REVIEW ARTICLE

Nodulation and Nitrogen Fixation of *Pongamia pinnata*

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Abstract

Nitrogen is one of the most important nutrients required by plants as a major component of all nucleic acids and proteins such as enzymes which control and enable their growth and reproduction. While much research has been conducted on the legume tree Pongamia (a candidate source for renewable biofuel), there is only a handful of studies on the mechanisms and regulation of nitrogen fixation, which is considered as one of the most important domestication traits that needs to be investigated. Steps to optimize the symbiotic nitrogen fixation of Pongamia is, firstly, to select the best rhizobial isolates as inoculum among the naturally-occurring pool of bacteria in soils across Queensland. There have been reports on rhizobia nodulating Pongamia isolated from Western Australia and India but not in Queensland, Australia. This study is the first to report such rhizobia isolates that nodulated Pongamia. Secondly, is to establish efficient nodulation by studying the factors such as nitrate and salinity. The published literature has provided extensive details on the effects of these factors in nodulation and their mechanisms in various legumes. However, only one preliminary study was published from our laboratory; the present study is the in-depth continuation of that effort. Lastly, nitrogen fixation in Pongamia must be assessed to determine if fixed nitrogen is sufficient to support its growth and reproduction. Acetylene reduction assay is the simplest and most common method of assessing fixed nitrogen but in this research, different methods were explored in order to compare both qualitative and quantitative results. This review summarises the current knowledge related to Pongamia, rhizobia, nodulation and nitrogen fixation.

Keywords: *Pongamia pinnata*, rhizobia, biofuel, legume tree, nodulation, nitrogen fixation

Introduction

Pongamia pinnata (L.) (also called *Millettia pinnata*), a member of the Millettieae tribe of the subfamily Papilionoidae and family Leguminosae, is a medium-sized arboreal legume tree indigenous to the Indian subcontinent and south-east Asia (including Papua-New Guinea, Fiji, Northern Australia). It has been grown in parts of Australia, New Zealand, China, Dubai, Hawaii and the continental USA after successful introduction to these countries and in other humid tropical lowlands around the world. Pongamia can be found in coastal areas, along limestone and rock coral outcrops, edges of mangrove forests and tidal streams and rivers (Scott et al., 2008).

Pongamia plants, like other legumes, form association resulting in root nodulation and symbiotic nitrogen fixation with bacteria present in soil which are generally called rhizobia. To optimise this nitrogen gain in Pongamia, it is of value to isolate the best possible rhizobia for Pongamia symbiosis. An indirect technique to isolate rhizobia from soil is to use a host plant as bait and then later on isolate from the surface-sterilised nodule. This baiting technique, which uses the nodulation process as effective trap for selective enrichment, allows isolation of rhizobia classified in different cross-inoculation groups (Gault and Schwinghamer, 1993). There were 29 rhizobia strains isolated by planting sterilised Pongamia seedlings into soils collected from Andhra Pradesh, Maharashtra and Karnataka in Southern India (Rasul et al., 2012). The ability of these strains to fix nitrogen was sadly not assessed; as there was no superior isolate identified in that study although evaluation of nodulation and molecular analysis were conducted. Another baiting experiment was conducted in Western Australia in which the researchers were able to isolate 40 strains of rhizobia from soil samples (Arpiwi et al., 2012). In this study, a superior rhizobia strain was determined as *Bradyrhizobium yuanmingense*. All rhizobia strains from both of these studies were able

to form 'creamy or white opaque' colonies on growth media with Congo Red, which is a broad characteristic of all rhizobia.

The association between Pongamia and rhizobia results in formation of nodules withbudlike structures in the roots. There are two main kinds of nodules; the determinate and indeterminate nodules, although there are other types of nodules that exist such as the collar-like nodules which are observed in *Lupinus albus* (Yates, 2007), leaf nodules which are common in plants of family Rubiaceae (Lemaire et al., 2012) and stem nodules such as in *Sesbania rostrata* (Bergersen et al., 1988). Determinate nodules, like those of soybean and *Lotus japonicus,* are characterised by a lack of a persistent meristem, which makes them spherical while indeterminate nodules which are observed in clover, pea, medics and woody legumes maintain an active apical meristem for longer period of growth or lifespan (Chaukiyal et al., 2000). This meristem produces new cells for growth resulting in the formation of generally cylindrical nodules. Scott et al. (2008) and Kazakoff et al. (2011) reported that Pongamia were able to produce spherical determinate nodules. However, during its later developmental stage, mature nodules exhibit indeterminate characteristics being cylindrical and coralloid in shape (Samuel et al., 2013).

