Nodulation and Symbiotic Nitrogen Fixation in the Biofuel Legume Tree *Pongamia pinnata*

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Abstract

The legume tree *Pongamia pinnata* (also called *Millettia pinnata*) is a non-food crop that can grow on marginal land not destined for the cultivation of food crops. It is an important candidate for the production of biofuel (bio-oil, biodiesel and aviation biofuel) from its oil-rich seeds. An important trait is its ability to grow in marginal, nitrogen-limited soils. This growth ability is attributed to the nitrogen-fixation activity of root nodules. Cutting both cotyledons in halves had little effect on shoot dry weight, nodule number and weight per plant at 8 weeks. These parameters were reduced when both cotyledons were removed; plants were visibly yellow. Surprisingly when no nutrients were supplied and with both cotyledons intact, plants grew as well as those with nutrients. To isolate rhizobial inoculants from soil, samples were collected from Meandu Mine, a coal-mining site near Kingaroy, Queensland (Australia), where pongamia is currently grown to rehabilitate the soil. Pongamia nodules were also collected from Mt. Coot-tha Botanic Gardens (Brisbane), where pongamia was introduced decades ago. Established 'Baiting technique' and 'One-drop-one-nodule technique' were applied to isolate potential rhizobia from the soil samples and nodules. All isolates were screened and characterised for symbiotic effectiveness and nitrogenase activity. A total of 21 putative rhizobial samples were isolated – five from soil samples and 16 from pongamia nodules. Out of these iso lates, seven were slow-growers while 14 were fast-growers. These isolates were coded as PR-UQ. Among the isolates, the fast-growers PR-UQ-03 and PR-UQ-05 were considered as the superior strains compared to the slow-growers PR-UQ-01 and PR-UQ-04, which were more effective than the rest of the isolates. The new isolates enhanced nodule number, shoot length and total plant dry weight and resulted in better plant growth than the available *Bradyrhizobium japonicum* strains CB1809, USDA110 and CB564. Acetylene reduction assay (short term) and Nitrogen Difference Analysis (long term) further confirmed that pongamia fixed more nitrogen upon inoculation with these rhizobia. Based on 16S rDNA sequence analysis, PR-UQ-01 and PR-UQ-04 are closely related to *Bradyrhizobium elkanii* and *Bradyrhizobium pachyrizi*, while PR-UQ-03 and PR-UQ-05 are both related to *Rhizobium mesoamericanum*. Cotyledons supply the seedling with nutrients for a considerable period of early seedling growth. The newly isolated, fast-growing rhizobia PR-UQ-03 and PR-UQ-05 and the slow-growing PR-UQ-01 and PR-UQ-04 are related to *Rhizobium* and *Bradyrhizobium* species and aid in the nodulation, nitrogen fixation and seedling/sapling growth of *Pongamia pinnata*.

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Introduction

Pongamia pinnata, a fast growing, outcrossing, papillionaceous tree legume with high oil content seeds found in northern Australia, Papua New Guinea, Indonesia and India, is a potential feedstock for biofuel production (Biswas et al., 2013; Gresshoff et al., 2015; Scott et al., 2008). It is salinity and drought tolerant (especially when established beyond the seedling stage), and grows well in low fertility soils (Kazakoff et al., 2011). This legume tree produces seeds, which contain about 40% (w/w) 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). 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, though only in small portions (10-20% of total feed; (Biswas and Gresshoff, 2014; Chandrasekaran et al., 1989; Konwar, 1987a, 1984, 1987b, c; Natanam et al., 1989a; Natanam et al., 1989b; Natanam et al., 1989c; Natanam, 1989; Ravi et al., 2000; Singh et al., 2006).

Multiple pod components (including the oil, seed cake, and pod walls) can be used for energy production via co-combustion, or fermentation. Pongamia oil is made up predominantly of triglycerides, which can be converted by transesterification to biodiesel (fatty acid methyl esters; FAME) or by hydrogenation to aviation A1 jet fuel (Kazakoff et al., 2011; Klein-Marcuschamer et al., 2013).

The potential of pongamia as a biofuel feedstock is now acknowledged (Dwivedi et al., 2011; Kazakoff et al., 2012; Kesari and Rangan, 2010; Klein-Marcuschamer et al., 2013; Murphy et al., 2012; Naik et al., 2008; Samuel et al., 2013). 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). 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 because it meets two criteria as an effective, second-generation biofuel, namely (1) it must be a non-food crop that can grow on marginal land not used for the cultivation of food crops, and (2) the use of vegetable oils from plants must 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 both North American and European industry standards (Scott et al., 2008).

Pongamia is yet to undergo any directed domestication that has accompanied the development of other modern annual and perennial crops. Some selection for yield and tree architecture may have occurred in India over the thousands of years of 'village' agriculture. There is no published record of breeding programs for pongamia. To initiate a directed domestication program, desirable traits need to be defined in concert with functional genomics studies to identify and characterise the relevant traits. The suggested domestication traits include nitrogen fixation efficiency, which is deemed as highly important, as it relates to the environmental and economic costs of nitrogen fertiliser supplementation. Other suggested traits include repeated annual cropping, crop uniformity, seed mass per tree (yield), extractable seed oil content, oil composition and stability, growth vigour at seedling and adult stage, erect growth and architecture, canopy density, seed abscission, resistance to insects, nematodes, fungi and bacteria, flowering time, water-use efficiency, hardiness to cold, acid soils, drought and salinity. Many of these traits will be multigenically controlled and breeding will be an on-going task for the emerging pongamia biofuel industry.

Pongamia nodulates well with a wide range of strains including *Bradyrhizobium* and *Rhizobium* such as *Bradyrhizobium* sp. strain CB564 and Rhizobia sp. strain NGR234 (Pueppke and Broughton, 1999; Rasul et al., 2012; Scott et al., 2008), *Bradyrhizobium japonicum* CB1809 and USDA110 (both slow growing rhizobia and widely used commercial inoculants for soybean) as well as fast growing rhizobia (Arpiwi et al., 2012; Samuel et al., 2013). Some reports indicate that trees, including pongamia, are mainly nodulated by fast-growing rhizobia than by slow-growing rhizobia (Moreira et al., 2006). However, the geographic and soil type distribution and taxonomy of rhizobia strains capable of nodulating pongamia and their effectiveness in symbiotic nitrogen fixation are little understood.

An indirect technique to isolate rhizobia from soil is to use an uninoculated host plant as 'bait' in sample soils and then later on isolate putative inoculant from the surface-sterilised nodule. This baiting technique, which uses nodules as effective traps for selective enrichment, allows isolation of rhizobia classified in different cross-inoculation groups (Gault and Schwinghamer, 1993). In a study by Rasul et al. (2012), 29 rhizobia strains were isolated by planting sterilised pongamia seedlings into soils collected from Andhra Pradesh, Maharashtra and Karnataka in Southern India. The ability of these strains to fix nitrogen was not assessed and there was no superior isolate identified in this study, although evaluation of nodulation and molecular analysis were conducted on these rhizobia. Another baiting experiment was conducted in Western Australia in which 40 strains were isolated from soil samples (Arpiwi et al. 2012). In this study, the 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 characteristic of all rhizobia.

On the basis of phenotypic, phylogenetic distinctiveness and molecular data, a novel species of the genus *Rhizobium*, *Rhizobium pongamiae* was isolated from nodules of pongamia in North Guwahati, Assam, India (Kesari et al., 2013). It is a gram-negative, non-motile, fast-growing, rod-shaped bacterium, which grew optimal at 28°C, pH 7.0 and in medium supplemented with 2% NaCl. It exhibits higher tolerance to the prevailing adverse conditions, for example, salt stress, elevated temperatures and alkalinity and was found to fix nitrogen using the acetylene reduction assay. PCR detected a nifH gene, encoding the iron component of nitrogenase (Kesari et al., 2013).

In the symbiotic relationship with host legumes, rhizobia can promote the host plants' growth by providing available nitrogen,

which would normally be restricted to them. Therefore, isolation, characterisation and selection of suitable rhizobia strains must be conducted to promote the growth of pongamia and increase its potential yields. This study aimed to identify effective rhizobia that can nodulate and fix nitrogen with pongamia in Queensland, Australia.

