago genome provides insight into the evolution of rhizobial symbioses**. *Nature* 2011, **480**:520-524.
80. Li Q-G, Zhang L, Li C, Dunwell JM, Zhang Y-M: **Comparative genomics suggests that an ancestral polyploidy event leads to enhanced root nodule**

symbiosis in the Papilionoideae. *Molecular biology and evolution* 2013, **30**:2602-2611.
81. Schranz ME, Mohammadin S, Edger PP: **Ancient whole genome duplications, novelty and diversification: the WGD Radiation Lag-Time Model**. *Current opinion in plant biology* 2012, **15**:147-153.
82. Bruneau A, Mercure M, Lewis GP, Herendeen PS: **Phylogenetic patterns and diversification in the caesalpinioid legumesThis paper is one of a selection of papers published in the Special Issue on Systematics Research**. *Botany* 2008, **86**:697-718.
83. Hallmann J, Quadt-Hallmann A, Mahaffee WF, Kloepper JW: **Bacterial endophytes in agricultural crops**. *Canadian Journal of Microbiology* 1997, **43**:895-914.
84. Garbeva P, van Overbeek LS, van Vuurde JWL, van Elsas JD: **Analysis of endophytic bacterial communities of potato by plating and denaturing gradient gel electrophoresis (DGGE) of 16S rDNA based PCR fragments**. *Microbial Ecology* 2001, **41**:369-383.
85. Reinhold-Hurek B, Hurek T: **Interactions of Gramineous Plants with Azoarcus spp. and Other Diazotrophs: Identification, Localization, and Perspectives to Study their Function**. *Critical Reviews in Plant Sciences* 1998, **17**:29-54.
86. Benhizia Y, Benhizia H, Benguedouar A, Muresu R, Giacomini A, Squartini A: **Gamma proteobacteria can nodulate legumes of the genus Hedysarum**. *Syst Appl Microbiol* 2004, **27**:462-468.
87. Rosenblueth M, Martinez-Romero E: **Rhizobium etli maize populations and their competitiveness for root colonization**. *Arch Microbiol* 2004, **181**:337-344.
88. Sprent JI, James EK: **N2-fixation by endophytic bacteria: questions of entry and operation**. In *Azospirillum VI and Related Microorganisms: Genetics — Physiology — Ecology*. Edited by Fendrik I, del Gallo M, Vanderleyden J, de Zamaroczy M: Springer Berlin Heidelberg; 1995:15-30.
89. Kuklinsky-Sobral J, Araujo WL, Mendes R, Geraldi IO, Pizzirani-Kleiner AA, Azevedo JL: **Isolation and characterization of soybean-associated bacteria and their potential for plant growth promotion**. *Environ Microbiol* 2004, **6**:1244-1251.
90. Conn VM, Franco CMM: **Analysis of the Endophytic Actinobacterial Population in the Roots of Wheat (Triticum aestivum L.) by Terminal Restriction Fragment Length Polymorphism and Sequencing of 16S rRNA Clones**. *Applied and Environmental Microbiology* 2004, **70**:1787-1794.
91. Hardoim PR, van Overbeek LS, Berg G, Pirttilä AM, Compant S, Campisano A, Döring M, Sessitsch A: **The Hidden World within Plants: Ecological and Evolutionary Considerations for Defining Functioning of Microbial Endophytes**. *Microbiology and Molecular Biology Reviews* 2015, **79**:293-320.
92. Coenye T, Vandamme P: **Diversity and significance of Burkholderia species occupying diverse ecological niches**. *Environmental Microbiology* 2003, **5**:719-729.

93. Parniske M: **Arbuscular mycorrhiza: the mother of plant root endosymbioses**. *Nat Rev Micro* 2008, **6**:763-775.
94. Schardl CL, Leuchtmann A, Spiering MJ: **Symbioses of grasses with seedborne fungal endophytes**. *Annu Rev Plant Biol* 2004, **55**:315-340.
95. Hardoim PR, van Overbeek LS, Elsas JDv: **Properties of bacterial endophytes and their proposed role in plant growth**. *Trends in Microbiology* 2008, **16**:463-471.
96. Hardoim PR, Hardoim CCP, van Overbeek LS, van Elsas JD: **Dynamics of Seed-Borne Rice Endophytes on Early Plant Growth Stages**. *PLoS ONE* 2012, **7**:e30438.
97. Tyler HL, Triplett EW: **Plants as a habitat for beneficial and/or human pathogenic bacteria**. *Annu Rev Phytopathol* 2008, **46**:53-73.
98. Kale SD, Tyler BM: **Entry of oomycete and fungal effectors into plant and animal host cells**. *Cellular Microbiology* 2011, **13**:1839-1848.
99. Saikkonen K, Wali PR, Helander M: **Genetic Compatibility Determines Endophyte-Grass Combinations**. *PLoS ONE* 2010, **5**:e11395.
100. Bacon CW, Glenn AE, Yates IE: **FUSARIUM VERTICILLIOIDES: MANAGING THE ENDOPHYTIC ASSOCIATION WITH MAIZE FOR REDUCED FUMONISINS ACCUMULATION**. *Toxin Reviews* 2008, **27**:411-446.
101. Rodriguez Estrada AE, Jonkers W, Corby Kistler H, May G: **Interactions between Fusarium verticillioides, Ustilago maydis, and Zea mays: An endophyte, a pathogen, and their shared plant host**. *Fungal Genetics and Biology* 2012, **49**:578-587.
102. Knief C: **Analysis of plant microbe interactions in the era of next generation sequencing technologies**. *Frontiers in Plant Science* 2014, **5**.
103. Pavlo A, Leonid O, Iryna Z, Natalia K, Maria PA: **Endophytic bacteria enhancing growth and disease resistance of potato (Solanum tuberosum L.)**. *Biological Control* 2011, **56**:43-49.
104. Bordiec S, Paquis S, Lacroix H, Dhondt S, Ait Barka E, Kauffmann S, Jeandet P, Mazeyrat-Gourbeyre F, Clément C, Baillieul F, et al.: **Comparative analysis of defence responses induced by the endophytic plant growth-promoting rhizobacterium Burkholderia phytofirmans strain PsJN and the non-host bacterium Pseudomonas syringae pv. pisi in grapevine cell suspensions**. *Journal of Experimental Botany* 2011, **62**:595-603.
105. van Loon LC, Bakker PAHM, van der Heijdt WHW, Wendehenne D, Pugin A: **Early Responses of Tobacco Suspension Cells to Rhizobacterial Elicitors of Induced Systemic Resistance**. *Molecular Plant-Microbe Interactions* 2008, **21**:1609-1621.
106. Hurek T, Handley LL, Reinhold-Hurek B, Piché Y: **Azoarcus Grass Endophytes Contribute Fixed Nitrogen to the Plant in an Unculturable State**. *Molecular Plant-Microbe Interactions* 2002, **15**:233-242.
107. Sessitsch A, Howieson JG, Perret X, Antoun H, Martínez-Romero E: **Advances in Rhizobium Research**. *Critical Reviews in Plant Sciences* 2002, **21**:323-378.
108. Sturz A, Christie B, Nowak J: **Bacterial endophytes: potential role in developing sustainable systems of crop production**. *Critical Reviews in Plant Sciences* 2000, **19**:1-30.