Nodule formation, whether determinate or indeterminate, requires a lot of energy and therefore must be regulated significantly to balance the amount of nitrogen acquired and the energy spent. One internal control mechanism of the plant is the Autoregulation of Nodulation (AON; (Ferguson et al., 2010), a regulatory process acting via long distance signalling. AON is initiated during early nodule development by the production of a rhizobia–induced signal in the root, which is mobilised to the shoot (Delves et al., 1986). Upon perception of the rhizobia-induced signal in the shoot, a novel factor called the Shoot-Derived Inhibitor (SDI) is produced (presumed to be a microRNA) and is predicted to be transported down to the root where it acts to inhibit continued nodule development (Ferguson et al., 2010; Ferguson et al., 2013; Gresshoff, 1993).

External factors also regulate legume nodule numbers. For example, the plant hormone ethylene is a strong inhibitor of nodulation and is produced following stress, enabling the plant to reduce nodule production, when growing conditions are sub-optimal. Many nitrogenous compounds such as nitrate and ammonia are also strong inhibitors of nodule formation (Carroll et al., 1985). Inhibition of nodule formation by nitrate has been known for over 100 years (Streeter and Wong, 1988). The presence of these nitrogenous compounds in the soil triggers the production of translocated CLE peptides, which inhibits nodulation (Reid et al., 2011). The application of low amounts of nitrate stimulated nodulation by increasing root growth yet avoiding nitrate toxicity (Eaglesham et al., 1983). On the other hand, soil acidity has been found to reduce nodule development and nitrogen fixation in legumes (Lin et al., 2012). Other factors that can indirectly affect nodulation include soil composition, water content and temperature (Ferguson et al., 2013).

Root nodules are the major site of symbiotic nitrogen fixation. Biological nitrogen fixation currently contributes 50-70 million tonnes annually to the global N for agriculture (Unkovich, 2008). Increasing the level of input requires a substantial investment in fundamental research for the optimization and application of the various nitrogen-fixing systems. Therefore, it is relevant to assess the nitrogen fixation process in Pongamia using the known techniques such as acetylene reduction assay, isotope techniques, N-difference method and ureide analysis (Hosseini Bai et al., 2012; Peoples et al., 1989).

Pongamia pinnata

Pongamia has a diploid chromosome number of 22 with an estimated nuclear DNA content of about 1.3 billion basepairs per haploid genome. It has compound leaves, which are pinnate and alternate. The mature leaves have glossy dark green color but they're pale green below (Figure 1). The stem has a diameter of 50-80 cm with smooth grey-brown bark with characteristic vertical fissuring. Because of its hardy stem, it can survive from -5 to 50 °C and 0-1200 m altitude and due to its deep root system, it can grow in most soil types particularly in extreme environmental conditions making it drought- and salinity-tolerant. It can survive in 200-2500 mm per year of rainfall and either in full sun or partial shade (Kazakoff et al., 2011). Pongamia is considered naturalized and posed a low risk to Queensland according to the Biosecurity Queensland weed risk assessment, which is based primarily on the fact that there is currently no evidence that the species has significant negative impacts as a weed elsewhere in the world (Csurhes and Hankamer, 2010).
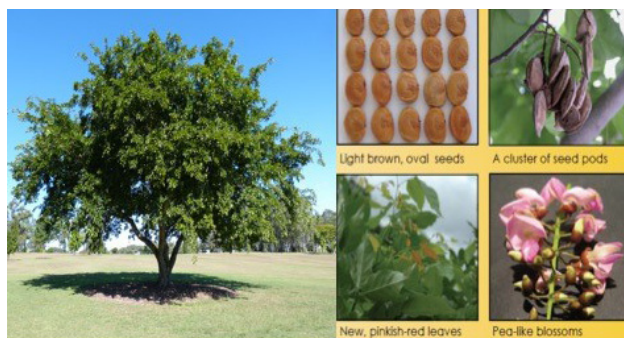
Figure 1. The Pongamia tree and its botanical features.

The Pongamia flower of white and pink colour, has an arrangement typical of other legumes with a standard petal, two identical wings and two identical keel petals (Scott et al, 2008). In general, about 25-35% of flowers set seeds and each seedpod begins development with two embryos which matures in approximately ten months. Each seed has an average mass of 1.8 g, while the pod wall also weight an average of 1.8 g (dry weight). A ten-year old tree, as seen in Brisbane parkland, can yield approximately 20000 seeds per year (Kazakoff, 2011). The tree grows to approximately 5 m in height and 15 cm stem diameter at ground level within five to seven years (Nair, 1993; Scott et al., 2008).

