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Comparative Assessment of Biological Nitrogen Fixation in *Pongamia pinnata*, a Biofuel Legume Tree

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Pongamia pinnata **has been established as a biofuel legume tree. Different methods such as acetylene reduction assay, ureide analysis, isotopic techniques, nitrogen difference method and isotope techniques (^{15}N natural abundance and ^{15}N enrichment technique) were employed to analyze, assess and estimate symbiotic nitrogen fixation in 16-wk-old pongamia seedlings. In the acetylene reduction assay, the uninoculated control had negligible ethylene produced while inoculated plants were shown to have increasing ethylene production from 0-min to 60-min incubation with a range of 0.03 to 2.76 mL. Pongamia was found to produce 1.19 x 10-6 mole per plant with a** ***Rhizobum*** **(PR-UQ-05) inoculation. Ureide analysis was also done, not only to estimate fixed nitrogen, but also to determine the presence of allantoin in the xylem of pongamia. The result showed that allantoin was present in pongamia at low levels of 143–150 nmole, which means pongamia utilizes ureides in the form of allantoin to transport its fixed nitrogen to other plant parts to support growth and reproduction. The nitrogen difference method and isotope techniques quantified the fixed nitrogen of pongamia inoculated with PR-UQ-05 which was estimated to be 100 mg/plant (based on the difference of total N yield between the nodulated and non-nodulated pongamia seedlings) and from 20.4 mg/plant (natural abundance) to 47.4 mg/plant (enriched). The different methods used in this study showed different results based on the amount of fixed nitrogen calculated for each method. However, all of the methods employed in this study demonstrated that Pongamia inoculated with PR-UQ-05 fixed more nitrogen than the uninoculated control. The symbiotic nitrogen fixation of Pongamia demonstrated in this study is very relevant to the biofuel industries.**

Key Words: *Pongamia pinnata*, biofuel, nitrogen fixation, ureide analysis, ^{15}N natural abundance, isotope techniques, nitrogen difference method
Abbreviation: ARA – acetylene reduction assay

INTRODUCTION

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 (Gresshoff et al. 2015). It is an important candidate for the production of biofuels (such as biodiesel and aviation fuel) from its oil-rich seeds (Gresshoff et al. 2017). Pongamia is resilient against abiotic stresses such as drought, salinity and acidity. An important trait that establishes Pongamia as a superior biofuel feedstock is its ability to grow in marginal, nitrogen-limited soils. This growth ability is attributed to the nitrogen-fixation activity of root nodules developed by symbiosis with bacterial microsymbionts broadly known as rhizobia (Nemenzo-Calica et al. 2016).

Nitrogen is one of the most important nutrients required by plants as a major component of all proteins and nucleic acids, which control and enable their growth and reproduction. While much research has been conducted in pongamia, there have been few completed studies on the mechanisms and regulation of nitrogen fixation. The symbiotic assimilation of nitrogen in root nodules of legumes is considered to be one of the most important domestication traits that needs to be investigated. The general objective of this study was to assess the nitrogen fixation of pongamia using various methods. Specifically, this study aimed to conduct acetylene reduction assay (ARA), N-difference method, ureide analysis and isotope techniques, and then compare the results or data.

The methods used to quantify and assess nitrogen

fixation are regarded as having some drawbacks due to the disadvantages or limitations that each method is reported to have (Unkovich et al. 2008). No specific method can give an exact measurement of fixed nitrogen, therefore exploring various methods such as acetylene reduction assay (ARA), N-difference, ureide analysis and isotope techniques, and then comparing the results or data, is the best way to assess symbiotic nitrogen fixation in pongamia.

The following information about acetylene reduction assay was derived from Unkovich et al. (2008). The enzyme nitrogenase, which reduces N_2 to NH_3, is also capable of reducing acetylene (C_2H_2) to ethylene (C_2H_4). Therefore, C_2H_2 can be used as an alternative substrate to N_2 (Hardy et al. 1968; 1973). In the case of legumes, the assayed nodules are representative of all nodules on the root system. Measurements of nitrogenase activity obtained under assay conditions are related to the rates of nitrogen fixation in-situ. The C_2H_2 reduction assay is a very sensitive diagnostic tool for detecting nitrogenase activity. It is simple, rapid and relatively inexpensive, and many measurements can be undertaken daily (Hardy et al. 1973).