109. Long HH, Schmidt DD, Baldwin IT: **Native Bacterial Endophytes Promote Host Growth in a Species-Specific Manner; Phytohormone Manipulations Do Not Result in Common Growth Responses**. *PLoS ONE* 2008, **3**:e2702.
110. Shi Y, Lou K, Li C: **Promotion of plant growth by phytohormone-producing endophytic microbes of sugar beet**. *Biology and Fertility of Soils* 2009, **45**:645-653.
111. Khan AL, Hamayun M, Kang SM, Kim YH, Jung HY, Lee JH, Lee IJ: **Endophytic fungal association via gibberellins and indole acetic acid can improve plant growth under abiotic stress: an example of Paecilomyces formosus LHL10**. *BMC Microbiol* 2012, **12**:3.
112. Bastián F, Cohen A, Piccoli P, Luna V, Baraldi R, Bottini R: **Production of indole-3-acetic acid and gibberellins A1 and A3 by Acetobacter diazotrophicus and Herbaspirillum seropedicae in chemically-defined culture media**. *Plant Growth Regulation* 1998, **24**:7-11.
113. Merzaeva OV, Shirokikh IG: **The production of auxins by the endophytic bacteria of winter rye**. *Applied Biochemistry and Microbiology* 2010, **46**:44-50.
114. Vadassery J, Ritter C, Venus Y, Camehl I, Varma A, Shahollari B, Novák O, Strnad M, Ludwig-Müller J, Oelmüller R: **The Role of Auxins and Cytokinins in the Mutualistic Interaction Between Arabidopsis and Piriformospora indica**. *Molecular Plant-Microbe Interactions* 2008, **21**:1371-1383.
115. Pirttilä AM, Joensuu P, Pospiech H, Jalonen J, Hohtola A: **Bud endophytes of Scots pine produce adenine derivatives and other compounds that affect morphology and mitigate browning of callus cultures**. *Physiologia Plantarum* 2004, **121**:305-312.
116. Ryu C-M, Farag MA, Hu C-H, Reddy MS, Wei H-X, Paré PW, Kloepper JW: **Bacterial volatiles promote growth in Arabidopsis**. *Proceedings of the National Academy of Sciences* 2003, **100**:4927-4932.
117. Ryu C-M, Farag MA, Hu C-H, Reddy MS, Kloepper JW, Paré PW: **Bacterial Volatiles Induce Systemic Resistance in Arabidopsis**. *Plant Physiology* 2004, **134**:1017-1026.
118. Ryu C-M, Hu C-H, Locy RD, Kloepper JW: **Study of mechanisms for plant growth promotion elicited by rhizobacteria in Arabidopsis thaliana**. *Plant and Soil* 2005, **268**:285-292.
119. Johnston-Monje D, Raizada MN: **Conservation and Diversity of Seed Associated Endophytes in *Zea* across Boundaries of Evolution, Ethnography and Ecology**. *PLoS ONE* 2011, **6**:e20396.
120. Taghavi S, Garafola C, Monchy S, Newman L, Hoffman A, Weyens N, Barac T, Vangronsveld J, van der Lelie D: **Genome Survey and Characterization of Endophytic Bacteria Exhibiting a Beneficial Effect on Growth and Development of Poplar Trees**. *Applied and Environmental Microbiology* 2009, **75**:748-757.
121. Niemi K, Julkunen-Tiitto R, Häggman H, Sarjala T: **Suillus variegatus causes significant changes in the content of individual polyamines and**

flavonoids in Scots pine seedlings during mycorrhiza formation in vitro. *Journal of Experimental Botany* 2007, **58**:391-401.
122. Elmerich C: **Historical perspective: from bacterization to endophytes**. In *Associative and endophytic nitrogen-fixing bacteria and cyanobacterial associations*. Edited by: Springer; 2007:1-20.
123. Santi C, Bogusz D, Franche C: **Biological nitrogen fixation in non-legume plants**. *Ann Bot* 2013, **111**:743-767.
124. Hurek T, Reinhold-Hurek B, Van Montagu M, Kellenberger E: **Root colonization and systemic spreading of Azoarcus sp. strain BH72 in grasses**. *Journal of bacteriology* 1994, **176**:1913-1923.
125. Pedrosa F, Elmerich C: **Regulation of nitrogen fixation and ammonium assimilation in associative and endophytic nitrogen fixing bacteria**. In *Associative and Endophytic Nitrogen-Fixing Bacteria and Cyanobacterial Associations*. Edited by: Springer; 2007:41-71.
126. Compant S, Clement C, Sessitsch A: **Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization**. *Soil Biology and Biochemistry* 2010, **42**:669-678.
127. Philippot L, Raaijmakers JM, Lemanceau P, van der Putten WH: **Going back to the roots: the microbial ecology of the rhizosphere**. *Nat Rev Micro* 2013, **11**:789-799.
128. Mercado-Blanco J, Lugtenberg BJJ: **Biotechnological Applications of Bacterial Endophytes**. *Current Biotechnology* 2014, **3**:60-75.
129. James EK, Gyaneshwar P, Mathan N, Barraquio WL, Reddy PM, Iannetta PPM, Olivares FL, Ladha JK: **Infection and Colonization of Rice Seedlings by the Plant Growth-Promoting Bacterium Herbaspirillum seropedicae Z67**. *Molecular Plant-Microbe Interactions* 2002, **15**:894-906.
130. Compant S, Mitter B, Colli-Mull JG, Gangl H, Sessitsch A: **Endophytes of grapevine flowers, berries, and seeds: identification of cultivable bacteria, comparison with other plant parts, and visualization of niches of colonization**. *Microb Ecol* 2011, **62**:188-197.
131. Fürnkranz M, Lukesch B, Müller H, Huss H, Grube M, Berg G: **Microbial Diversity Inside Pumpkins: Microhabitat-Specific Communities Display a High Antagonistic Potential Against Phytopathogens**. *Microbial Ecology* 2012, **63**:418-428.
132. Madmony A, Chernin L, Pleban S, Peleg E, Riov J: **Enterobacter cloacae, an obligatory endophyte of pollen grains of Mediterranean pines**. *Folia Microbiologica* 2005, **50**:209-216.
133. Truyens S, Weyens N, Cuypers A, Vangronsveld J: **Bacterial seed endophytes: genera, vertical transmission and interaction with plants**. *Environmental Microbiology Reports* 2015, **7**:40-50.
134. van Overbeek LS, Franke AC, Nijhuis EHM, Groeneveld RMW, da Rocha UN, Lotz LAP: **Bacterial Communities Associated with Chenopodium album and Stellaria media Seeds from Arable Soils**. *Microbial Ecology* 2011, **62**:257-264.

135. Nelson EB: **MICROBIAL DYNAMICS AND INTERACTIONS IN THE SPERMOSPHERE**. *Annual Review of Phytopathology* 2004, **42**:271-309.
136. Berg T, Tesoriero L, Hailstones D: **PCR - based detection of Xanthomonas campestris pathovars in Brassica seed**. *Plant Pathology* 2005, **54**:416-427.
137. Schaad NW, Cheong S, Tamaki S, Hatziloukas E, Panopoulos NJ: **A combined biological and enzymatic amplification (BIO-PCR) technique to detect Pseudomonas syringae pv. phaseolicola in bean seed extracts**. *Phytopathology* 1995, **85**:243-246.
138. Stahnke A, Hayes M, Meyer K, Witt K, Weiderman J, Fernando AP, Reese RN: **Prairie Turnip *Pediomelum esculentum* (Pursh) Rydb. historical and modern use, propagation, and management of a new crop**. *Native Plants Journal* 2008, **9**:12.
139. Johnson JR, Larson GE: *Grassland plants of South Dakota and the Northern Great Plains*. Brookings, SD: South Dakota State University; 2007.
140. Stahnke A, Reese RN: **Morphological Examination of Prairie Turnip (*Psoralea esculenta* Pursh) Root**. *Proceeding of the South Dakota Academy of Science* 2003, **82**:5.
141. Perera G, Reese RN: **Nutrional value of *Psoralea esculenta***. *SD Acad. Sci.* 2002, **81**.
142. WIlson JK: **Leguminous plants and their associated organisms**. Edited by Station CUAE. Ithaca, New York; 1939.
143. Bushnell OA, Sarles WB: **Studies on root nodule bacteria of wild leguminous plants in Wisconsin**. *Soil Science* 1937, **40**:409-423.
144. Egan AN, Crandall KA: **Divergence and diversification in North American Psoraleeae (Fabaceae) due to climate change**. *BMC Biol* 2008, **6**:55.
145. Petrini O, Dreyfuss M: **Endophytische pilze in epiphytischen Araceae, Bromeliaceae und Orchidaceae**. In *Sydowia Annales mycologici*: 1981.
146. Murashige T, Skoog F: **A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures**. *Physiologia Plantarum* 1962, **15**:473-497.
147. Mirza BS, Rodrigues JL: **Development of a direct isolation procedure for free-living diazotrophs under controlled hypoxic conditions**. *Appl Environ Microbiol* 2012, **78**:5542-5549.
148. Subramanian S, Fu Y, Sunkar R, Barbazuk WB, Zhu JK, Yu O: **Novel and nodulation-regulated microRNAs in soybean roots**. *BMC Genomics* 2008, **9**:160.
149. Rettedal EA, Clay S, Brozel VS: **GC-clamp primer batches yield 16S rRNA gene amplicon pools with variable GC clamps, affecting denaturing gradient gel electrophoresis profiles**. *FEMS Microbiol Lett* 2010, **312**:55-62.
150. Muyzer G, de Waal EC, Uitterlinden AG: **Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA**. *Appl Environ Microbiol* 1993, **59**:695-700.
151. Stackebrandt E, Goodfellow M: *Nucleic acid techniques in bacterial systematics*: Wiley; 1991.