According to Kazakoff et al. (2011), Pongamia trees have low weed potential because of the following reasons: (1) its seeds require warm and wet conditions to germinate; (2) reduced frequency of germination preventing soil-based seed bank build-up; (3) non-invasive and moderate formation of suckers from roots; and, (4) low attraction of seeds as forage for animals.

Each part of the Pongamia tree has many uses. Pongamia inflorescence shows its advantage as ornamental plant and is now commonly used for shade streetscapes. Its wood can be used as stove fuels, poles and ornamental carvings (Scott et al., 2008). The bark can be made into paper and pulp twine. In India, Pongamia flowers, seeds or leaves have been utilised for medicinal purposes. Many medical research studies showed that Pongamia has antimicrobial, therapeutic and spermicidal properties (Brijesh et al., 2006; Elanchezhiyan et al., 1993; Simonsen et al., 2001; Uddin et al., 2003; Srinivasan et al., 2001; 2003; Bandivdekar et al., 2002).

Pongamia is becoming important nowadays as a potential biofuel feedstock (Scott et al., 2008; Biswas et al., 2013). This legume tree produces seeds, which contain 40% oil, predominantly 50% of which is the mono-unsaturated oleic acid (C18:1) that can be used for biodiesel production (after transesterification with methanol or ethanol) (Kazakoff et al., 2011). Pongamia oil is made up predominantly of triglycerides, which can be converted by transesterification to biodiesel (fatty acid methyl esters) or by hydrogenation to aviation A1 jet fuel (Kazakoff et al., 2011; Klein-Marcuschamer et al., 2013). The oil is low in saturated palmitic (C16:0) and stearic (C18:0) acids, and is non-edible. Following oil extraction the resultant seedcake finds utility as supplemental animal feed for poultry, cattle and sheep, but only in small portions (10-20%); (Chandrasekaran et al., 1989; Konwar and Banerjee, 1987a; Konwar et al., 1984; 1987b; 1987c; Natanam and Chandrasekaran, 1989; Natanam et al., 1989a; 1989b; 1989c; Ravi et al., 2000; Amarjit et al., 1985; Biswas and Gresshoff, 2014). Multiple pod components (including the oil, seed cake, and pod walls) can be used for energy production via co-combustion, or fermentation.

The potential of Pongamia as a biofuel feedstock is now acknowledged (Naik et al., 2008; Kesari and Rangan, 2010; Dwivedi et al., 2011; Kazakoff et al., 2012; Klein-Marcuschamer et al., 2013; Samuel et al., 2013; Murphy et al., 2012). According to the US Department of Energy (International Energy Outlook, 2009), the current demand for oil from fossil fuels is around 85 million barrels per day (approximately 13.5 billion litres) and in 2030, it is expected that oil demand will be around 106 million barrels per day (approximately 16.9 billion litres). Pongamia and other biofuel feedstocks can contribute substantially to the future energy demands of the domestic and industrial economies.

Pongamia is a strong candidate as a biofuel producing plant, because it meets two criteria as an efficient biofuel (Kazakoff et al., 2012): (1) must be a non-food crop that can grow on marginal land not destined to be used for the cultivation of food crops, and (2) the use of vegetable oils from plants such as *P. pinnata* has the potential to provide an environmentally acceptable fuel, the production of which is greenhouse gas neutral, with reductions in current diesel engine emissions. Moreover, the composition of seed oil and the properties of fatty acid methyl esters (FAMEs) of Pongamia meet North American and European industry standards (Scott et al., 2008) (Table 1).

Pongamia is yet to undergo any directed domestication that has accompanied the development of modern annual and perennial crops. Some selection for yield and tree architecture may have occurred in India over thousands of years of 'village' agriculture (Kazakoff, 2011). There have been no genetic breeding programs for Pongamia. In order to initiate a directed breeding

Table 1. Properties of Pongamia oil, which makes it an excellent biofuel feedstock based on the Northern American and European industry standards (Scott et al., 2008).