ARA does not work for field-grown plants. Here are some recognized limitations of ARA (based on Unkovich et al. 2008): (i) The measurements reflect nitrogenase activity for only the duration of the assay. There are marked diurnal and seasonal changes in enzyme activity, and many individual measurements are required to provide an estimate of nitrogen fixation for an entire growing season. (ii) The relationships between C_2H_2 reduction and nitrogen fixation may change during plant growth, and can differ across legume–rhizobial strain combinations (Witty and Minchin 1988). (iii) It is virtually impossible to quantitatively recover whole root systems from field-grown legumes (and non-legumes), thereby sometimes resulting in gross underestimates of total nitrogenase activity (Vikman and Vessey 1992). (iv) C_2H_2 can induce a decline in nitrogenase activity in some legume species, while plant handling, nodule detachment and excision of nodulated roots have all been demonstrated to interfere with nodule gas exchange, leading to lower nitrogenase activity (Minchin et al. 1983, 1986). (v) Although measurements on undisturbed plants are likely to be more reliable than the traditional closed-incubation system, the flow-through C_2H_2 reduction chambers that have been developed for use in the field are cumbersome. This and other issues have prevented their widespread use (Giller 2001). (vi) C_2H_2 is explosive and poses a possible hazard to the experimenter. (vii) While the C_2H_2 reduction assay may be quantitative for pot studies under some conditions, and can provide a useful tool for detecting nitrogen fixation activity in both leguminous and non-leguminous plants, the calculated rates of nitrogen fixation cannot be extrapolated beyond the incubation vessel. As a consequence, the method is unsuitable for measuring nitrogen fixation at field scales (Unkovich et al. 2008).

The following information about the N-difference method were derived from Unkovich et al. (2008). N-difference compares total N of the N_2-fixing species with that of a neighboring non-N_2-fixing species, with the difference between the two measures assumed to be due to nitrogen fixation. N accumulated by the non-nitrogen fixing control is derived only from soil N, and its N content represents the amount of soil mineral N available for plant growth. The nitrogen-fixing plants use the same amount of soil mineral N as the non-nitrogen fixing control. It is a simple, low-cost method that can be applied when facilities for only dry matter determinations and total N analyses are available. This method requires a non-nitrogen fixing control to be included in the experimental design. Differences between nitrogen-fixing and non-nitrogen-fixing plants in root morphology and rooting depth can result in different capacities to use soil N (Chalk 1998). There may be errors in accurately quantifying total N accumulated by the nitrogen-fixing plants and control plants. The technique is likely to be most reliable under conditions of low plant available N and where there are large differences in N yield between the nitrogen-fixing plants and the non-nitrogen fixing control (Unkovich et al. 2008).

Unkovich et al. (2008) further added that in many tropical and subtropical legumes, the N-solute composition in xylem sap and stem segments changes from one dominated by the ureides allantoin and allantoic acid in nitrogen-fixing plants, to one dominated by nitrate and amino acids in plants utilizing soil N. The substantial differences in the principal forms of N transported in the xylem between symbiotic and non-symbiotic plants allows incoming fixed N and soil N to be distinguished. The N-solute composition of xylem sap and stem segments reflects current N assimilation by the legume. The abundance of ureides relative to other N solutes can be used as an indirect measure of the percentage of legume N derived from the atmosphere, i.e., %Ndfa. The procedures used to sample xylem sap and stem segments of field-grown legumes are not technically demanding. Ureides, nitrate and amino acids in xylem sap and stem segments can be easily and rapidly analyzed using simple

colorimetric assays in a test tube. There is no need for expensive or sophisticated equipment. No special experimental design is required, so the method can be used for on-farm measurement of nitrogen fixation. Many samples can be collected and analyzed in a single day. Plants can be sampled non-destructively from the top of the stem, or from lateral branches, with the base intact to continue to grow (Herridge 1988). The nitrogen fixation method provides an estimate of %Ndfa, which can be used to assess the N_2 fixation response of the legume to experimental treatments, without the need to measure legume total N yield. %Ndfa, when combined with a measure of legume total N, can be used to calculate the amounts of N fixed during a period of growth (e.g., Herridge et al. 1990). Its use is restricted to ureide-exporting legume species (e.g., *Glycine, Vigna, Phaseolus*, and *Macroptilium*). The nitrogen fixation method provides an indirect measure of %Ndfa, necessitating calibration against another method, e.g., 15N isotope dilution (Herridge and Peoples 1990; Alves et al. 2000).