152. Poly F, Ranjard L, Nazaret S, Gourbiere F, Monrozier LJ: **Comparison of nifH gene pools in soils and soil microenvironments with contrasting properties**. *Appl Environ Microbiol* 2001, **67**:2255-2262.
153. Pruesse E, Peplies J, Glöckner FO: **SINA: Accurate high-throughput multiple sequence alignment of ribosomal RNA genes**. *Bioinformatics* 2012, **28**:1823-1829.
154. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, et al.: **Clustal W and Clustal X version 2.0**. *Bioinformatics* 2007, **23**:2947-2948.
155. Tamura K, Nei M: **Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees**. *Mol Biol Evol* 1993, **10**:512-526.
156. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S: **MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0**. *Molecular Biology and Evolution* 2013, **30**:2725-2729.
157. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, et al.: **Fiji: an open-source platform for biological-image analysis**. *Nat Meth* 2012, **9**:676-682.
158. Thomas P, Sekhar AC: **Live cell imaging reveals extensive intracellular cytoplasmic colonization of banana by normally non-cultivable endophytic bacteria**. *AoB Plants* 2014, **6**:plu002.
159. Bergersen FJ: *Methods for evaluating biological nitrogen fixation*: J. Wiley; 1980.
160. Goos RJ, Abdraimova N, Johnson BE: **A Method for Determination of Ureides in Soybean Tissues**. *Communications in Soil Science and Plant Analysis* 2014:null-null.
161. Gaby JC, Buckley DH: **A comprehensive aligned nifH gene database: a multipurpose tool for studies of nitrogen-fixing bacteria**. *Database (Oxford)* 2014, **2014**:bau001.
162. Trinick M: **Symbiosis between Rhizobium and the non-legume, Trema aspera**. *Nature* 1973, **244**:459-460.
163. Callaham D, Deltredici P, Torrey JG: **Isolation and cultivation in vitro of the actinomycete causing root nodulation in Comptonia**. *Science* 1978, **199**:899-902.
164. Perret X, Staehelin C, Broughton WJ: **Molecular Basis of Symbiotic Promiscuity**. *Microbiology and Molecular Biology Reviews* 2000, **64**:180-201.
165. Kloepper JW, Ryu CM, Zhang S: **Induced Systemic Resistance and Promotion of Plant Growth by Bacillus spp**. *Phytopathology* 2004, **94**:1259-1266.
166. Kaga H, Mano H, Tanaka F, Watanabe A, Kaneko S, Morisaki H: **Rice seeds as sources of endophytic bacteria**. *Microbes Environ* 2009, **24**:154-162.
167. Kloepper JW, Ryu CM: **Bacterial Endophytes as Elicitors of Induced Systemic Resistance**. In *Microbial Root Endophytes*. Edited by Schulz BJE, Boyle CJC, Sieber TN: Springer Berlin Heidelberg; 2006:33-52.
168. Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feitelson J, Zeigler DR, Dean DH: **Bacillus thuringiensis and its pesticidal crystal proteins**. *Microbiol Mol Biol Rev* 1998, **62**:775-806.

169. Rinu K, Pandey A: **Bacillus subtilis NRRL B-30408 inoculation enhances the symbiotic efficiency of Lens esculenta Moench at a Himalayan location**. *Journal of Plant Nutrition and Soil Science* 2009, **172**:134-139.
170. Yi YJ, Luo K, Liu EM: **Endophyte B-001 (*Bacillus subtilis*) colonization and its biological characters**. *Journal of Nuclear Agricultural Science* 2007, **21**:349-352.
171. Huang B, Lv C, Zhuang P, Zhang H, Fan L: **Endophytic colonisation of Bacillus subtilis in the roots of Robinia pseudoacacia L**. *Plant Biol (Stuttg)* 2011, **13**:925-931.
172. Sharon L. Doty BO, Gang Xin, Jun Won Kang, Glenda Singleton, Zareen Khan, Azra Vajzovic, James T. Staley: **Diazotrophic endophytes of native black cottonwood and willow**. *Symbiosis* 2009, **47**:23-33.
173. Ozawa T, Ohwaki A, Okumura K: **Isolation and Characterization of Diazotrophic Bacteria from the Surface-Sterilized Roots of Some Legumes**. *Scientific Report of the Graduate School of Agriculture and Biological Sciences, Osaka Prefecture University* 2003, **55**:29-36.
174. Gadkari D, Morsdorf G, Meyer O: **Chemolithoautotrophic assimilation of dinitrogen by Streptomyces thermoautotrophicus UBT1: identification of an unusual N2-fixing system**. *J Bacteriol* 1992, **174**:6840-6843.
175. Christiansen J, Cash VL, Seefeldt LC, Dean DR: **Isolation and Characterization of an Acetylene-resistant Nitrogenase**. *Journal of Biological Chemistry* 2000, **275**:11459-11464.
176. Dart P: **Infection and development of leguminous nodules**. *A treatise on dinitrogen fixation* 1977, **3**:367-472.
177. Allen EK, Allen O, Newman A: **Pseudonodulation of leguminous plants induced by 2-bromo-3, 5-dichlorobenzoic acid**. *American Journal of Botany* 1953:429-435.
178. Hirsch A, Bhuvaneswari T, Torrey J, Bisseling T: **Early nodulin genes are induced in alfalfa root outgrowths elicited by auxin transport inhibitors**. *Proceedings of the National Academy of Sciences* 1989, **86**:1244-1248.
179. Dudley ME, Jacobs TW, Long SR: **Microscopic studies of cell divisions induced in alfalfa roots by Rhizobium meliloti**. *Planta* 1987, **171**:289-301.
180. Mathesius U, Weinman JJ, Rolfe BG, Djordjevic MA: **Rhizobia Can Induce Nodules in White Clover by "Hijacking" Mature Cortical Cells Activated During Lateral Root Development**. *Molecular Plant-Microbe Interactions* 2000, **13**:170-182.
181. Yang WC, Katinakis P, Hendriks P, Smolders A, de Vries F, Spee J, van Kammen A, Bisseling T, Franssen H: **Characterization of GmENOD40, a gene showing novel patterns of cell-specific expression during soybean nodule development**. *Plant J* 1993, **3**:573-585.
182. Bauer P, Ratet P, Crespi MD, Schultze M, Kondorosi A: **Nod factors and cytokinins induce similar cortical cell division, amyloplast deposition and MsEnod12A expression patterns in alfalfa roots**. *The Plant Journal* 1996, **10**:91-105.