Criteria or Requirement for biofuel	Physico-chemical properties of Pongamia oil
Composition of seed oil	Oleic acid (C18:1; 40-55%) Palmitic acid (C:16; 5-20%) Stearic acid (C18:0; 5-10%) Linoleic acid (C18:2; 15-20%) Rachitic acid (C20:0) Eicosanoid acid (C20:1) Beenak acid (C22:0) Lignoceric acid (C24:0).
Saponification number - the relative fatty acid chain length of the FAMEs	196.7
Iodine value - a measure of the total number of double bonds amongst the respective fatty acids	80.9
Cetane number - an indication of ignition quality of the fuel	55.84
Viscosity of FAMEs	3.8 to 4.8 $mm^2.s^{-1}$ at 40 °C
Flash point of FAMEs	135 to 150 °C
Pour point - the lowest temperature at which oil will flow	2.1°C
Cloud point - the temperature that will lead to separation of dissolved solids from the oil	8.3 °C
Land area for sufficient production to meet the 10% replacement of fossil fuel derived biodiesel	1.99 x 10^6 ha

or domestication program, desirable traits need to be established in concert with functional genomics studies to identify and characterize the relevant traits. The suggested domestication traits include nitrogen fixation efficiency, which is deemed as the most important, as it relates to the environmental and economic costs of nitrogen fertilizer supplementation. Other suggested traits include repeated annual cropping, crop uniformity, seed viability over time, seed mass per tree (yield), seed oil content (extractable), oil composition and stability, growth vigour at seedling and adult stage, erect growth and architecture, seed abscission, resistance to insects, nematodes, fungi and bacteria, flowering time, water-use efficiency, hardiness to cold, acid soils, drought and salinity (Kazakoff et al., 2011).

Bradyrhizobium japonicum and other Rhizobia

Bacteria that fix nitrogen in agricultural systems can be classified into two groups: the heterotrophs and the autotrophs (Unkovich, 2008). The heterotrophic groups can be further classified into four types: the free-living, root-associated, endophytic and symbiotic while the autotrophs (such a photosynthetic cyanobacteria) can either be free-living or symbiotic. *Bradyrhizobium japonicum* is a symbiotic heterotroph

with a broad range of plant hosts that includes many legumes (such as cowpea and soybean) and even the non-legume *Parasponia.* Some examples of bacterial genera that are also symbiotic and fix nitrogen in symbioses are *Rhizobium, Mesorhizobium,* and *Sinorhizobium.*

In the Handbook of Rhizobia (Somasegaran and Hoben, 1994), genetically diverse and physiologically heterogeneous bacteria of the genus Rhizobium (family Rhizobiaceae) are classified together according to their growth characteristics and their ability to nodulate plants of the family Leguminosae. This classification scheme is usually referred to as "cross-inoculation" grouping, in which one species of Rhizobium nodulates all the legumes within that group. The fast growing acid producers, which develop pronounced turbidity in liquid media within two to three days and have a mean doubling time of two to four hours, belong in Group I. The cells are rod-shaped to pleomorphic, 0.5-0.9 µ in diameter and 1.2-3.0 µ in long, and are motile by two to six peritrichous flagella. They can grow on a wide range of carbohydrates and usually grow best on glucose, mannitol, or sucrose. They are generally infective on temperate legumes. Examples of Group I rhizobia are *R. leguminosarum,* which nodulates peas, vetch and lentils; *R. phaseoli*

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which nodulates beans and scarlet runner bean; *R. trifolii* which nodulates clovers and *R. meliloti* which nodulates alfalfa (medics) and fenugreek. On the other hand, the slow growing, alkali producing rhizobia, which require three to five days to produce moderate turbidity in liquid media and have a mean doubling time of six to seven hours, belong in Group II. These rhizobia utilise pentoses best as their carbon source. The cells are predominantly rod-shaped, and motile by a single polar or subpolar flagellum; they mainly nodulate tropical legume species. Examples of Group II include *R. lupini*, which nodulates lupins and serradella; *Bradyrhizobium japonicum*, which nodulates soybeans and *Bradyrhizobium* spp., which nodulates cowpea and other legumes.

According to Somasegaran and Hoben (1994), rhizobia are generally aerobic chemo-organotrophs, which allows them to be easily grown *in vitro.* Most rhizobia are aerobic although some strains are able to grow well at low oxygen conditions (microaerophilic). Rhizobia utilize relatively simple carbohydrates and amino compounds while some strains require vitamins for growth. These bacteria grow best in a temperature range of 25-30 °C, and a near neutral pH of 6-7 (Somasegaran and Hoben, 1994). The ability to be physiologically versatile is an important trait of rhizobia in order to adapt to the competitive and complex soil environment. They must outcompete other rhizobia for infection sites on legume roots aside from exhibiting saprophytic competence among other soil microorganisms.