Materials and Methods

Cotyledon Nutrient Carry-Over

To determine the role of cotyledons in nodulation and avoid the carry-over of nutrients from cotyledons, 40 pongamia seedlings growing in sterile vermiculite at 20 days old were divided into five treatments: (1) inoculated with CB1809 but no nutrient solution added; (2) uninoculated and no nutrient solution added; (3) inoculated with CB1809 with nutrient solution (B&D N-free solution); (4) uninoculated but with nutrient solution; and, (5) uninoculated but with nitrogen (2 mM ammonium nitrate solution) and nutrient solution. Two replicates per treatment were used. Their cotyledons were either cut, removed, or left intact as soon as primary leaves uncurl; all of the seedlings for each treatment were coded as: 0COT = Both cotyledons were removed; 1COT = One cotyledon removed; 2COT = Both cotyledons intact; 1/2 COT = Both cotyledons cut into halves. After eight weeks, the plants were uprooted and nodulation was assessed.

Isolation of Rhizobia

The soil samples were characterised by pH and nutrient component analysis. To get the pH of the soil samples, samples were air-dried and homogenised to <1 mm diameter. Six grams of each sample were weighed and put inside a 50-ml Falcon tube. Two replicates were made for each sample. Then, 30 ml of deionised water was added into each tube (1:5 soil solution ratio). The soil samples were mixed for an hour on an end-over-end shaker at room temperature. The pH was then measured using the pH meter by directly dipping the pH electrode into each tube. For total Carbon and Nitrogen level analysis, dried samples were subjected to combustion and analysed using a LECO TruSpec analyser. For total nutrient analysis, samples were extracted and analysed using a Varian Vista Pro ICPOES instrument.

Baiting technique was employed to isolate the rhizobia from soil samples from the Meandu Mine (six different sites). Soil samples were filled into sterile pots. Pongamia seeds were size-sorted and weighed to select the best seeds, surface-sterilised with 5% commercial bleach for one minute and rinsed with 70% ethanol twice followed by washing seven times sterile distilled water, after imbibition and sown directly into the soil. Plants were maintained in the glasshouse at 27-30^{0}C daytime and 17-20^{0}C night time temperature during summer season and watered with 100-ml B&D nutrient solution (stored at room temperature) once and 100-ml sterile distilled water afterwards as needed. Plants were protected from cross-contamination by covering the surface with white sterile beads with a pipe inserted for watering purposes. The plants were uprooted after 10 weeks. Larger,

pink nodules that were close to the crown or primary roots were selected for the isolation of rhizobia in the laboratory. Data were gathered for shoot length, number of nodes, number of leaflets, number of nodules, root, shoot, nodule and total plant dry weights, nodule fresh weight (preserved), soil type and properties, soil analysis and nodule morphology.

To isolate rhizobia from the pongamia nodules collected from Mt. Coot-tha (Brisbane, Queensland) and from the nodules produced from baited plants in soil samples, the one-drop-one-nodule technique was employed, in which five nodules (large and pink inside) per replicate plant were selected, placed inside sterile tubes and then surface-sterilised with 95% ethanol for 5 seconds and 3% commercial bleach for 5 minutes. Each nodule was crushed into a drop of sterile distilled water in an empty sterile Petri dish using sterile forceps. The nodule extract was then streaked three-ways into the yeast extract mannitol agar with Congo Red (YEMA+CR). Plates were incubated for 6-10 days at 28^{0}C. Well-isolated colonies that appeared on plates after 3-5 days of incubation were sub-streaked into another yeast extract mannitol agar plate and were designated as "fast-growers", while colonies that grew within 6-10 days of incubation were designated as "slow-growers". All isolates were purified by serial sub-streaking into YEMA+CR until pure cultures (judged by consistent colony morphology) were achieved.

Nodulation and Symbiotic Effectiveness Under Glasshouse Conditions

The rhizobia that produced the highest number of 'active' nodules in pongamia were determined based on the assessment of nodulation and symbiotic effectiveness tests. Pure cultures of the isolates and the three available rhizobia strains in the laboratory known to nodulate soybeans (i.e., CB1809, USDA110 and CB564) were inoculated into YMB for 48 hours with shaking. The culture suspensions were then inoculated into surface-sterilised pongamia sown in sterile vermiculite in 15 cm pots which were thinned out later into one plant per pot. Five replicate plants were done for each treatment. Plants were maintained under glasshouse conditions, watered with N-free nutrient solution (B&D nutrient solution) and sterile distilled water and were harvested after 12 weeks. Surfaces of the pots were covered with sterile white beads, in which a pipe was used as opening for watering purposes. The holes of the pipes were covered with sterile aluminium foil to prevent cross-contamination. The data gathered include: shoot length, number of nodes, number of leaflets, number of nodules, root, shoot, nodule and total plant dry weights, nodule fresh weight (preserved), and nodule morphology.

All data were statistically analysed and the symbiotic effectiveness was computed using the formula (Bergersen, 1988):

$$\text{Symbiotic Effectiveness} = \frac{\text{Dry weight (Inoculated)} \times 100}{\text{Dry weight (Uninoculated)}}$$

Time Course of Nodulation

Surface-sterilised seeds were sown into sterile vermiculite and inoculated with the best rhizobia isolates. An uninoculated control was also included. Five replicates were used per treatment and the plants were uprooted at 4 to 8 weeks for data collection: nodule number, nodule fresh weight, shoot and root dry weights, total N level in dried shoots and roots, area of zone of infection using a dissecting microscope attached to NIS-Elements software program for image analysis and leghemoglobin concentration using spectrophotometry (Bio-Rad SmartSpec™ 3000 spectrophotometer, USA, 2014).

Assessment of Symbiotic Nitrogen Fixation

Plants inoculated with the best isolates were subjected to Acetylene Reduction Assay (ARA) to assess the nitrogen fixation activity in the nodulated roots and compared to soybeans. The root system was carefully shaken to remove the vermiculite and were cut and quickly transferred into sealed bottle containers with Suba-seals (Sigma-Aldrich, Australia). The 10% of total gas volume was removed and replaced with 10% acetylene. At 0 and 60 minutes, the gas samples were obtained by a syringe and were directly assayed to Gas Chromatography (injected into a Shimadzu GC-17A gas chromatograph (FID detector, 2 m self-packed PoraPak N 60 column) set with a column oven temperature as 90 °C, detector and injection chamber at 110 °C and gas pressure 90 kPa.). The short assay time was used to avoid root cutting associated drop of measurable ARA. Meanwhile, the Nitrogen Difference method was also employed to estimate the amount of fixed nitrogen in all dried plants inoculated with rhiziobia and the reference treatments.

Characterisation of Rhizobia

The isolates were morphologically characterised based on their colony size in diameter, colour, shape, margin, elevation and texture. Growth curves in liquid culture of all isolates were also determined. One millilitre of pure culture was inoculated to 99 ml of yeast extract mannitol broth and incubated with shaking at 28°C. At 0, 18, 24, 45, 48, 68 and 72-hour incubation, 0.1 ml of the culture suspension was diluted to 10-6 to 10-8 using sterile dilution blanks and plated on YM agar at 28°C. Viable count was determined after 5-10 days of incubation. Growth curves were plotted based on the viable count for each incubation time. The antibiotic resistance of each isolate was also determined. Isolates were grown in YM broth (YMB) with shaking until the titre of about 108 cells/ml was achieved. A dilution of 10-6 was made and then plated into YEMA+CR containing different antibiotics. After 6-12 days at 28°C, viable count of the isolates was determined implicating their antibiotic resistance or sensitivity, while their morphological characteristics on the media with antibiotics were also noted. The isolates were also grown in various growth conditions to determine their tolerance in different temperature (22°C, 37°C and 45°C), pH (4.0, 7.0 and 9.0), and salinity (0.1%, 0.5% and 1% (w/v) NaCl).

Identification of the Rhizobia Strains

Rhizobia were grown in YMB in flasks and then transferred into 1.5 ml centrifuge tubes. Tubes were centrifuged at 16,000 g for 10 min (using an Eppendorf centrifuge 5415D, F45-24-11, Germany). The resulting supernatant was decanted. The rhizobia were resuspended in 467 µl of 10 mmol/L Tris-HCl, pH 8 and 1 mmol/L EDTA. Then 30 µl of 10% SDS and 3 µl of 20 mg/ml proteinase K was added into the tubes.

Tubes were incubated for 1 hour at 37°C. Phenol:chloroform :isoamylalcohol (25:24:1; 500 µl) was added. Tubes were centrifuged in an Eppendorf centrifuge (5415 D) at 16,000 g for 2 min. Sodium acetate (3 M, 0.1 ml, pH 5.2) and 0.6 ml of isopropanol were added to precipitate the DNA. Tubes were incubated at least 30 minutes on ice. The tubes were centrifuged in an Eppendorf centrifuge (5415 D) for 15 minutes at 16,000 g. The DNA pellet was washed in 500 µl of 70% ethanol. The tubes were centrifuged again for 15 minutes. The DNA was dried and resuspended in 100 µl of sterile MilliQ water.