183. Gasser I, Cardinale M, Müller H, Heller S, Eberl L, Lindenkamp N, Kaddor C, Steinbüchel A, Berg G: **Analysis of the endophytic lifestyle and plant growth promotion of Burkholderia terricola ZR2-12**. *Plant and Soil* 2011, **347**:125-136.
184. Atkins C, Dilworth M, Glenn A: **Ammonia assimilation and export of nitrogen from the legume nodule**. *Biology and biochemistry of nitrogen fixation.* 1991:293-319.
185. Herridge DF, Atkins CA, Pate JS, Rainbird RM: **Allantoin and Allantoic Acid in the Nitrogen Economy of the Cowpea (Vigna unguiculata [L.] Walp.)**. *Plant Physiol* 1978, **62**:495-498.
186. Rainbird RM, Thorne JH, Hardy RWF: **Role of Amides, Amino Acids, and Ureides in the Nutrition of Developing Soybean Seeds**. *Plant Physiology* 1984, **74**:329-334.
187. Diaz-Leal JL, Galvez-Valdivieso G, Fernandez J, Pineda M, Alamillo JM: **Developmental effects on ureide levels are mediated by tissue-specific regulation of allantoinase in Phaseolus vulgaris L**. *J Exp Bot* 2012, **63**:4095-4106.
188. Tegeder M: **Transporters involved in source to sink partitioning of amino acids and ureides: opportunities for crop improvement**. *Journal of Experimental Botany* 2014, **65**:1865-1878.
189. Lucinski R, Polcyn W, Ratajczak L: **Nitrate reduction and nitrogen fixation in symbiotic association Rhizobium-legumes**. *Acta Biochim Pol* 2002, **49**:537-546.
190. Serrano A, Chamber M: **Nitrate Reduction in Bradyrhizobium s p.(Lupinus) Strains and its Effects on their Symbiosis with Lupinus luteus**. *Journal of plant physiology* 1990, **136**:240-246.
191. Polcyn W, Luciński R: **Functional similarities of nitrate reductase from yellow lupine bacteroids to bacterial denitrification systems**. *Journal of plant physiology* 2001, **158**:829-834.
192. Chamber-Perez MA, Serrano A: **C-N balance, Nitrate and Nitrite Reductases in Bradyrhizobium sp. (Lupinus) Nodules Exposed to Nitrate, and the Relationships to Nitrogenase Activities**. *Journal of Plant Physiology* 1991, **139**:70-75.
193. Rigaud J: **Comparison of the efficiency of nitrate and nitrogen fixation in crop yield**. In *Nitrogen and carbon metabolism*. Edited by: Springer; 1981:17-48.
194. Tiedje JM: **Ecology of denitrification and dissimilatory nitrate reduction to ammonium**. *Biology of anaerobic microorganisms* 1988, **717**:179-244.
195. Hermann SM: **The Life History and Evolutionary Biology of a Long-Lived Herbaceous Legume, Psoralea esculenta**. In *Biological Sciences*. Edited by. Chicago: University of Illinois at Chicago; 1983.
196. Castle LM: **The Prairie Turnip Paradox: Contributions of Population Dynamics, Ethnobotany, and Community Ecology to Understanding *Pediomelum Esculentum* Root Harvest on the Great Plains**. In *Ecology and Evolutionary Biology*. Edited by: Universty of Kansas; 2006. vol Doctor.

APPENDIX

A.1 Isolate tables

Table A1. Isolates obtained from mature plants.

MATURE ISOLATES	CLOSEST RELATIVE	PERCENT SIMILARITY	NIFH	LOCATION	TISSUE
OP31	*Bacillus megaterium* CP009920	99.5	+	Oak Lake	Peel
OP33	*Xanthobacteraceae* bacterium AB245351	100		Oak Lake	Peel
OP34	*Mycobacterium* sp. HE616181	97.9		Oak Lake	Peel
OT11	*Rhizobium* sp. uncultured FN421842	100	+	Oak Lake	Top
OT12	*Pseudomonas chlororaphis* HF952693	99.8		Oak Lake	Top
OT13	*Rhizobium* sp. uncultured HM340314	99	+	Oak Lake	Top
OT21	*Rhizobium* sp. uncultured FN421842	100	+	Oak Lake	Top
OU21	*Pseudomonas marginalis* AB021401	100	+	Oak Lake	Tuber
PP13	*Rhizobium* sp. uncultured FN421842	99.5		OLC Plot	Peel
PT22	*Curtobacterium flaccumfaciens* DQ065751	100		OLC Plot	Top
PU11	*Pseudomonas fluorescens* JX885768	99.8		OLC Plot	Tuber
PU21	*Bacillus pumilus* KC692165	100		OLC Plot	Tuber
PU31	*Curtobacterium flaccumfaciens* DQ065751	100		OLC Plot	Tuber
VP11	*Rhizobium* sp. uncultured FN421842	99.4	+	Root Stock	Peel
VP21	*Rhizobium gallicum* AM922177	99.7		Root Stock	Peel
VP31	*Rhizobium gallicum* AM922177	99.7		Root Stock	Peel
VT11	*Xanthomonas translucens* AY994100	100		Root Stock	Top
VT21	*Erwinia persicina* AM294946	100		Root Stock	Top
VT22	*Rahnella aquatilis* CP003244	99.8	+	Root Stock	Top
VT23	*Erwinia persicina* AM294946	99.2	+	Root Stock	Top
VT31	*Erwinia persicina* AM294946	100		Root Stock	Top
VT33	*Rahnella aquatilis* CP003244	99.8	+	Root Stock	Top
VU11	*Bacillus megaterium* CP009920	100		Root Stock	Tuber
VU13	*Rahnella aquatilis* CP003244	99.8		Root Stock	Tuber
VU21	*Dyella japonica* AB682150	99.3	+	Root Stock	Tuber
VU31	*Rahnella aquatilis* CP003244	99.8	+	Root Stock	Tuber
VU32	*Rhodococcus* sp. JX949803	99		Root Stock	Tuber
VU33	*Burkholderia* sp. GU385802	100		Root Stock	Tuber
VU36	*Bacillus megaterium* CP009920	99.3		Root Stock	Tuber
WP11	*Rhizobium gallicum* AM922177	99.5		Pine Ridge	Peel
WP12	*Xanthomonas translucens* AY994100	100		Pine Ridge	Peel
WP13	*Inquilinus limosus* JANX01000434	99.2	+	Pine Ridge	Peel
WP31	*Inquilinus limosus* JANX01000434	99	+	Pine Ridge	Peel
WP31A	*Pantoea agglomerans* JNGC01000002	99.1		Pine Ridge	Peel
WU11	*Labrys monachus* AJ535707	97.2		Pine Ridge	Tuber

WU21	*Bacillus pumilus* KC692165	100	+	Pine Ridge	Tuber
WU22	*Rhizobium leguminosarum* CP001622	99.7		Pine Ridge	Tuber
WU31	*Rahnella sp.* KF153219	100		Pine Ridge	Tuber

Table A2. Bacteria isolated from plants inoculated with the 3 different applied inoculums comprised of isolates from mature *P. esculentum*.