The conventional classification was subjected to many criticisms and molecular and taxonomic studies were done to classify the group of rhizobia based on different molecular markers such as 16S rDNA, *NodC* genes, and *NifH* genes. Figure 2 shows different phylogenetic trees based on these different molecular markers (Laguerre et al., 2001). The current taxonomy of rhizobia according to Laguerre et al. (2001) is based on the 16S rDNA sequences and reveals that there are six genera of rhizobia: *Rhizobium, Sinorrhizobium, Mesorhizobium, Bradyrhizobium, Azorhizobium*, and the recently described *Allorhizobium*. Rhizobia species that have distinct symbiotic (*Sym*) genotypes and phenotypes can be genetically related as defined by their 16S rDNA sequences (Figure 2 A). On the other hand, Figures 3 (B) and 3 (C) further revealed that species of rhizobia that are genetically distinct can have the same *Sym* genes (Laguerre et al., 2011). There are also instances of inconsistencies between the classifications and phylogenies resulting from comparative analyses of *Sym* genes and 16S rDNA.
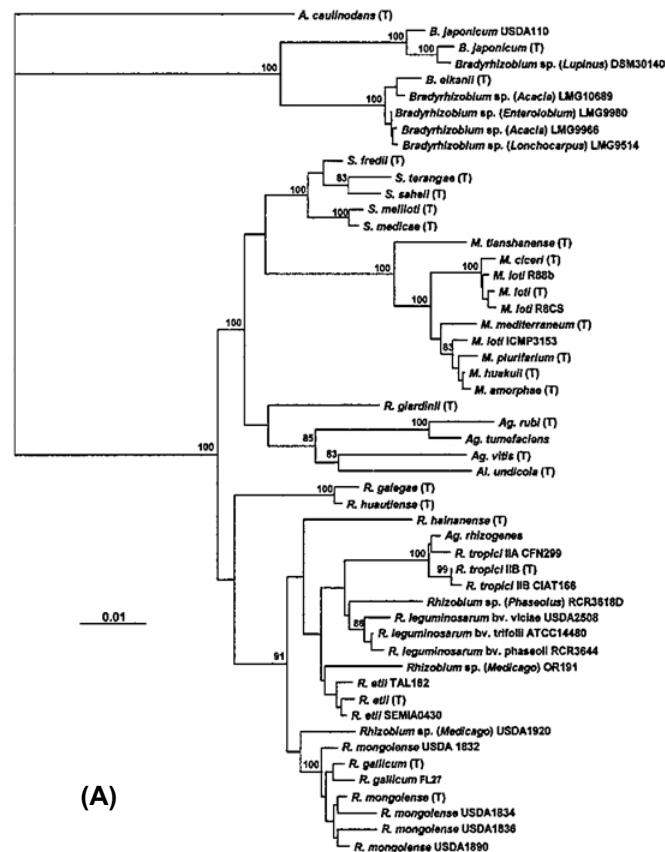