A total of 100 µl of PCR cocktail was used with the following recipe: 200 µM each of dATP, dCTP, dGTP and dTTP; 0.4 µM of each universal primer 27F: GAGTTTGATCCTGGCTCAG (Dorsch and Stackebrandt, 1992) and 1492R: TACGGTTACCTT-GTTACGACTT (Goodfellow and Stackebrandt, 1991); 1x DNA polymerase and 50-500 ng of template DNA and covered with 50 µl of sterile mineral oil. The PCR profile used was initial denaturation at 94°C for 2 min; denaturation at 94°C for 1 min at 40 cycles; annealing at 52°C for 30 seconds; extension at 72°C for 30 seconds and final extension for 2 min.

Five µl of each of the PCR products were loaded to the wells of a 1% agarose gel with 2 µl loading dye along with a 1 kb molecular ladder. The resulting molecular bands were isolated from the gel and sent to AGRF (Brisbane) for DNA sequencing and identification. The sequences were aligned using MUSCLE 3.8.31 and refined using Gblocks 0.91b. The phylogeny was run using PhyML 3.1/3.0 aLRT and rendered using TreeDyn 198.3 (Dereeper et al., 2008).

Results

Cotyledon Nutrient Carry-Over

All of the uninoculated treatments did not produce nodules. For the treatments without N-free nutrient solution added, either inoculated with CB1809 or not, the data between replicates had higher variation while replicates for treatments watered with nutrient solution had closer values. Treatment with ammonium nitrate and with both cotyledons cut in halves had the longest average shoot length of 52 cm, while the same treatment but with intact cotyledons had the most number of nodes and leaflets, with mean values of about 8 and 16, respectively. See supplemental Table S1.

Figure 1 shows the nodulation assessment of all the treatments which revealed that the highest number of nodules was observed in the inoculated treatments added with nutrient solution and with both cotyledons intact. Both inoculated treatments were able to produce several nodules but higher number was
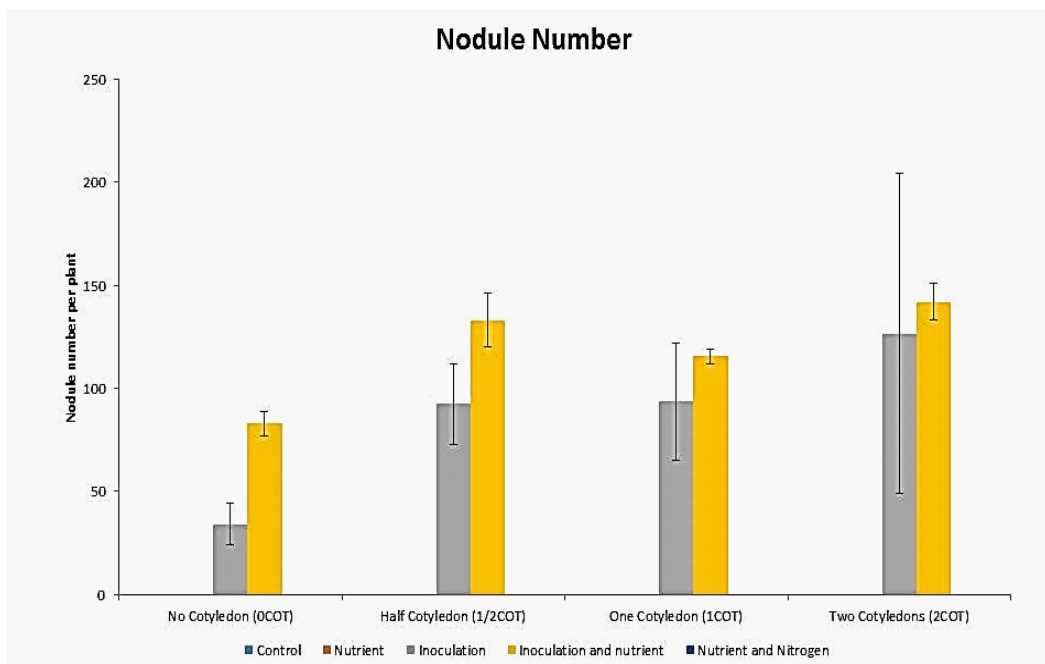
Figure 1. Nodule number vs the five different treatments: inoculated with CB1809 but no nutrient solution added; uninoculated and no nutrient solution added; inoculated with CB1809 with nutrient solution (B&D N-free solution); uninoculated but with nutrient solution; and, uninoculated but with nitrogen (2 mM ammonium nitrate solution) and nutrient solution.

observed in the treatments with nutrient solution.

There were also no huge differences in the plant structure, nodulation and growth conditions among treatment plants with intact cotyledons, one cotyledon removed and both cotyledons cut into halves. See supplemental Figure S1. Treatment plants with ammonium nitrate had the best growth and health conditions as evidenced by their dark green shoots. Uninoculated plants without both cotyledons were able to survive after 16 weeks and showed differences from the rest of the treatments, as they had the poorest plant growth and symbiosis parameters, such as the lowest number of nodes, leaflets, shoot height, nodule number, fresh and dry nodule weight, and shoot and root dry weights. The appearance of visible difference in growth conditions among the inoculated, uninoculated, with or without nutrient solution and with nitrogen treatment plants had been obvious earlier in plants with both cotyledons halved. Growth variations were noted in just 3-4 weeks after inoculation while variations for other plants (either both cotyledons intact, one removed or both cotyledons removed), were observed at later stages of around 7-8 weeks after inoculation or at harvesting.

Isolation of Rhizobia

A total of 21 potential rhizobia were successfully isolated from the different soil samples and pongamia nodules collected in Queensland, Australia (see supplemental Table S2). Meanwhile, Supplemental Table S3 shows the results of soil analysis for each soil sample. Soil 1 and 3 were sandy while the rest were loamy. Soil 1 (shale/sandstone) had the highest N level of 0.062 %wt, while Soil 2 (Farmhouse Control Site) had the lowest N level of 0.497 %wt. Only Soil 1 had a neutral pH while the
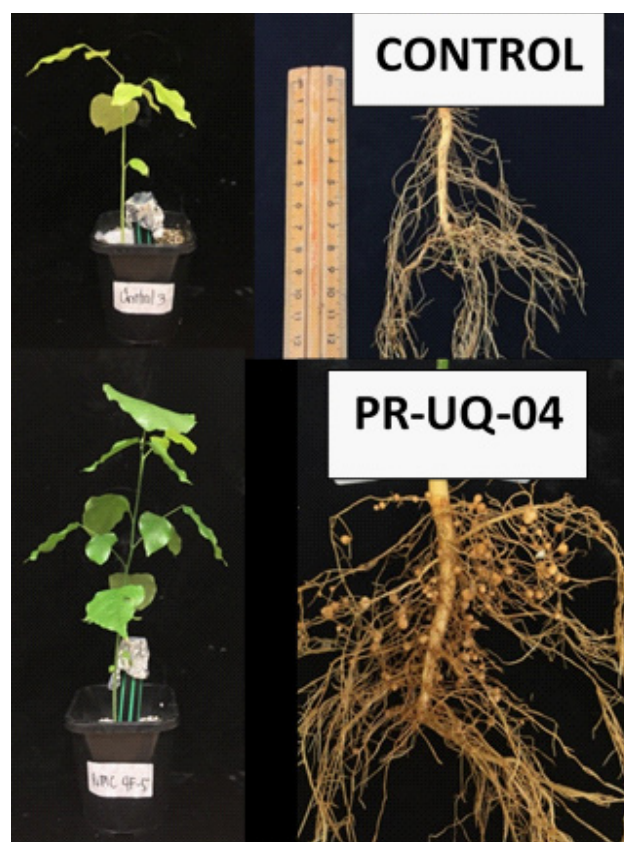
Figure 2. Nodulation of *Pongamia pinnata* plants. Uninoculated plants (A) were observed to grow poorly having yellowish leaves and stunted growth without nodules (B). Inoculated plants (C) using the isolates from baiting techniques grew better with nodulated roots (D).

rest were slightly acidic. The mineral levels in Soil 1 were mostly higher than the rest of the soil samples, while Soil 4 and 6 had average levels.

Nodulation and Symbiotic Effectiveness

Uninoculated plants (control) grew poorly compared with inoculated plants which had higher shoot and root dry weights (see supplemental Table S4). No nodules were found on the uninoculated plants which confirmed their 'no contamination' status. Inoculated plants showed the following: (i) PR-UQ-01 had the highest plant dry weight with 2nd largest nodule number; (ii) PR-UQ-03 had highest shoot length, 2nd highest shoot dry weight among the nodule isolates and 2nd highest N content; (iii) PR-UQ-04 had the largest nodule number among the soil isolates (Figure 2) and (iv) PR-UQ-05 had the highest nodule number among the nodule isolates. These four strains were therefore selected for further studies.