INOCULATED EXPERIMENT	CLOSEST RELATIVE	PERCENT SIMILARITY	NIFH	INOCULUM	TISSUE
BN2	*Bacillus circulans* strain 202PP KM349204 *Bacillus nealsonii* strain MER 126 KT719704	100		*Burkholderia*	Nodule
RN5	*Bacillus nealsonii* strain UBMPTV 04 KT265233	100	+	*Rhizobium*	Nodule
RN4	*Bacillus nealsonii* strain UBMPTV 04 KT265233	100	+	*Rhizobium*	Nodule
RN3	*Bacillus nealsonii* strain UBMPTV 04 KT265233	100		*Rhizobium*	Nodule
RN2	*Bacillus nealsonii* strain UBMPTV 04 KT265233	100	+	*Rhizobium*	Nodule
CN3	*Bacillus nealsonii* strain UBMPTV 04 KT265233	100		No Inoculum	Nodule
CN11	*Bacillus nealsonii* strain UBMPTV 04 KT265233	100	+	No Inoculum	Nodule
CN12	*Bacillus nealsonii* strain UBMPTV 04 KT265233	100	+	No Inoculum	Nodule
BN1	*Pantoea agglomerans* strain TSC KT075206	100		*Burkholderia*	Nodule
CN1	*Rhizobium* sp. CR 5-1 LN867314	100		No Inoculum	Nodule
RN1	*Rhizobium* sp. CR 5-1 LN867314	100		*Rhizobium*	Nodule
RT22	*Bacillus circulans* strain 202PP KM349204	100		*Rhizobium*	Tuber
BT2	*Bacillus circulans* strain 202PP KM349204	100		*Burkholderia*	Tuber
BT1	*Bacillus circulans* strain 202PP KM349204	100		*Burkholderia*	Tuber
RT12	*Bacillus nealsonii* strain UBMPTV 04 KT265233	100		*Rhizobium*	Tuber

CT24	*Bacillus nealsonii* strain UBMPTV 04 KT265233	100	+	No Inoculum	Tuber
CT12	*Bacillus nealsonii* strain UBMPTV 04 KT265233	100		No Inoculum	Tuber
RT21	*Bacillus megaterium* strain ED786 KT354282	100		*Rhizobium*	Tuber
RT11	*Bacillus megaterium* strain ED786 KT354282	100	+	*Rhizobium*	Tuber
CT23	*Microbacterium* sp. O-3 HG796193	100		No Inoculum	Tuber
CT11	*Sphingobium yanoikuyae* strain LD29 HQ660519	100		No Inoculum	Tuber
CT21B	*Sphingomonadaceae* bacterium SAP53 3 JN872544	100		No Inoculum	Tuber
CT21A	*Methylobacterium* sp. 25CI KT347471	100		No Inoculum	Tuber
CT22	*Rhizobium* sp. StTD710 LC025448	100	+	No Inoculum	Tuber

Table A3. Bacteria isolated from surface sterilized seeds that were germinated under sterile condition on water agar.

SEED ISOLATES	CLOSEST RELATIVE	PERCENT SIMILARITY	NIFH	SEED SOURCE
S1	*Bacillus circulans* strain 202PP KM349204 *Bacillus nealsonii* strain MER 126 KT719704	100		Prairie Moon Nursery
S2	*Bacillus circulans* strain 202PP KM349204 *Bacillus nealsonii* strain MER 126 KT719704	100		Nebraska
S3	*Bacillus nealsonii* strain UBMPTV 04 KT265233	98.6		Nebraska
S4	*Bacillus circulans* strain 202PP KM349204 *Bacillus nealsonii* strain MER 126 KT719704	98.2		Collected (CRST)
S5	*Bacillus circulans* strain 202PP KM349204 *Bacillus nealsonii* strain MER 126 KT719704	100		Collected (CRST)

A.2 Microscope images

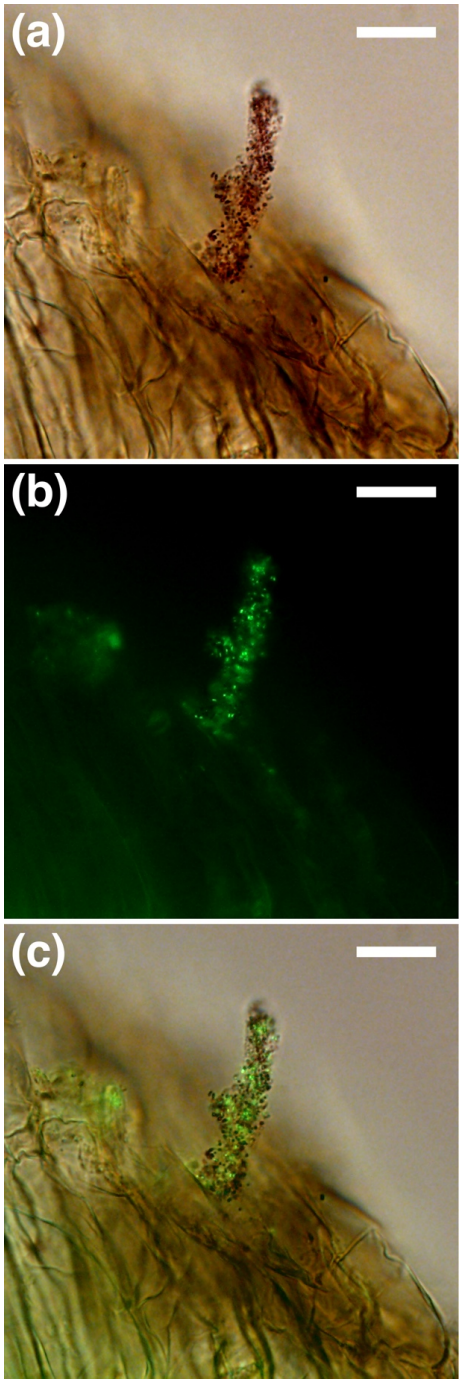

Figure A1. Infected root hair like structure of a seed germinated in sterile conditions. (a) TTC (red) shows areas of respiration. (b) SYTO13 (green) stains nucleic acids. (c) Overlay shows co-localization of the two stains. Bars are 20 μM.

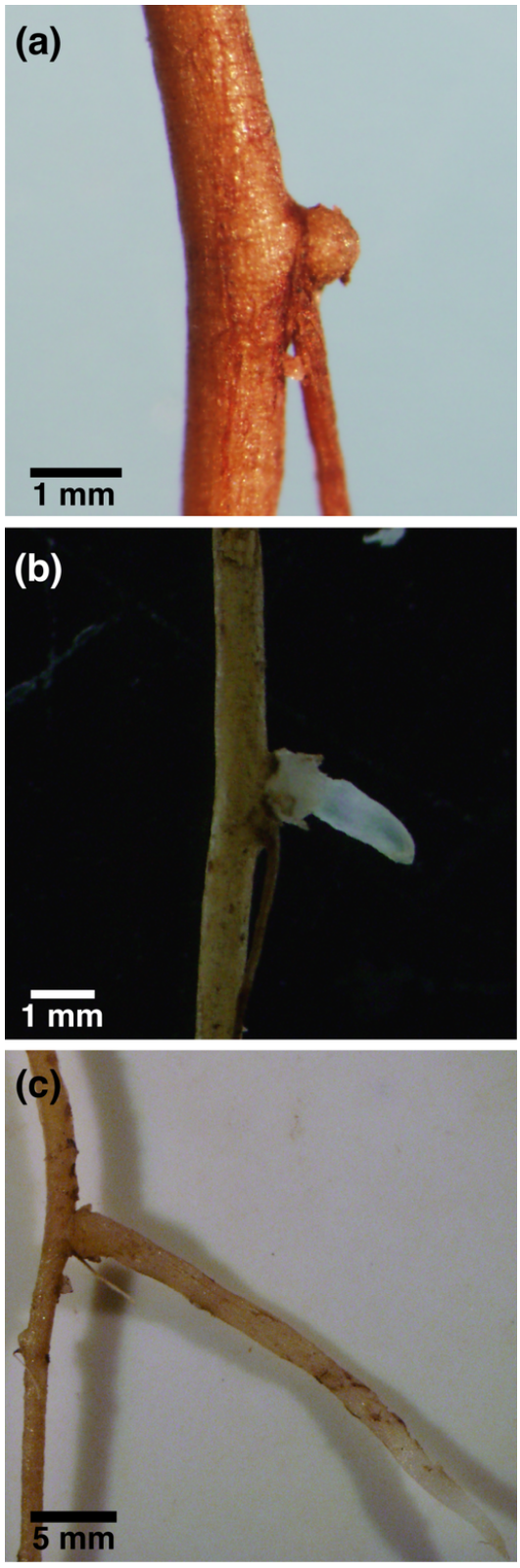

Figure A2. Nodule development in *P. esculentum*. (a) Determinate type nodule. (b) Intermediate type nodule. (c) Elongated intermediate type nodule.