According to Unkovich et al. (2008), different calibrations may be needed during the vegetative and reproductive stages of development. The nitrogen fixation technique provides only a 'point-in-time' estimate of the legume's symbiotic dependence at, or shortly before, the time of sap sampling, so repeated sampling of xylem composition and plant N may be required during a growing season. Volumes of xylem sap and solute composition vary diurnally, and can be affected by delays in sap collection (Herridge 1988). It is a versatile and useful technique that can be applied in glasshouse and field experiments, or used in farmers' fields, to assess nitrogen fixation by ureide-exporting tropical and subtropical legumes. It can provide estimates of nitrogen fixation (%Ndfa and total fixed N_2) for field-grown legumes similar to those based on more sophisticated techniques.

The following information about isotope techniques was sourced from Unkovich et al. (2008). There are two main stable isotopes of N—^{14}N and ^{15}N—, with ^{14}N being naturally more abundant than ^{15}N. In absolute terms, the isotopic abundance of ^{15}N is usually expressed as a percentage of the total N (atom% ^{15}N). The isotope ^{15}N occurs in atmospheric N_2 at a constant abundance of 0.3663 atom% (Mariotti 1983). If the ^{15}N concentration in atmospheric N_2 differs significantly from that of plant-available soil N, and these values are known, it is possible to calculate nitrogen fixation on the basis of ^{15}N analyses of the putative nitrogen-fixing plant and a non-nitrogen fixing. Either there is no discrimination or identical discrimination between ^{14}N and ^{15}N during the uptake and metabolism of plant-available soil N and N_2, or the discrimination can be accounted for. Any variability in the ^{15}N composition of the air and soil is small compared to the difference between them.

Unkovich et al. (2008) further mentioned that the ^{15}N natural abundance allows nitrogen fixation to be monitored in almost any location where both nitrogen-fixing and non-nitrogen fixing plants are present, as nothing needs to be added before measurements can be undertaken. The potential exists to measure nitrogen fixation in farmers' fields and in experiments not originally designed with nitrogen fixation in mind. The ^{15}N composition of plant-available soil N can change with soil depth and time during the growing season, particularly where ^{15}N-enriched materials have been applied to the soil (Chalk 1985). ^{15}N-enriched materials (when used) have a high cost. The ^{15}N abundance of plant -available soil N can be either too low and/or too variable for the methodology to be applied. Isotopic discrimination during nitrogen fixation is generally assumed to be zero in ^{15}N enrichment studies, but needs to be considered when using ^{15}N natural abundance. Nitrogen isotope techniques are arguably the benchmarks for quantifying plant-associated nitrogen fixation against which other techniques are compared and calibrated.

MATERIALS AND METHODS

Time and Place of Study

This study was conducted from January to August 2016. The potted experiments were done at the Central Glasshouse located at 89E Glasshouse Road, St. Lucia, University of Queensland, Australia. The laboratory experiments were done at the Centre for Integrative Legume Research Laboratory in the same university. Analysis for isotope experiments was done at the Stable Isotope Facility, University of California, USA.

Treatments and Experimental Design

Pongamia pinnata seeds (same mother tree as source of seeds) were surface-sterilized and were sown in sterile vermiculite medium. Plants were grown for 8 wk. Plants with uniform growth were selected for nitrogen fixation experiments. Control plants were uninoculated and they remained non-nodulated throughout the conduct of the study while the experimental plants (8-wk-old) were inoculated with *Bradyrhizobia* species (PR-UQ-03 and PR-

UQ-05). The plants were further grown for another 8 wk after inoculation and were watered with sterile distilled water (150 mL per pot) thrice a week. The plants were watered with N-free nutrient solution (Broughton and Dilworth) once a week at 150 mL per plant. Plants were protected from cross contamination by covering the surface with white sterile beads with a sterile pipe inserted for watering purposes.

This study utilized the Randomized Complete Block Design (RCBD) and all treatments were replicated four times. All plants were assessed 8 wk after inoculation (16-wk-old pongamia seedlings).

Acetylene Reduction Assay (ARA)

Uninoculated control and pongamia seedlings inoculated with the selected isolate from the University of Queensland (CILR laboratory): PR-UQ-05 (*Rhizobium* species previously isolated from soil samples in Queensland, Australia; Nemenzo-Calica et al. 2016) were subjected to acetylene reduction assay (ARA) to assess the nitrogen fixation activity in the nodulated roots and compared with 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). Vermiculite is a yellow or brown mineral found as an alteration product of mica and other minerals, and used for insulation or as a moisture-retentive medium for growing plants. The 10% of total gas volume was removed and replaced with 10% acetylene. At 0 and 60 min, the gas samples were obtained by a syringe and were directly injected into a Shimadzu GC-17A gas chromatograph (FID detector, 2 m self-packed PoraPak N 60 column) set with a column oven temperature at 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.