Time Course of Nodulation

All control plants had no nodules from week 4 to 8 as shown in supplemental Table S5. Total plant dry weight increased from 1.23 g at week 4 up to 2.11 g in week 8. The total N level in shoots and roots increased from 5.12% Wt at week 4 to 6.22% Wt at week 7 but decreased to 5.89% Wt on week 8. PR-UQ-03 plants nodulated at week 4 with 38 nodules per plant and continue to produce nodules until week 8 with 80 nodules per plant. The total plant dry weight increased from week 4 at 1.859 g to week 6 at 2.406 g but almost without any changes at weeks 7 and 8. Leghemoglobin concentration at week 4 was 0.132 mg/ml and increased to 0.640 mg/ml in week 8 (see Figure 3). The area of zone of infection of the biggest active nodule was 1.58 mm2 at week 4 and continued to increase its

area up to 3.89 mm2 at week 8. The total N level in shoots and roots was 6.56% Wt at week 4 and no significant changes until week 8. On the other hand, PR-UQ-05 plants produced 56 nodules per plant at week 4 which continued to increase until week 8 producing 78 nodules per plant. The total plant dry weight also doubled from 1.139 g in week 4 up to 2.283 g at week 8. Leghemoglobin concentration increased from week 4 at 0.235 mg/ml to week 8 at 0.663 mg/ml. The area of zone of infection of its biggest nodule started at 1.86 mm^2 at week 4 and increased to 3.08 mm^2 at week 8. The total N level in shoots and roots was 4.30% Wt at week 4 and continued to increase at week 8 measuring 5.12% Wt.

The inoculation of PR-UQ-05 showed significant increase in nodule number, total plant dry weight, leghemoglobin concentration, area of zone of infection and total N level in shoots and roots from week 4 to week 8 while inoculation with PR-UQ-03 increased nodule number, leghemoglobin concentration and area of zone of infection from week to week 8 and no significant increase for total plant dry weight and total N level in shoots and roots over the weeks.

Assessment of Nitrogenase Activity

All nodulated pongamia and soybean (as control) plants inoculated with the best isolates were observed to have nitrogenase activities in their nodules as implicated in the increased acetylene-dependent ethylene production from 0 to 60 minutes sampling time incubated at room temperature as shown in Table 1. In contrast, the non-nodulated, uninoculated controls had zero to almost negligible values. Pongamia plants were observed to have higher nitrogen fixing (i.e., acetylene reduction) activity compared with soybeans when inoculated with PR-UQ-05, but show no significant difference when inoculated with PR-UQ-03.

The N difference method further confirmed that the new in-
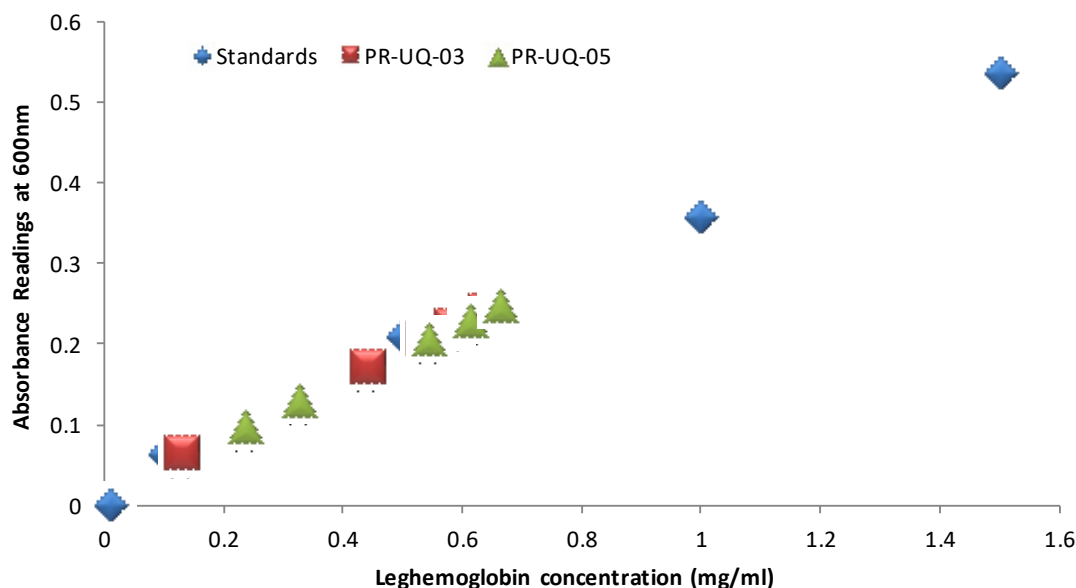
Figure 3. Leghemoglobin content (mg/ml) versus the absorbance readings at 600 nm of the standard haemoglobin, PR-UQ-03 nodules and PR-UQ-05 nodules from week 4 to week 8.

Table 1. Volume of ethylene (ml) produced by pongamia and soybeans inoculated with PR-UQ-03 vs PR-UQ-05 with uninoculated control after 60-minute incubation at room temperature. Values shown were the average of three replicates used per treatment.

Treatments	Volume of ethylene (ml)/plant	Number of nodules/plant	Nodule Fresh Weight (g)/plant
PR-UQ-03 – Soybeans	0.21±0.01 ns	14±2 c	0.01±0 c
PR-UQ-03 – Pongamia	0.30±0 ns	65±2.03 b	0.50±0.01 b
PR-UQ-05 – Soybeans	0.06±0.03 ns	17±7.36 c	0.02±0.01 c
PR-UQ-05 - Pongamia	2.76±1.18 ns	86±3.06 a	0.868±0.03 a

* Values followed by the same letter are not significantly different at 5% significance level. ns= not significantly different (Standard Error).

Table 2. The nitrogen difference method to estimate fixed nitrogen of *Pongamia pinnata* inoculated with the isolates compared with CB564 and uninoculated control for 12-week growth period under glasshouse conditions.

Treatments	Shoot Nitrogen Content (wt %)	Root Nitrogen Content (wt %)	Total Nitrogen Content (wt %)	N Fixed* (wt%)	Total Plant Dry Weight (Grams)	N Fixed/g Dry Wt
Uninoculated Control	1.24±0.05 c	1.18±0.06 c	2.42±0.10 b	0 b	1.991	0
CB564	2.87±0.30 ab	2.2±0.26 ab	5.07±0.53 a	2.65 a	2.157	1.229
PR-UQ-03	3.55±0.16 a	2.59±0.18 a	6.15±0.32 a	3.73 a	2.452	1.521
PR-UQ-05	3.46±0.47 a	2.56±0.42 a	6.02±0.88 a	3.6 a	2.777	1.296

* Values followed by the same letter are not significantly different at 5% significance level. Shown are the mean values and SE. Legend: yellow highlights – high values; blue highlights – low values. *N fixed (wt%/g DW) = N yield by inoculated plant (wt%) – N yield by uninoculated plants (wt%).

oculant isolates PR-UQ-03 and PR-UQ-05 aid in the nitrogen fixation of pongamia as seen in the increase of N level content in the shoots and roots of pongamia seedlings in a 12-week growth period (See Table 2). Inoculated plants had higher N level content in shoots and root compared with uninoculated control and CB564. Consistently, pongamia plants inoculated with PR-UQ-05 had the highest nodule number, nodule fresh weight and nitrogen fixation activity.

Characterisation of the 'Best' Rhizobial Inoculant

The selected rhizobia PR-UQ-01 and PR-UQ-03 were able to utilise mannitol as the only carbon source. PR-UQ-03 grew within 5 days of incubation, thus considered a "fast grower", while PR-UQ-01 appeared within 6-10 days of incubation making it a "slow grower". Suppplemental Table S2 summarises the characteristics of all the 21 isolates on YEMA+CR and with six different types of antibiotics (10 mg/L in media).

The rhizobia PR-UQ-03 and PR-UQ-05 were selected as "best" among the 21 isolates due to their symbiotic performance and being the fast-growers. Both strains were grown into Yeast extract mannitol agar with Congo Red with varying incubation temperatures (22°C, 37°C, and 45°C); different pH (4, 7 and 9) and on different NaCl concentration (0.1%, 0.5% and 1%). Both PR-UQ-03 and PR-UQ-05 can grow on pH 7.0 media only. Also both strains were able to survive at 22°C incubation, but did not grow at 37°C and 45°C. Both PR-UQ-03 and PR-UQ-05 were able to grow on 0.1% and 0.5% NaCl supplementation

of the medium. Both rhizobia isolates did not grow on 1% NaCl supplementation.