A.3 Crude protein content of *P. esculentum*

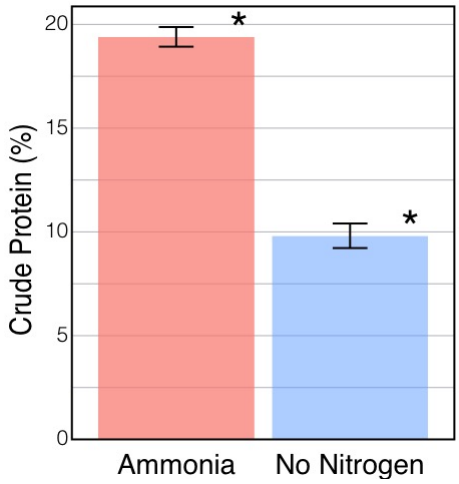

Figure A3. Crude protein of *P. esculentum* with and without ammonia as a nitrogen source. T-test confirmed significantly different means ($p < 0.05$). N= 12.

A.4 Acetylene reduction assay

A.4.1 R Script for area under curve calculation

```
library (flux)
data <- read.csv("2015_10_02 NH4 SAMPLE 5.txt", header = TRUE)
subdata <- subset(data, x >= 2.995)
subdata2 <- subset(subdata, x <= 3.101)
x <- subdata2$x
y <- subdata2$y

auc(x,y, thresh = 0, dens = 100)
```

A.4.2 R Script for calculation of ethylene reduced from acetylene

```
ppm <- 0.5*10000 #convert percent v/v to ppm
vol <- ppm*10^-6 #convert ppm to volume of C2H4 L per L of air
n <- ((101325)*(vol))/((8.31441)*(298.15)) #ideal gas law to calc mol of gas/L where n = PV/RT
umol <- (n/1000)*1000000 # mol/L converted to umol/mL
c2h4 <- umol*40 #total c2h4 in umol for the entire container (40ml) or total c2h4 produced by
 the plant

c2h4 #output
```

A.4.3 R Script for ethylene standard curve

```
> data
      C2H4     AUC
1  16.34972  651.08
```

```
2    81.74861  1166.25
3   817.48610  9505.56
4  1634.97200 11530.12
5  8174.86100 61754.46
6    16.34972   620.95
7    81.74861  1138.23
8   817.48610  9449.33
9  1634.97200 11509.23
10 8174.86100 61731.39
11   16.34972   671.81
12   81.74861  1130.89
13  817.48610  9432.98
14 1634.97200 11575.01
15 8174.86100 61701.11
> model <- lm(formula = AUC ~ 0 + C2H4)
> summary(model)

Call:
lm(formula = AUC ~ 0 + C2H4)

Residuals:
   Min     1Q Median     3Q    Max
-869.7 -178.2  511.9  547.7 3316.1

Coefficients:
     Estimate Std. Error t value Pr(>|t|)
C2H4   7.5713     0.1105    68.5   <2e-16 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 1604 on 14 degrees of freedom
Multiple R-squared:  0.997,      Adjusted R-squared:  0.9968
F-statistic:  4692 on 1 and 14 DF,  p-value: < 2.2e-16
> newx <- seq(1,15)
> prd <- predict(model, newdata=data.frame(x=newx), interval = c("confidence"), level = 0.95,
 type = "response")
> prd
         fit       lwr       upr
1    123.7889   119.9130   127.6648
2    618.9446   599.5653   638.3239
3   6189.4460  5995.6528  6383.2393
4  12378.8906 11991.3041 12766.4770
5  61894.4604 59956.5279 63832.3929
6    123.7889   119.9130   127.6648
7    618.9446   599.5653   638.3239
8   6189.4460  5995.6528  6383.2393
9  12378.8906 11991.3041 12766.4770
10 61894.4604 59956.5279 63832.3929
11   123.7889   119.9130   127.6648
12   618.9446   599.5653   638.3239
13  6189.4460  5995.6528  6383.2393
```

```
14 12378.8906 11991.3041 12766.4770
15 61894.4604 59956.5279 63832.3929

plot(x,y)
abline(model)
lines(C2H4[1:5],prd[1:5,2])
lines(C2H4[1:5],prd[1:5,3])
```

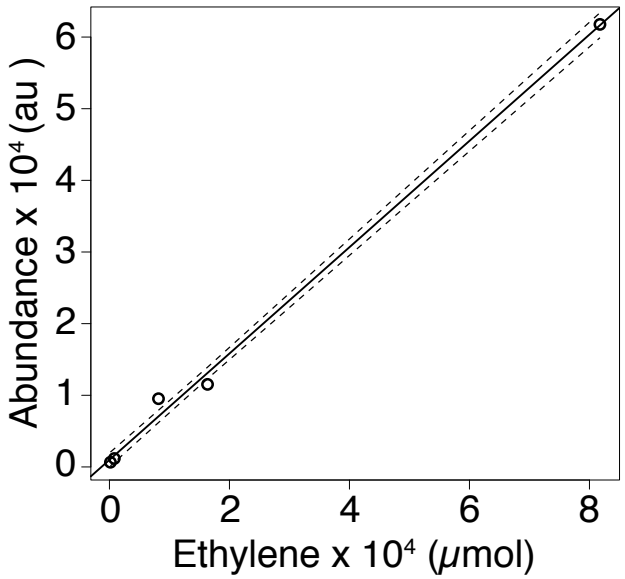

Figure A4. Ethylene standard curve. Dashed lines represent a 95% confidence interval.

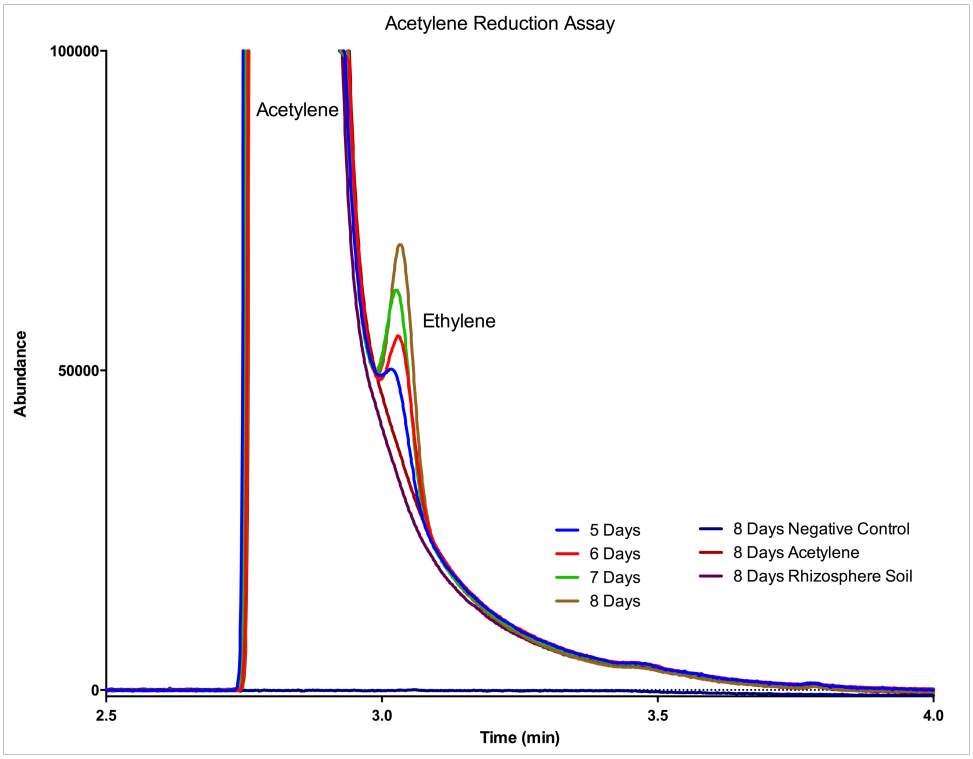

Figure A5. Times course of acetylene reduction in a mature *P. esculentum*. The time course covers day five through 8 of incubation with measurements collected every 24 hours. Controls included an empty vessel, a vessel holding acetylene, and a vessel with the rhizosphere plus acetylene.