Figure 2. Phylogenetic relationship among rhizobia based on (A) 16S rDNA sequences; (Laguerre et al., 2001).
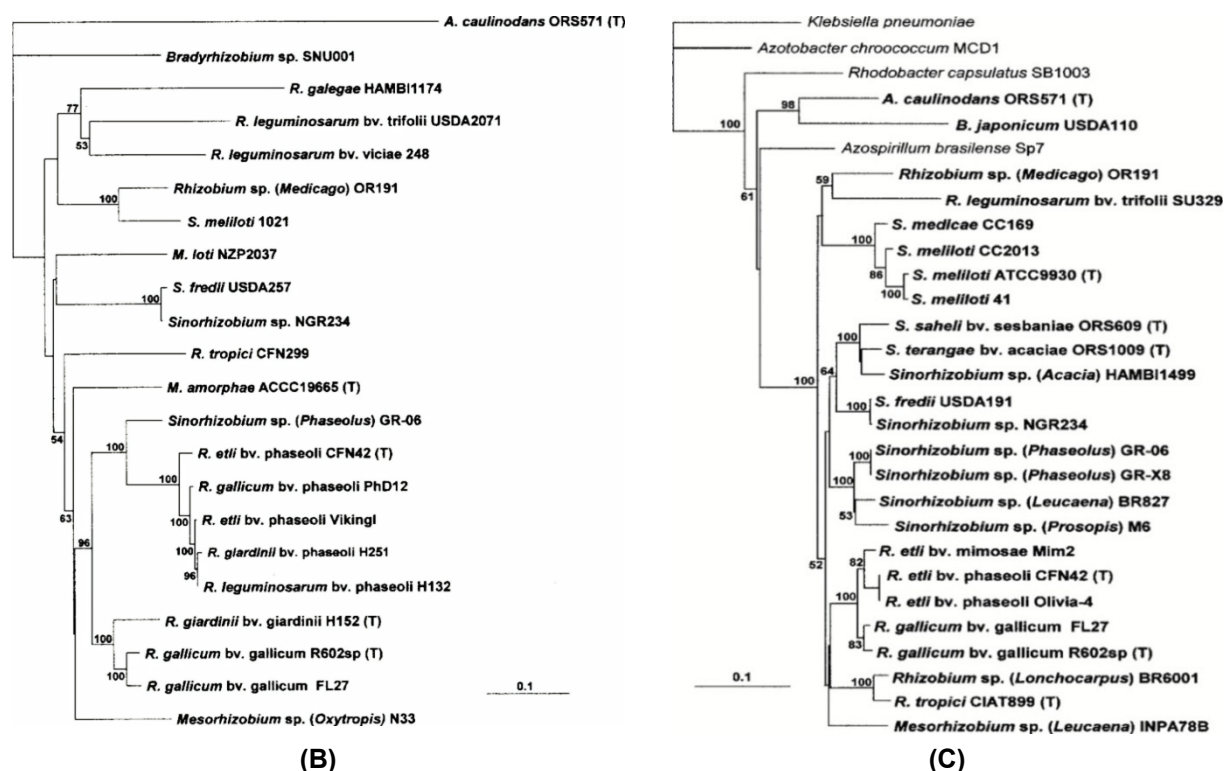
Figure 3. Phylogenetic relationship among rhizobia based on (B) NodC genes; and (C) NifH genes (Laguerre et al., 2001).

In any legume-rhizobium studies, the preservation of nodules after field or glasshouse sampling or even during long-term field trips is crucial. Most of the time, nodules become dried. Some experiments also require drying of nodules to obtain their mean dry weight, which causes a problem, if there is a need to isolate rhizobia or study their diversity in those nodules. The application of DNA fingerprinting technique to dried nodules of *Phaseolus vulgaris* (L.) for strain differentiation was considered a solution (Santasup et al., 2000). A simple protocol was developed in this study to extract and purify rhizobial genomic DNA from individual dried nodules for strain differentiation by PCR-based fingerprinting. The protocol consists of preparation of a cell suspension from rehydrated dried nodules, heat extraction of genomic DNA from rhizobial cells, crude purification of DNA by ethanol precipitation, and Sephadex G-50 column purification of DNA. This protocol, which was strongly suggested for soybeans and other legume nodules might work well in dried Pongamia nodules.

Nodulation in Legumes

Ferguson et al. (2010) in his study reported that the process of nodulation requires a coordinated exchange of signals between the host legume and the rhizobia, as the roots of legumes release different types of phenolic flavonoid compounds creating a gradient or cocktail of signals that are perceived by the compatible rhizobia. Only a specific type of this flavonoid signal attracts a specific rhizobia species and the concentration of these signals also differs along the gradient from the root tip in the rhizosphere (Ferguson et al., 2010).

The following mechanism of nodulation in legumes was derived from Biswas and Gresshoff (2014). Once the rhizobia detect the flavonoids released by the roots of the legumes, their *nodD* genes will activate the Nod box promoter driving transcriptionally several (over 50 have been identified) Nod genes such as *nodABC*, *nodSU* genes which in turn further activate other operons (for transcription factors, etc.) in the rhizobia. The activation of the Nod genes results in the synthesis of the Nod Factors (NF). The *nodC* gene links the five N-acetyl D-glucosamine units creating the oligosaccharide backbone of NF. Other Nod genes connect the fatty acyl group and fucose – a non-reducing sugar, or sulphate onto the oligosaccharide backbone. These decorations provide specificity for interaction with specific host legumes.

The Nod Factors from the rhizobia are being perceived by the legumes through their Nod Factor Receptor (NFR) protein kinases, which are found in their root hairs or epidermis within the "window" of nodulation – the site in the roots of legumes which

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contains receptors and is susceptible to rhizobial infection and later progress to nodule formation. A study on the characterization of the zone of nodulation by Bhuvaneswari et al. (1980) has shown that successful infections occur around the root tip at the time of inoculation, in the region of newly developing root hairs. This 'susceptible zone' or 'window' of nodulation is transient and moves along with the growth of the root (Kinkema et al., 2006).