The size of the colonies also observed to vary among strains grown on the different media. At pH7.0 and 0.1% NaCl supplemented media incubated at 28°C, colonies of PR-UQ-05 were bigger in size (1-2 mm diameter) compared to colonies on 0.5% NaCl media (at 28°C) and on normal media at 22°C incubation. The number of colonies of PR-UQ-05 at 22°C was also decreased by 25%. The cold incubation affects the growth of PR-UQ-05 but did not totally inhibit the rhizobia growth. PR-UQ-03 colonies were lesser in number but definitely bigger in size (3 mm diameter) except those that grew on 0.5% NaCl (1-2 mm diam) compared with PR-UQ-05. No growth was observed in all control plates (inoculated with sterile distilled water only). Supplemental Table S6 shows the characteristics of the selected rhizobia PR-UQ-03 and PR-UQ-05.

Identification and Molecular Characterisation of the Best Rhizobia

DNA was extracted successfully from the four selected rhizobia strains: PR-UQ-01, PR-UQ-03, PR-UR-04 and PR-UQ-05 and was subjected to PCR using primers 27F and 1492R. Results showed that 16S rDNA of the four isolates was amplified which was further used for DNA sequencing. See supplemental Figure S2.

The predicted molecular band (1,250 bp) from the PCR gel was extracted and purified. Another gel electrophoresis using the purified PCR products was done to confirm one single
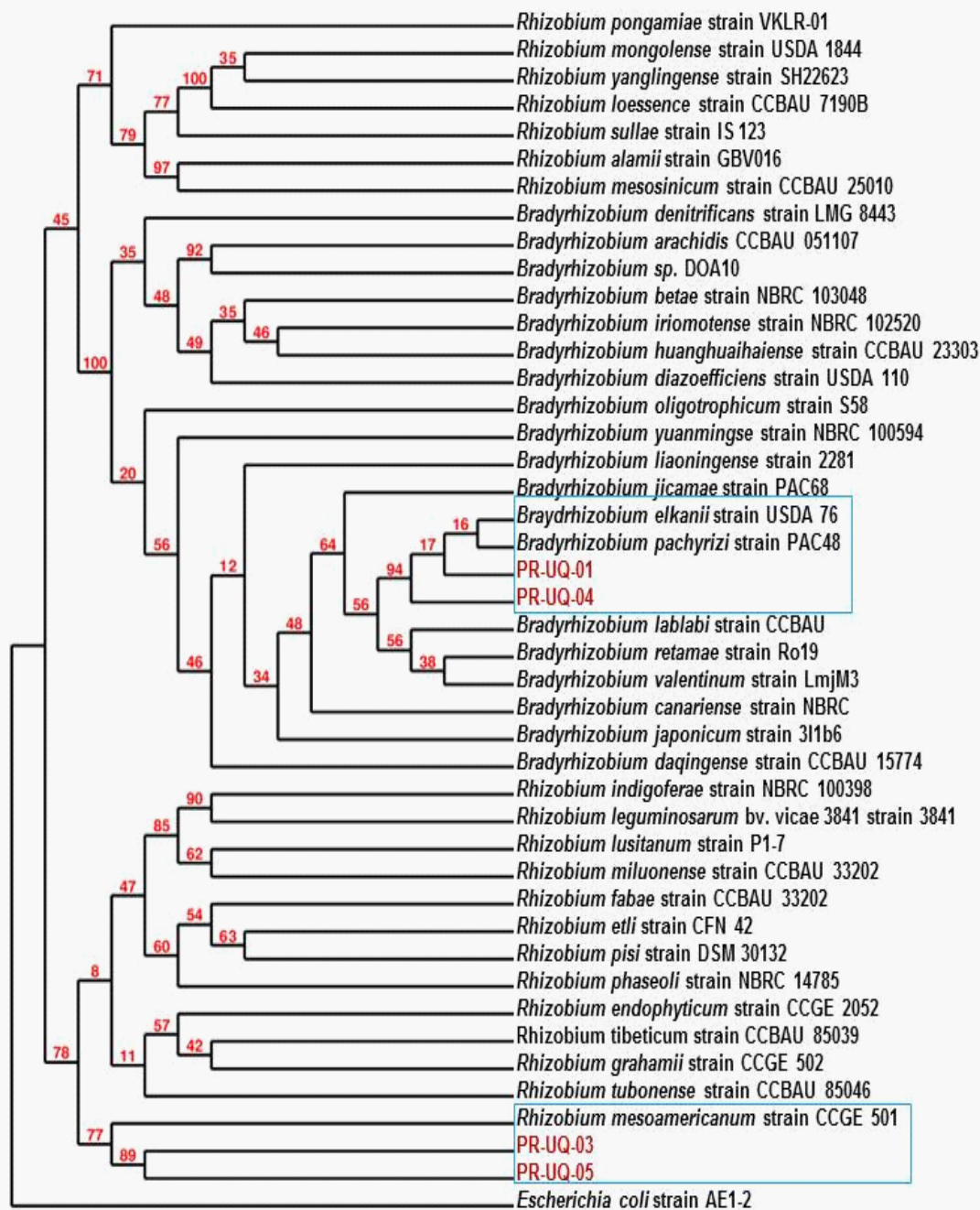
Figure 4. Phylogenetic relationship among the four rhizobial isolates nodulating pongamia (Dereeper et al., 2008).

band amplified. All four rhizobia strains produced only a single molecular band; thus, the PCR products were purified. Restriction enzyme digestion using the three enzymes-AclI, DpnIII and HaeIII, was done to confirm that the four rhizobia strains belong to different genera. Results showed that the pattern of band fragments differed among the four strains implicating that they belong to different genera. Purified PCR products were then sequenced at AGRF (Brisbane, Australia) and the resulting sequences were subjected to BLAST and phylogenetic analysis.

BLAST results: (e values=0) revealed that PR-UQ-01 is *Bradyrhizobium elkanii*, PR-UQ-03 is *Rhizobium mesoamericanum*, PR-UQ-04 is *Bradyrhizobium elkanii* and *B. pachyrizi* and PR-UQ-05

is *Rhizobium mesoamericanum* (all at 99% certainty). Comparison of sequences using BLAST alignment between PR-UQ-01 and 04 as well as PR-UQ-03 and 05 revealed that these two sets of strains were highly similar (>99%), although the restriction nuclease digestion pattern and symbiotic tests results differ among them.

Figure 4 shows the phylogenetic analysis of the four selected isolates compared with other rhizobia strains which revealed that both PR-UQ-01 and PR-UQ-04 were closely related to *Bradyrhizobium elkanii* and *B. pachyrizi* with bootstrap value of 94 while PR-UQ-03 and PR-UQ-05 were both closely related to *Rhizobium mesoamericanum* with a boostrap value of 89.

Discussion

The basis of all the experimental setups in this study was derived from the cotyledon experiment which was performed to determine the role of cotyledon in nodulation and growth of pongamia seedlings and to avoid the carry-over of nutrient from the cotyledon to the plants which might affect the results of the succeeding experiments. Cotyledon experiment had shown that all of the uninoculated treatments did not produce nodules which implied good glasshouse maintenance. The addition of nutrient solution lessens the variation between replicate plants in each treatment. Both inoculated treatments were able to produce several nodules but higher number was observed in the treatments with nutrient solution. Hence, the addition of nutrient solution at the described rate (weak in normal horticulture) improved the nodulation of pongamia. The appearance of visible difference in growth conditions among the inoculated, uninoculated, with or without nutrient solution and with nitrogen treatment plants had been obvious earlier in plants with both cotyledons halved. Growth variations were noted in just 3-4 weeks after inoculation while variations for other plants (either both cotyledons intact, one removed or both cotyledons removed), were observed at later stages of around 7-8 weeks after inoculation or at harvesting. This implied that the cotyledons affect the nodulation and growth of pongamia. By cutting both cotyledons into halves, the nutrient carry-over will be controlled and thus the variation of result can be now attributed to the different treatments and not to nutrients in cotyledon. Therefore, the cotyledon factor will now be eliminated in future studies by simply cutting the cotyledons into halves. Moreover, the pongamia cotyledon can also provide enough nutrients for the plants to grow even up to 16 weeks with or without nutrient solution added.

This study was able to successfully isolate a total of 21 potential rhizobia from the different soil samples in a mining site in Kingaroy, Queensland where pongamia had been grown to rehabilitate the area while the pongamia nodules used in the isolation were collected from Botanical Gardens in Mt. Coot-tha, Queensland where pongamia were introduced several years ago. Out of the six soil samples located in different areas in the mining sites, only two produced nodules in the baited pongamia seedlings which can be attributed to the presence of average level of nutrients (not too high or low), pH and loamy soil characteristics which can support rhizobia growth. The baiting experiment using these soil samples from mining sites further revealed that rhizobia that can nodulate pongamia are present in the soil with poor nutrient conditions and where pongamia are not previously grown.