A.5 R Script for ANOVA and bar plot construction

```
> data <- read.table("nodule count.txt", header = TRUE)
> data
   Mock Rhizobium Burkholderia
1     2         0          11
2     4         2           4
3     0         4          11
4     5         4           9
5     4         1           2
6     8         7           6
7     3         5          10
8     7         2           1
9     6         5           0
10    4         2           3
11   10         1           2
12    7         2          10
```

```
> sdata <- stack(data)
> summary(data)
     Mock         Rhizobium      Burkholderia
 Min.   : 0.00   Min.   :0.000   Min.   : 0.00
 1st Qu.: 3.75   1st Qu.:1.750   1st Qu.: 2.00
 Median : 4.50   Median :2.000   Median : 5.00
 Mean   : 5.00   Mean   :2.917   Mean   : 5.75
 3rd Qu.: 7.00   3rd Qu.:4.250   3rd Qu.:10.00
 Max.   :10.00   Max.   :7.000   Max.   :11.00
> sdata
   values         ind
1       2        Mock
2       4        Mock
3       0        Mock
4       5        Mock
5       4        Mock
6       8        Mock
7       3        Mock
8       7        Mock
9       6        Mock
10      4        Mock
11     10        Mock
12      7        Mock
13      0   Rhizobium
14      2   Rhizobium
15      4   Rhizobium
16      4   Rhizobium
17      1   Rhizobium
18      7   Rhizobium
19      5   Rhizobium
20      2   Rhizobium
21      5   Rhizobium
22      2   Rhizobium
23      1   Rhizobium
24      2   Rhizobium
25     11 Burkholderia
26      4 Burkholderia
27     11 Burkholderia
28      9 Burkholderia
29      2 Burkholderia
30      6 Burkholderia
31     10 Burkholderia
32      1 Burkholderia
33      0 Burkholderia
34      3 Burkholderia
35      2 Burkholderia
36     10 Burkholderia
> oneway.test(values~ind)

 One-way analysis of means (not assuming equal variances)
```

```
data:  values and ind
F = 1.2219, num df = 2.000, denom df = 21.094, p-value = 0.3147

> oneway.test(values~ind, var.equal = TRUE)

 One-way analysis of means

data:  values and ind
F = 0.9367, num df = 2, denom df = 35, p-value = 0.4015
> library(Rmisc)
Loading required package: lattice
Loading required package: plyr

> sumdata <- summarySE(sdata, measurevar = "values", groupvars = c("ind"), na.rm=TRUE)
> sumdata
         ind  N   values       sd       se       ci
1 Burkholderia 12 5.750000 4.223850 1.2193205 2.683706
2         Mock 12 5.000000 2.763397 0.7977240 1.755779
3    Rhizobium 12 2.916667 2.065224 0.5961789 1.312181
> sumdata$ind <- factor(sumdata$ind)
> library(ggplot2)
> ggplot(sumdata, aes(x=ind, y=values, fill = ind)) + geom_bar(position=position_dodge(), stat =
"identity") + geom_errorbar(aes(ymin = values-se, ymax = values+se), width = .2, position =
position_dodge(.9))
```

A.6 R Script for ureide assay standard curve

```
> avgdata <- read.csv("ureide standard data averages.csv", header = TRUE)
> avgdata
  mg absorbance
1 40 0.11733333
2 30 0.09966667
3 20 0.08833333
4 10 0.07166667
5  0 0.06166667
> plot(avgdata)
> rawdata <- read.csv("ureide standard data.csv", header = TRUE)
> rawdata
   mg  aborbance
1  40 0.11000000
2  40 0.11800000
3  40 0.12400000
4  30 0.10166667
5  30 0.09766667
6  20 0.08833333
7  20 0.08633333
8  20 0.09033333
```

```
9  10 0.07100000
10 10 0.07166667
11 10 0.07233333
12  0 0.06200000
13  0 0.06200000
14  0 0.06100000
> attach(rawdata)
> model <- lm(aborbance ~ mg)
> summary(model)

Call:
lm(formula = aborbance ~ mg)

Residuals:
      Min        1Q    Median        3Q       Max
-0.0058963 -0.0020426  0.0001148  0.0021259  0.0081037

Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept) 0.0598667  0.0016189   36.98 9.81e-14 ***
mg          0.0014007  0.0000673   20.81 8.76e-11 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 0.00362 on 12 degrees of freedom
Multiple R-squared:  0.973,      Adjusted R-squared:  0.9708
F-statistic: 433.2 on 1 and 12 DF,  p-value: 8.757e-11

> abline(model)
> newx <- seq(1,14)
> prd <- predict(model, newdata = data.frame(x=newx), interval = c("confidence"), level = 0.95,
 type = "response")
> prd
         fit       lwr       upr
1  0.11589630 0.11219900 0.11959359
2  0.11589630 0.11219900 0.11959359
3  0.11589630 0.11219900 0.11959359
4  0.10188889 0.09925986 0.10451792
5  0.10188889 0.09925986 0.10451792
6  0.08788148 0.08577097 0.08999200
7  0.08788148 0.08577097 0.08999200
8  0.08788148 0.08577097 0.08999200
9  0.07387407 0.07136461 0.07638354
10 0.07387407 0.07136461 0.07638354
11 0.07387407 0.07136461 0.07638354
12 0.05986667 0.05633945 0.06339388
13 0.05986667 0.05633945 0.06339388
14 0.05986667 0.05633945 0.06339388
> lines(mg, prd[,2])

> lines(mg, prd[,3])
```

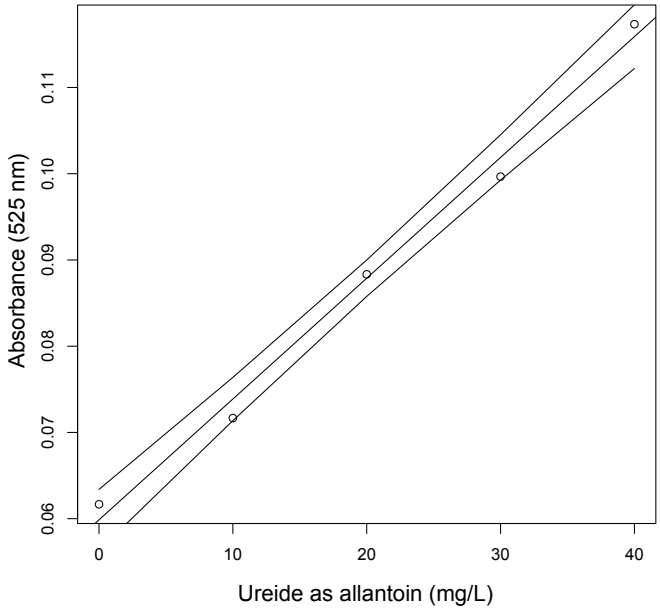

Figure A6. Ureide Standard Curve. Confining lines indicate the 95% confidence interval.