In soybeans, there are two interacting receptors, NFR1 and NFR5 which are positioned closely with each other and are made up of three domains, the LysM domain located outside the epidermis, the transmembrane domain and the intracellular kinase domain. (Indrasumunar et al, 2010; 2011). In a study by Indrasumunar and Gresshoff (2011), NFR1 does not have the functional LysM domain and therefore needs the NFR 5 to act as 'fingers', which hold the Nod Factors released by the compatible rhizobia. The transmembrane domains of both receptors are embedded in the double membrane of fatty acids in the root epidermis and facilitate the activation of kinases located inside the plant cytoplasm. NFR5 has a non-functional kinase domain and therefore requires NFR1. Kinases facilitate the phosphorylation of threonine or serine in plant proteins changing their activity while using ATP. In approximately 15 minutes after the NFs are perceived or attached into the NFR complex, potassium ion channels are activated creating a spike of calcium ions inside the cell. This calcium spiking triggers the activation of the calcium and calmodulin-dependent kinase (CCaMK) leading to the activation of succeeding transcription factors such as Nodulation Signalling Pathway 1 (NSP1). NSP1 further activates the Early Nodulation Expression (ERN) genes. Meanwhile, the CCaMK/Cyclops interaction results in the activation of synthesis of cytokinin (CK), which attach to the cytokinin receptor (LHK1/CRE1), located in the root cortex and pericycle. Cytokinin is known to facilitate cell division in plants (Indrasumunar and Gresshoff, 2011).

The rhizobia is attached to the portion of the root called "window of nodulation" or Zone of Nodulation (ZON) which is found below the zone of root extension and just above the root meristem (ref)It is the region of differentiation of root cells and is the only part of the roots that is responsive to rhizobial inoculation. The rhizobia multiply and breakdown a portion of the plant cell wall causing a reaction of the plant resulting to root hair deformation or curling. They continue to divide inside the root hair causing a pressure inside, thus pushing the dividing rhizobia towards the cortical cells. The invading rhizobia ramify through or along the cortical cells creating an infection thread. Once the rhizobium enters the cortical cell, a peribacteroid membrane (PBM) is formed surrounding the dividing rhizobium, which is now called a symbiosome (Gonzalez-Guerrero et al., 2014; Udvardi and Poole, 2013). In soybeans and probably also in Pongamia, the rhizobium multiplies up to 25000 viable cells per infected cell while in medicago or clover, the rhizobia still multiply to 1000 cells per infected cell but do not separate completely (Biswas and Gresshoff, 2014). This difference in symbiosome development among legumes might be due to the presence of nodule cysteine-rich peptides, which makes medicago symbiosome three times larger than soybeans and are non-viable if mature (Biswas, 2014).

Most small herbaceous shrub legumes completely die at the end of the growing season or after flowering stage; hence they do not require nitrogen supply for extended periods of time. These legumes tend to form determinate nodules with definite shape and lifespan (Sprent and Parsons, 2000). The formation of indeterminate nodules by woody legumes on the other hand, maybe due to their different seasonal nitrogen requirement as they require more nutrient for their nodulation and nitrogenase activities during the summer rainy season but lower nutrient requirement for these activities during winter (Baird et al., 1985; Sprent and Parsons, 2000; Ng and Hau, 2008).

Some legumes produce determinate nodules, which are spherical but later on become cylindrical or coralloid during maturation. For instance, all samples of wild legumes in China produced spherical nodules, which were considered undifferentiated and immature ones since other shapes of nodules were also observed especially at later stage (Ng and Nau, 2009). *Cajanus cajan* (pigeon pea) also initially produced determinate nodules, which later on transformed into indeterminate ones during its later stage (Brown and Walsh, 1994). Similar observations were reported in *Pithecellobium dulce*, in which all examined species of young nodules were spherical while mature nodules were elongated, branched and coralloid (Qadri et al., 2007).
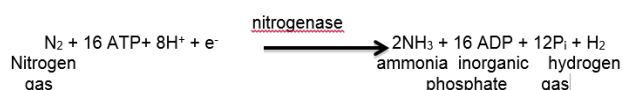
Biological Nitrogen Fixation

Nitrogen is the main limiting nutrient in any agricultural systems. The addition of fertilizers to the soil provides sufficient nitrogen to the crops either through legume nitrogen fixation or residual soil fertility (Jensen et al, 2013). The energetic and environmental costs of production of these fertilizers are remarkably great regardless of whether the fertilizers are produced chemically or by mining. Since the earliest days of agriculture, biological nitrogen fixation (BNF) serves as an alternative way to add nitrogen to the

soil and is still the basis of some cropping systems (Unkovich, 2008). In this process, legumes, such as the biofuel tree *Pongamia pinnata*, and rhizobia form a symbiosis, in which nitrogen from the air is fixed into the nodules and later released to the soil after plant death (Unkovich, 2008; Peoples et al., 1989).