To determine the best strains, the 21 isolates from the baiting experiment and isolation from pongamia nodule were screened for strain symbiotic effectiveness by inoculating into pongamia under uniform growth conditions. Four strains PR-UQ-01, PR-UQ-03, PR-UQ-04 and PR-UQ-05 showed promising results in the screening test but the fast growers PR-UQ-03 and PR-UQ-05 were selected and were used as inoculants for the other experiments beause PR-UQ-03 had highest shoot length, 2nd highest shoot dry weight among the nodule isolates and 2nd highest N content while PR-UQ-05 had the highest nodule number among the nodule isolates.

In the time course of nodulation experiment, the inoculation of PR-UQ-05 showed significant increase in nodule number, total plant dry weight, leghemoglobin concentration, area of zone of infection and total N level in shoots and roots from week 4 to week 8 while inoculation with PR-UQ-03 increased nodule number, leghemoglobin concentration and area of zone of infection from week 4 to week 8 and no significant increase for total plant dry weight and total N level in shoots and roots over the weeks. This implied that both strains improved nodulation in pongamia. This results is further supported by the acetylene reduction assay and N difference method which revealed that nodulated pongamia inoculated with PR-UQ-03 and PR-UQ-05 were observed to have nitrogenase activities in their nodules. The nitrogen fixation present in the nodules of the inoculated plants contributed to the better growth conditions and nodulation of these plants. Pongamia plants were observed to have higher nitrogen fixing (i.e., acetylene reduction) activity compared with soybeans when inoculated with PR-UQ-05, but show no significant difference when inoculated with PR-UQ-03. This means that PR-UQ-03 and PR-UQ-05 have low symbiotic effectiveness with soybeans as soybeans are known to be strain specific legumes while pongamia on the other hand, is known to be promiscuous legume and can be nodulated with any species of rhizobia. The N difference method further confirmed that the new inoculant isolates PR-UQ-03 and PR-UQ-05 aid in the nitrogen fixation of pongamia as seen in the increase of N level content in the shoots and roots of pongamia seedlings in a 12-week growth period.

PR-UQ-03 and PR-UQ-05 were both isolated from pongamia nodules. PR-UQ-03 had 2-3 mm diam colonies which were white/creamy, high convex and slimy while PR-UQ-05 had 3-4 mm diam colonies with white but prolong incubation observed to produced red center, high convex and very slimy. Both utilised mannitol as the only carbon source, "fast growers", had optimum growth at pH 7.0, temperature of 28°C although it can survive at 22°C, and in yeast extract mannitol agar with 0.1% to 0.5% NaCl. Both strains were resistant to AMP, SPEC and TET. 16S rDNA amplification of both strains yielded 1250 bp using primers 27F and 1492R. BLAST results: (e values=0) revealed that both PR-UQ-03 and PR-UQ-05 are related to *Rhizobium mesoamericanum* (all at 99% certainty). Comparison of sequences using BLAST PR-UQ-03 and 05 revealed that these strains were highly similar (>99%), although the restriction nuclease digestion pattern and symbiotic tests results differ between them. Phylogenetic analysis revealed that both PR-UQ-03 and PR-UQ-05 were both closely related to *Rhizobium mesoamericanum* with a boostrap value of 89.

Conclusions and Future Work

Pongamia plants can fix nitrogen from their efficient symbiosis with either fast or slow-growing rhizobia with nodules starting to form within 2-3 weeks after inoculation and continuing throughout the tested 10-12 weeks growth period. Cutting both cotyledons in halves had little effect on shoot dry weight, nodule number and weight per plant at 8 weeks, but were reduced when both cotyledons were removed and plants were visibly yellow.

By cutting both cotyledons into halves, the nutrient carry-over will be controlled and thus the variation of result can be now attributed to the different treatments and not to nutrients in cotyledon. Therefore, the cotyledon factor will now be eliminated in future studies by simply cutting the cotyledons into halves. Surprisingly when no nutrients were supplied and with both cotyledons intact, plants grew as well as those with nutrients. Cotyledons supply the seedling with nutrients for a considerable period of early growth (8 to 16 weeks after germination), while symbiotic nitrogen fixation supports pongamia on its later growth and eventual reproduction (flowering and seed formation).

The fast growers, *Rhizobium*-related strains PR-UQ-03 and PR-UQ-05, isolated from pongamia nodules are likely the best rhizobia strains among the 21 isolates with confirmed nodulation and symbiotic effectiveness in pongamia and with an established active nitrogenase activity.

Further study is still recommended to test the survival of these rhizobia strains in the field to be used in the improvement of plant performance in soil where native rhizobia nodulating pongamia are sparse or absent.

Abbreviations

PR-UQ: Pongamia Rhizobia-University of Queensland
CB: CSIRO Brisbane
USDA: United States Department of Agriculture
COT: Cotyledon
B&D: Broughton and Dilworth
YEMA: Yeast extract mannitol agar
YMB: Yeast extract mannitol broth
CR: Congo red
ARA: Acetylene reduction assay

Competing Interests

The author declares that they have no competing interests.

Authors Contributions

PNC conducted experiments, evaluated results and wrote the manuscript. AI, PS, PD and PMG contributed to the conception, interpretation and supervision of the research. PMG and PNC revised and edited the manuscript. AI performed the statistical analysis.

Acknowledgements

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Table S1. Effect of cotyledon in nodulation of Pongamia pinnata seedlings under glasshouse conditions for 16 weeks growth period.

Treatments	Shoot length (cm)	# of Nodes	# of Leaflets	Nodule Number	Nodule Fresh Weight (g)	Shoot Dry Weight (g)	Root Dry Weight (g)	Nodule Dry Weight (g)	Total Plant Dry Weight (g)
+ CB1809 and −Nutrients									
2COT	41±2	6±1	10±1	123±78	1.00±0.4	2.70±0.8	1.72±0.3	0.15	4.57±1.1
1COT	25±2	6±1	6±1	94±29	0.41±0.2	1.23±0.1	0.93±0.1	0.06	2.22±0.2
1/2COT	29±2	7±2	11±2	93±20	0.97±0.4	1.57±0.1	1.27±0.2	0.13	2.97±0.3
0COT	16±1	3	3	34±10	0.15	0.39	0.19	0.02	0.60
-CB1809 and −Nutrients									
2COT	37±1	5±1	7±1	0	0	2.75	1.70±0.7	0	4.45±0.7
1COT	26±4	3±1	3±1	0	0	1.13±0.3	0.58±0.2	0	1.72±0.4
1/2COT	35±3	6±1	8±2	0	0	2.13±0.2	1.51±0.1	0	3.64±0.3
0COT	25	5±1	6±2	0	0	0.94	0.50	0	1.44
+ CB1809 and +Nutrients									
2COT	42±3	8	15	142±9	0.87±0.1	2.13±0.1	0.62	0.21	2.96±0.2
1COT	39±2	5±1	10±1	116±4	0.89±0.1	1.56±0.1	0.50	0.18	2.24
1/2COT	37±2	5±1	11±1	133±13	0.73	1.25±0.1	1.25±0.1	0.17±0.5	2.68±0.8
0COT	31	3±1	7±1	83±3	0.45	0.73	0.26	0.01	1.04±0.1
-CB1809 and +Nutrients									
2COT	41±1	6±1	14±1	0	0	2.79	0.94±0.1	0	3.73±0.1
1COT	37±2	6±1	9±2	0	0	1.13±0.3	0.48±0.1	0	1.62±0.2
1/2COT	37±4	5±1	8±2	0	0	1.22±0.3	0.65±0.2	0	1.87±0.5
0COT	35±1	4±1	10±2	0	0	0.71±0.2	0.26±0.1	0	0.97±0.2
-CB1809, +nutrients and +N									
2COT	43	8±1	16±1	0	0	2.66±0.1	1.00	0	3.65±0.1
1COT	35±3	7	10	0	0	2.86±0.5	0.96±0.3	0	3.81±0.8
1/2COT	52±2	8	15	0	0	1.70	1.13±0.1	0	2.82±0.1
0COT	38±1	6±1	12±2	0	0	1.37±0.2	0.53	0	1.90±0.2

Shown are the mean values and standard errors for the two replicates used in each treatment. Legend: 0COT = Both cotyledons were removed; 1COT = One cotyledon removed; 2COT = Both cotyledons intact; 1/2 COT = Both cotyledons cut into halves.