A.7 R Script for hierarchical clustering of isolates and their origin

```
library(gplots)
library(RColorBrewer)
library(vegan)
frequency.table <- read.table("Phylotype Per Tissue.txt", header = TRUE)
row.names(frequency.table) <- frequency.table$Phylotype
heatmapdata <- frequency.table[,-1]
scale <- colorRampPalette(c("lightyellow", "red"), space = "rgb")(100)
heatmapdata2 <- vegdist(heatmapdata, method = "bray")

row.clus <- hclust(heatmapdata2, "aver")
heatmapdata3 <- vegdist(t(heatmapdata), method = "bray")
col.clus <- hclust(heatmapdata3, "aver")
heatmap(as.matrix(heatmapdata), Rowv = as.dendrogram(row.clus), Colv =
  as.dendrogram(col.clus), col = scale, margins = c(12,10))
chisq.test(heatmapdata)


heatmap.2(as.matrix(heatmapdata), Rowv = as.dendrogram(row.clus), Colv =
as.dendrogram(col.clus), col = scale, margins = c(15,15), trace = "none", density.info = "none",
xlab = "Location", ylab = "Phylotype", main = "Phylotype Distribution", lhei = c(2,8))
```

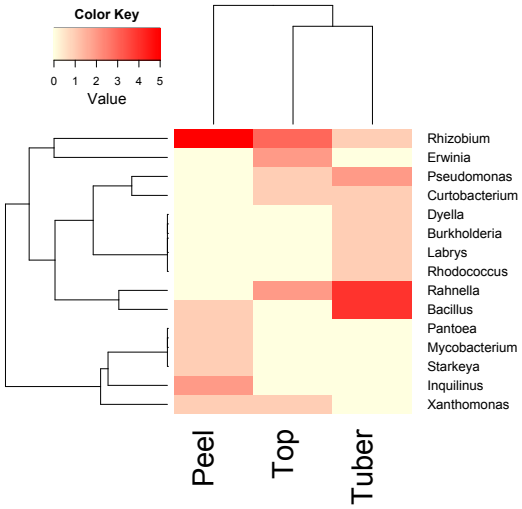

Figure A7. Hierarchical cluster comparing isolate genus to tissue type

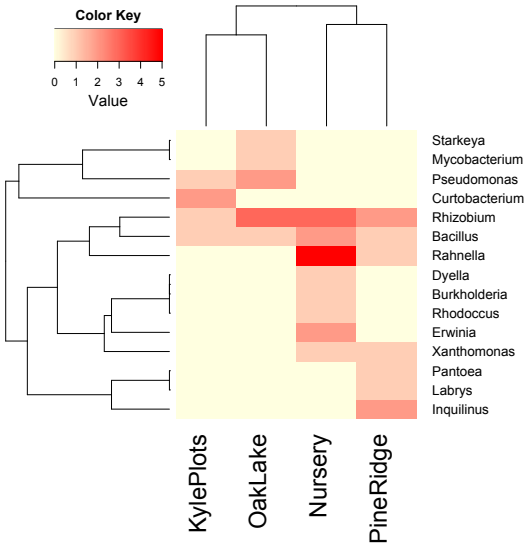

Figure A8. Hierarchical cluster comparing isolate genus to location.

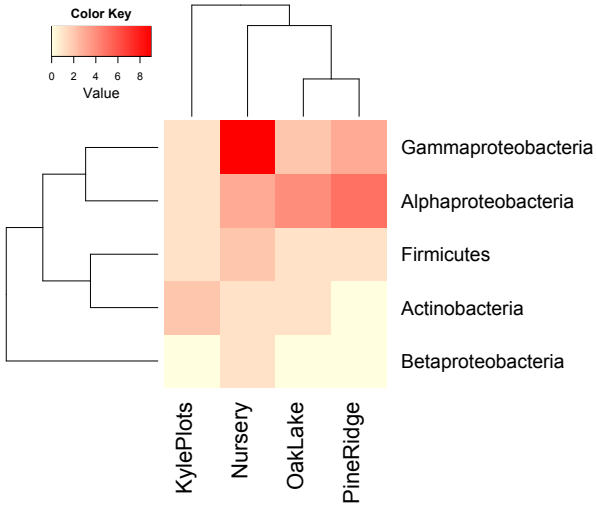

Figure A9. Hierarchical cluster comparing Isolate phylotype to location.

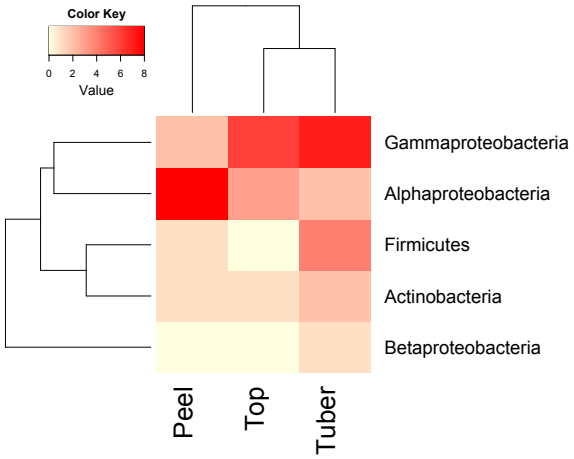

Figure A10. Hierarchical cluster comparing isolate phylotype to tissue type.

A.8 Primer design for qPCR

Specific *16S rRNA* primer sets were designed for use in a qPCR assay to quantify *Bacillus* spp. versus Rhizobia in nodule tissue. A pair was specifically designed to target only *Bacillus* spp. and tested against multiple other isolates, including Rhizobia. The forward and reverse primers were TGAGGTAACGGCTCACCAAG and CGTCCATTGCGGAAGATTCC, respectively, with a

product of 125 bp. The primer set amplified only *Bacillus* species. The pair also amplified the target sequences from nodules, tubers, and seeds (Figure A14).

Multiple pairs were also designed to target only Rhizobia. The primer sets functioned properly *in silico* but amplified sequences from non-target species on the bench top.

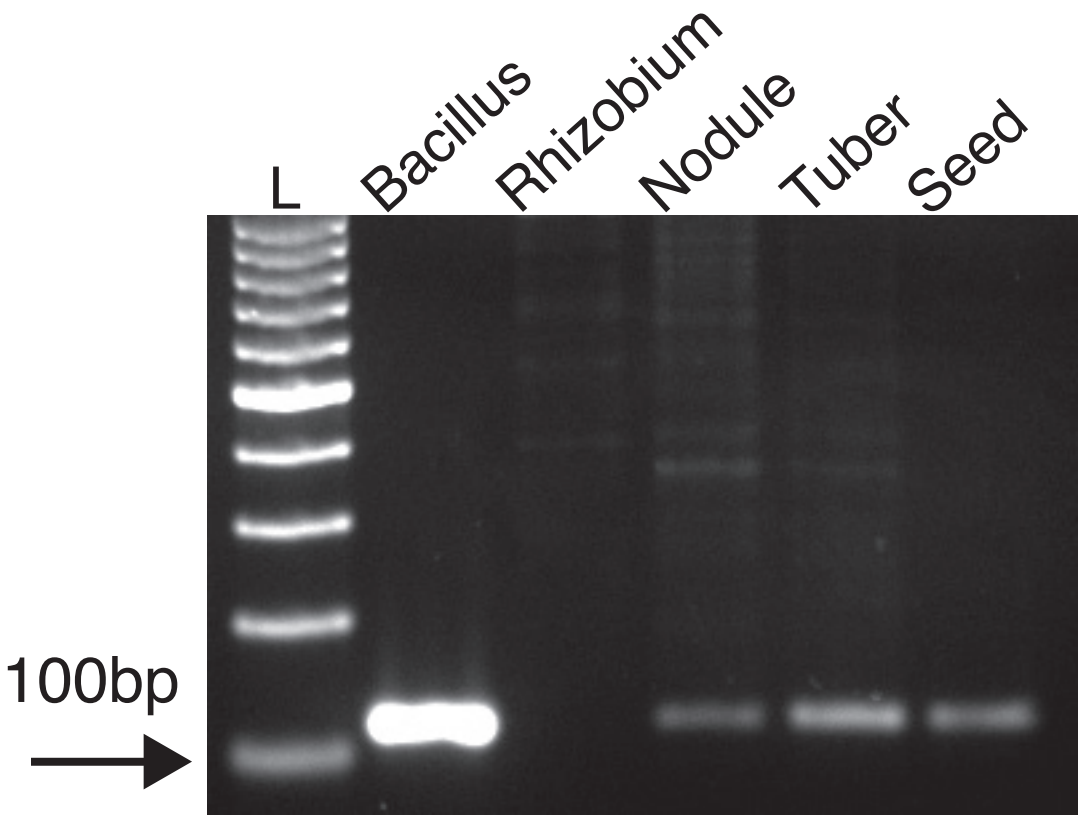

Figure A11. PCR products of the *Bacillus* specific primer pair. The 125bp product was amplified from nodule, tuber, and seed tissue.