According to Biswas and Gresshoff (2014), the infected cell, which contains several bacteroids is positioned adjacent to uninfected cells and close to a xylem pole. Nitrogen from the atmosphere is fixed into the infected cell, and subsequently converted to ammonia by the enzyme nitrogenase whichconsists of iron protein complex and a molybdenum-iron polypeptide. It requires the oxidative phosphorylation of malate, which comes from the Krebs Cycle in plants with the aid of a small microaerobic amount of oxygen that enters the infected cell and binds to leghemoglobin (Biswas and Gresshoff, 2014).

The nitrogen fixation process requires 16 ATPs to provide sufficient energy to break the strong triple bond between the two nitrogen atoms of N_2 gas: $N\equiv N$. (Note that protons (H^+) are also utilized and combined to produce hydrogen gas). Indeed, some rhizobia have a separate hydrogenase, by which this hydrogen gas is reutilized to make ATP. Ironically, this process requires the limited oxygen of the nodule interior, restricting oxygen supply for much needed oxidative phosphorylation (respiration), itself producing critical ATP (Biswas and Gresshoff, 2014). This is the chemical equation for the process of nitrogen fixation:

$$N_2 + 16\ ATP + 8H^+ + e^- \xrightarrow{\text{nitrogenase}} 2NH_3 + 16\ ADP + 12P_i + H_2$$

Nitrogen gas → ammonia, inorganic phosphate, hydrogen gas

As Biswas and Gresshoff (2014) described, ammonia (NH_3) in aqueous solution attracts a proton and forms ammonium (NH_4^+). Ammonium is toxic to plants thus it must be converted to non-toxic form such as glutamine using the enzyme glutamine synthase. Glutamine is exported to the peroxisome of the adjacent uninfected cell where it is converted to allantoin or allantoic acid, which are ureides. Ureides are efficient N transport compounds as they have a C:N ratio of 1:1, compared to glutamine's 2:1; thus less carbon skeletons are needed to move N within the plant. These ureides then go to the xylem vessel and are transported from the roots to the shoots of the plants.

Regulation of nitrogen fixation involves the role of cotyledons in vegetative growth and nitrogen assimilation of soybeans. Removal of one or both cotyledons increased the amount of nitrogen in the shoots of nodulated soybeans at flowering doubling

the nitrogen content of their leaves (Peat et al., 1981). A similar study on Pongamia is therefore relevant in order to determine, if cotyledons can also affect nitrogen fixation of nodulated Pongamia using a compatible rhizobia strain such as the *Bradyrhizobium japonicum* CB1809.

Biological Nitrogen Fixation (BNF) offers both economic and ecological significance by reducing external nitrogen input and improving the quality and quantity of internal resources (Weisany et al., 2013). Mineral nutrients present in soil may influence nitrogen fixation in legumes at various stages of symbiotic process such as infection and nodule development, nodule function, and host plant growth. Major soil elements like phosphorus are needed for nodulation as well as potassium and sulphur. Potassium is also needed for osmoadaptation of plants particularly in saline soils while micro-elements such asboron and calcium are needed in nodule maturation, and required during early symbiotic events respectively. In addition, iron and molybdenum are components of the nitrogenase complex and copper is needed in proteins involved in nitrogen fixation. Nickel and cobalt are similarly important as nickel is a component of nickel-dependent hydrogenase in active rhizobia and cobalt is required for synthesis of leghemoglobin, essential during nitrogen fixation.

Biofuel industries will be interested in the establishment of the potential of the Pongamia tree to fix nitrogen as this would mean optimum plant growth and seed production with less fertilizer utilization, hence reduces cost of production.

Legumes have been known to facilitate nitrogen transfer through the root exudates or connections between root systems thus providing plant-available nitrogen for other non-nitrogen-fixing plants. Therefore, incorporating Pongamia in crop plantations may be a good agricultural practice since, ecologically, the plant has the ability to fix nitrogen, thus impacts climate change adaptation and maintains ecosystem nitrogen balance.

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