Table S2. Putative rhizobia isolated from different soil samples and pongamia nodules.

Code	Origin	Morphological/Physiological Characteristics
PR-UQ-01	Meandu Mine Soil 6	Slow-grower; Resistant to AMP, SPEC, TET & KAN; 1-1.5 mm diam; whitish-pinkish; flat; circular; slimy
PR-UQ-02	Pongamia Nodule (Mt. Coot-tha)	Fast-grower; Resistant to AMP and TET; 1mm diam; white; circular; low convex; slimy
PR-UQ-03	Pongamia Nodule (Mt. Coot-tha)	Fast-grower; Resistant to AMP, SPEC & TET; 2-4 mm diam; white; high convex; very slimy
PR-UQ-04	Meandu Mine Soil 4	Slow-grower; Resistant to AMP, SPEC & TET; 1-1.5 mm diam; whitish-pinkish; flat; circular; slimy
PR-UQ-05	Pongamia Nodule (Mt. Coot-tha)	Fast-grower; Resistant to AMP, SPEC & TET; 2-4 mm diam; white; high convex; very slimy
PR-UQ-06	Meandu Mine Soil 4	Fast-grower; Resistant to AMP, SPEC & TET; 1 mm diam; white-pinkish; low convex; circular; slimy
PR-UQ-07	Meandu Mine Soil 6	Fast-grower; Resistant to AMP, SPEC & TET; 2.5-3 mm diam; pinkish-reddish; flat to low convex; irregular shape; very slimy
PR-UQ-08	Meandu Mine Soil 6	Slow-grower; Resistant to AMP & TET; 0.5 mm diam; white-pinkish; flat to low convex; circular; slimy
PR-UQ-09	Pongamia Nodule (Mt. Coot-tha)	Fast-grower; Resistant to RIF and AMP; 3-4 mm diam; white; high convex; circular; very slimy
PR-UQ-10	Pongamia Nodule (Mt. Coot-tha)	Fast-grower; Susceptible to all tested antibiotics; 3-4 mm diam; semi-transluscent; flat; circular; slimy
PR-UQ-11	Pongamia Nodule (Mt. Coot-tha)	Fast-grower; Resistant to RIF, AMP, STR, SPEC, TET and KAN; 2 mm diam; white; flat; circular; slimy
PR-UQ-12	Pongamia Nodule (Mt. Coot-tha)	Fast-grower; Resistant to RIF, AMP, STR, SPEC, TET and KAN; 3-4 mm diam; reddish; low convex; circular; slimy
PR-UQ-13	Pongamia Nodule (Mt. Coot-tha)	Fast-grower; Resistant to RIF, AMP, STR, SPEC and TET; 1 mm diam; white; flat to low convex; circular; slimy
PR-UQ-14	Pongamia Nodule (Mt. Coot-tha)	Fast-grower; Resistant to RIF and AMP; 3-4 mm diam; white; high convex; circular; very slimy
PR-UQ-15	Pongamia Nodule (Mt. Coot-tha)	Fast-grower; Resistant to RIF, AMP, STR, SPEC and KAN; 2mm diam; white; low convex; circular; slimy
PR-UQ-16	Pongamia Nodule (Mt. Coot-tha)	Fast-grower; Resistant to RIF, AMP, STR, SPEC, TET and KAN; 3-4 mm diam; opalescent; flat; circular to irregular; slimy
PR-UQ-17	Pongamia Nodule (Mt. Coot-tha)	Fast-grower; Resistant to RIF, AMP, STR and KAN; 3-4 mm diam; reddish; irregula; flat; slimy
PR-UQ-18	Pongamia Nodule (Mt. Coot-tha)	Slow-grower; Resistant to RIF, AMP, STR, SPEC and TET; 1 mm diam; white; circular; flat/low convex; slimy
PR-UQ-19	Pongamia Nodule (Mt. Coot-tha)	Slow-grower; Resistant to RIF, AMP, STR, SPEC and TET; 1 mm diam; white; circular; flat/low convex; slimy
PR-UQ-20	Pongamia Nodule (Mt. Coot-tha)	Slow-grower; Resistant to RIF, AMP, STR, SPEC and TET; 1 mm diam; white; circular; flat/low convex; slimy
PR-UQ-21	Pongamia Nodule (Mt. Coot-tha)	Slow-grower; Resistant to RIF, AMP, STR, SPEC and TET; 1 mm diam; white; circular; flat/low convex; slimy

Table S3. The physical property, pH, N level and mineral content of the soil samples.

Parameters	MM Soil 1	MM Soil 2	MM Soil 3	MM Soil 4	MM Soil 5	MM Soil 6
Source	Kingaroy 2 East Shale/ Sandstone	Farmhouse Control Site D3	Southwest Control Site D5	Southwest Spoil	Southwest Topsoil	Kingaroy 2 East Topsoil
Physical Property	sandy	Loamy	sandy/ loamy	loamy	loamy	loamy
pH	7.01	5.07	5.15	5.24	4.97	5.18
N level Wt%	0.062	0.497	0.238	0.262	0.179	0.101
Phosphorus [Olsen]* (ppm)	3	46	37	9	14	10
Potassium [Am. Acet.] (meq/100g)	0.21	0.43	0.46	0.23	0.39	0.30
Calcium [Am. Acet.] (meq/100g)	0.92	5.92	3.66	1.67	1.35	3.69
Magnesium [Am. Acet.] (meq/100g)	4.59	1.62	1.17	3.50	1.56	1.91
Sulphur [MCP]* (ppm)	376	30	13	74	24	15
Boron [CaCl2]* (ppm)	0.3	0.7	0.4	0.4	0.5	0.5
Copper [DTPA] (ppm)	1.1	0.7	0.3	1.1	0.6	0.2
Iron [DTPA] (ppm)	33	71	54	69	124	66
Manganese [DTPA] (ppm)	8.7	25.9	6.2	13.3	6.9	4.8
Zinc [DTPA] (ppm)	3.7	7.1	1.8	4.3	0.8	0.4
Sodium [Am. Acet.] (meq/100g)	2.0	<0.1	0.1	1.2	0.4	0.2
Aluminum [KCl] (meq/100g)	0.15	0.44	0.39	0.26	1.05	0.76
Al base saturation (%)	1.9	5.2	6.7	3.8	22.2	11.1
Aluminum (ppm)	14	40	35	23	95	68
Sodium (ppm)	449	<18.4	25	285	87	37
Calcium (ppm)	184	1184	732	334	270	738
Magnesium (ppm)	551	194	140	420	187	229
Potassium (ppm)	82	168	179	90	152	117

Legend: Yellow highlights – high values; blue highlights – low values.

Table S4. Strain symbiotic effectiveness of the 21 isolates compared with USDA110, CB1809, CB564 and uninoculated control with *Pongamia pinnata* at 12-week growth period under glasshouse conditions.

TREATMENTS	SHOOT LENGTH (cm)	# OF NODES	# OF LEAVES	# OF NODULES	DRY WEIGHTS (grams)			
					SHOOT	ROOT	NODULE	TOTAL PLANT
CONTROL	28±2	5±1	8±1	0	1.26±0.2	0.73±0.1	0	2.00±0.2
CB1809	42±8	8	15	142±9	2.13±0.2	0.62	0.21	2.75±0.2
USDA110	40±4	5±1	9±1	81±13	2.12±0.4	0.70±0.1	0.16	2.98±0.3
CB564	32±3	6±1	13±2	63±24	1.35±0.3	0.81±0.2	0.10	2.16±0.5
PR-UQ-01	47±1	6	13±1	148±18	2.65±0.2	0.80±0.1	0.28±0.1	3.73±0.4
PR-UQ-02	33±2	7±1	14±2	71±7	1.47±0.1	0.99±0.2	0.16	2.45±0.3
PR-UQ-03	42±6	6±1	13±2	84±19	2.02±0.5	0.94±0.2	0.16	2.96±0.7
PR-UQ-04	43±3	5	13±2	152±19	2.10±0.1	0.67	0.21	2.78±0.1
PR-UQ-05	36±2	5±1	11±2	143±29	1.33±0.1	0.76±0.1	0.15	2.09±0.2
PR-UQ-06	44±2	6±1	15±2	99±17	2.07±0.3	0.70±0.1	0.27	3.05±0.3
PR-UQ-07	41±1	6±1	13	118±12	2.12±0.2	0.78±0.1	0.22	2.98±0.1
PR-UQ-08	42±3	6	14±2	127±15	2.03±0.2	0.91±0.1	0.24	3.18±0.3
PR-UQ-09	34±2	6±1	15±2	61±26	1.56±0.2	1.13±0.1	0.15±0.1	2.69±0.2
PR-UQ-10	38±4	7±1	10±2	48±20	1.54±0.3	0.98±0.1	0.09	2.51±0.4
PR-UQ-11	36±2	8±1	16±2	115±9	1.70±0.3	1.08±0.1	0.15	2.77±0.3
PR-UQ-12	28±2	5±1	11±2	35±15	0.98±0.1	0.76	0.05	1.74±0.2
PR-UQ-13	39±1	7±1	15±1	50±8	2.05±0.2	1.19	0.13	3.24±0.2
PR-UQ-14	39±2	8±1	16±2	66±6	1.97±0.2	1.05	0.14	3.02±0.2
PR-UQ-15	41±3	7	16	81±16	1.79±0.2	0.82±0.1	0.16±0.1	2.61±0.2
PR-UQ-16	29±2	6±1	10±1	25±12	0.81±0.2	0.46±0.2	0.05	1.27±0.4
PR-UQ-17	34±4	6	10±1	25±15	0.83±0.2	0.23	0.04	1.06±0.2
PR-UQ-18	30±3	6±1	13±1	19±12	0.93±0.2	0.35±0.1	0.03	1.28±0.3
PR-UQ-19	31±3	7	11±1	44±9	1.18±0.2	0.71±0.1	0.09	1.89±0.4
PR-UQ-20	29±4	6±1	11±1	54±6	0.85±0.2	0.44±0.1	0.07	1.29±0.3
PR-UQ-21	30±4	6±1	10±1	32±11	0.83±0.1	0.37±0.1	0.04	1.20±0.2

Shown are the mean values (with SE) for all the five replicates used in each treatment. Legend: yellow highlights — high values; blue highlights — low values.

Table S5. Time course of nodulation for pongamia inoculated with the best rhizobia PR-UQ-03 and PR-UQ-05 compared with uninoculated control from week 4 to week 8 growth periods under glasshouse conditions. Values are average for five replicates.

TREATMENTS	NODULE #	NODULE FRESH WT (g)	ROOT DW (g)	SHOOT DW (g)	Total Plant DW (g)	ABSORBANCE (600nm)	LEGHEMOGLOBIN (mg/ml)	ROOT N LEVEL (Wt %)	SHOOT N LEVEL (Wt %)	Total N Level (Wt %)	Area of Zone of Infection of Largest Nodule (mm²)
CONTROL Week 4	0	0	0.488	0.742	1.23	0	0	2.13	2.99	5.12	0
CONTROL Week 5	0	0	0.605	1.159	1.764	0	0	2.40	3.51	5.91	0
CONTROL Week 6	0	0	0.703	1.219	1.922	0	0	2.23	3.71	5.94	0
CONTROL Week 7	0	0	1.013	0.997	2.010	0	0	2.63	3.59	6.22	0
CONTROL Week 8	0	0	0.974	1.138	2.112	0	0	2.59	3.30	5.89	0
PR-UQ-03 Week 4	38	0.289	0.960	0.899	1.859	0.064	0.132	2.80	3.76	6.56	1.58
PR-UQ-03 Week 5	53	0.406	1.130	0.172	2.382	0.172	0.442	3.09	3.76	6.85	2.07
PR-UQ-03 Week 6	63	0.497	1.486	0.92	2.406	0.205	0.537	2.96	3.51	6.47	2.63
PR-UQ-03 Week 7	80	0.605	1.028	1.120	2.148	0.222	0.586	2.57	3.51	6.06	2.81
PR-UQ-03 Week 8	80	0.722	0.966	1.128	2.094	0.241	0.640	2.48	3.31	5.79	3.89
PR-UQ-05 Week 4	56	0.515	0.471	0.668	1.139	0.100	0.235	2.11	2.19	4.30	1.86
PR-UQ-05 Week 5	59	0.546	0.66	0.956	1.622	0.132	0.327	2.43	3.15	5.58	2.14
PR-UQ-05 Week 6	73	0.579	0.862	0.901	1.764	0.207	0.543	2.78	3.29	6.07	3.11
PR-UQ-05 Week 7	72	0.744	0.715	0.558	1.273	0.231	0.612	2.16	2.82	4.99	2.55
PR-UQ-05 Week 8	78	0.773	1.004	1.278	2.283	0.249	0.663	2.16	2.96	5.12	3.08

Table S6. Characteristics of PR-UQ-03 and PR-UQ-05 based on various parameters.

Parameters	PR-UQ-03	PR-UQ-05
Origin	Pongamia nodule, Botanical Gardens, Mt. Coot-tha, Brisbane, Australia	Pongamia nodule, Botanical Gardens, Mt. Coot-tha, Brisbane, Australia
Colony morphology	2-3 mm diam; white; high convex; slimy	3-4 mm diam; white but later observed to have red center in longer incubation; high convex; excessively slimy
Nodulation	Positive	Positive
pH	7	7
NaCl tolerance/salinity	0.1 – 0.5 %	0.1 – 0.5 %
Temperature	22-28°C	22-28°C
Carbon utilisation	Mannitol	Mannitol
Antibiotic resistance	Resistant to AMP, SPEC & TET	Resistant to AMP, SPEC & TET
Turbidity in YM broth	$\geq$72 hours	<48 hours
Incubation	3 days	<3 days

Figure S1. Pongamia seedlings at different treatments. All pongamia plants with both cotyledons removed (0COT) had consistent lowest shoot length, shoot dry weight, nodule number and nodule dry weight (yellow). Uninoculated plants without nutrients but both cotyledons intact (2COT) grew as well as those with nutrients implicating that cotyledons supply the seedling with nutrients for a considerable period of early seedling growth.
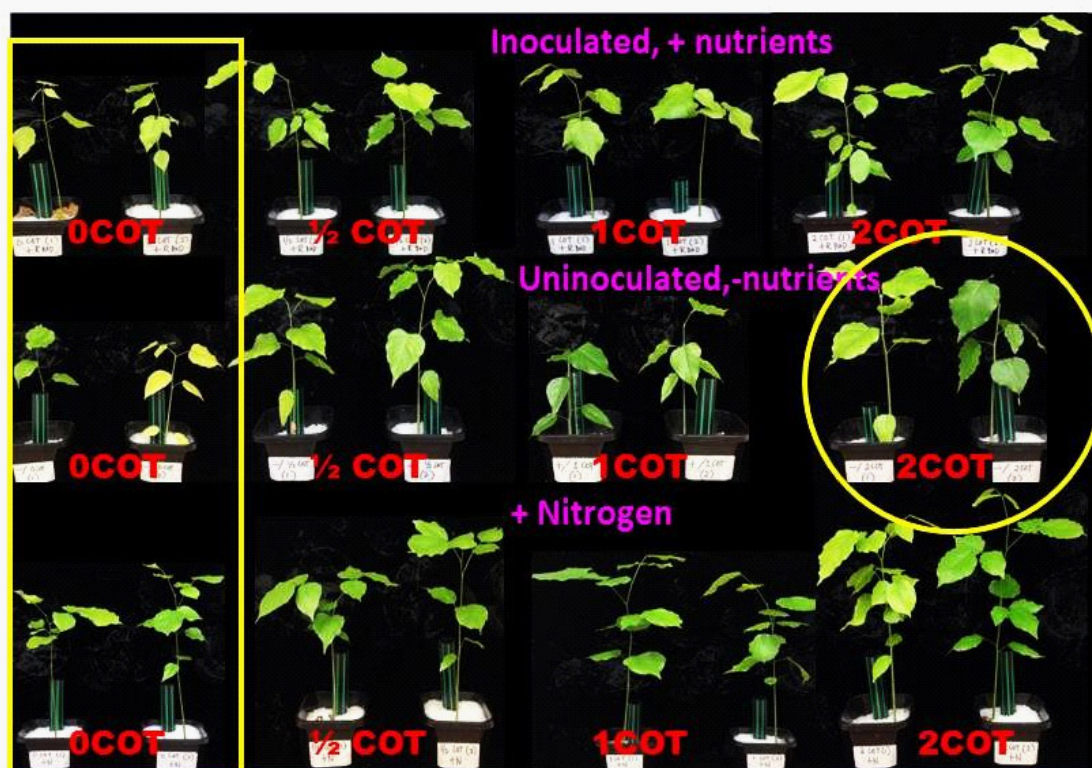
Figure S2. Amplified 16S rDNA (1,250 bp) of the four best rhizobia isolates: Lane 1 = Negative Control; Lanes 2 and 3 = PR-UQ-01; Lanes 4 and 5 = PR-UQ-03; Lanes 6 and 7 = PR-UQ-04; and, Lanes 8 and 9 = PR-UQ-05. (blue arrows indicate the predicted PCR product).