Nitrogen Difference Method

Meanwhile, the Nitrogen Difference method was also employed to estimate the amount of fixed nitrogen in all dried plants inoculated with rhizobia and the reference treatments. Pongamia seeds were surface-sterilized and sown into sterile pots with sterile vermiculite. 150 mL of rhizobia suspension was inoculated into each pot except for the uninoculated control. All plants were maintained under glasshouse conditions for 8 wk and were harvested for data gathering. All plants were dried in the oven for 3 d and were analyzed for total nitrogen level. Inoculated plants were then compared with uninoculated plants (non-nodulating). Nitrogen difference was used to estimate the amount of fixed N using the formula:

N fixed = N yield by inoculated plant – N yield by uninoculated plants

Ureide Analysis

The xylem sap was extracted from Pongamia seedlings (inoculated with PR-UQ-05 and uninoculated control) by boiling the shoots. Meanwhile, a standard curve describing the optical density (O.D.) response to increasing concentrations of allantoin was constructed within the range 0–0.10 mM allantoin. A 2.5 mL of each of the five concentrations were added to duplicate test tubes. 0.5 mL of 0.5 N sodium hydroxide (NaOH) was then added. The NaOH was dispensed in such a way that additional mixing was unnecessary. The rack of tubes was placed in a boiling water bath for 10 min. The level of the boiling water was kept above the contents of the tubes. The rack of tubes was removed from the water bath and was placed on a bench.

A 1.0 mL 0.65 N HCl/phenylhydrazine mix was added. The rack of tubes was placed in the boiling water bath for exactly 2 min. The boiling water was higher than the contents of the tubes. The rack was removed from the boiling water bath and was immediately plunged into an ice bath (a plastic tub containing ice may be used) and left for 15 min.

The rack of tubes was removed from the ice bath and 2.5 mL HCl/KFeCN was added using Dispenser 3. The HCl/KFeCN should be cold. The tubes were left on the rack placed on top of the bench for 10 min and the O.D. at 600 nm was read on a spectrophotometer. It is important to read the optical densities as quickly as possible because the color will fade after a further 15 min.

Isotope Techniques

Eight sterile Pongamia seedlings were inoculated with PR-UQ-05 and divided into two setups: for ^{15}N natural abundance and for ^{15}N enrichment method. Eight uninoculated control were also included to compare results between nitrogen-fixing and non-nitrogen fixing (uninoculated, non-nodulated plants). All plants were maintained under glasshouse conditions for 8 wk while plants for ^{15}N enrichment were enriched with ^{15}N-labeled ammonium nitrate (98% atom); 10 mM concentration was watered once to the plants at 250 mL per plant or pot. The

16 plants were uprooted and data were gathered after 8 wk which included shoot length, nodule number, total nodule fresh weight, number of nodes, number of leaflets, biomass and root dry weights. Dried plants were ground and weighed according to the protocol of sample preparation described in the UC Davis Stable Isotope Facility. Samples were shipped to the USA for analysis and results were sent back via email.

Data Analysis

For acetylene reduction assay, the gas chromatograph data (peak area) were converted into nmole per plant. For the N-difference method, the dry weights were measured and the amount of fixed nitrogen was estimated based on the formula:

N fixed = N yield by inoculated plant – N yield by uninoculated plants

For ureide analysis, the volume of allantoin (ureide) was derived from the standard curve of allantoin standards. For the isotope techniques, the amount of nitrogen and other relevant data of dried pongamia samples were provided by the Stable Isotope Facility. To determine the significance of the data, Tukey's Honestly Significant Difference (HSD) Tests were utilized.

RESULTS

Acetylene Reduction Assay (ARA)

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 min sampling time during incubation at room temperature. In contrast, the non-nodulated, uninoculated controls had zero or negligible ethylene production values. Pongamia plants were observed to have higher nitrogen-fixing (i.e., acetylene reduction) activity compared with soybeans when inoculated with PR-UQ-05 which produced 1.16 x 10-4 mole of ethylene per plant.

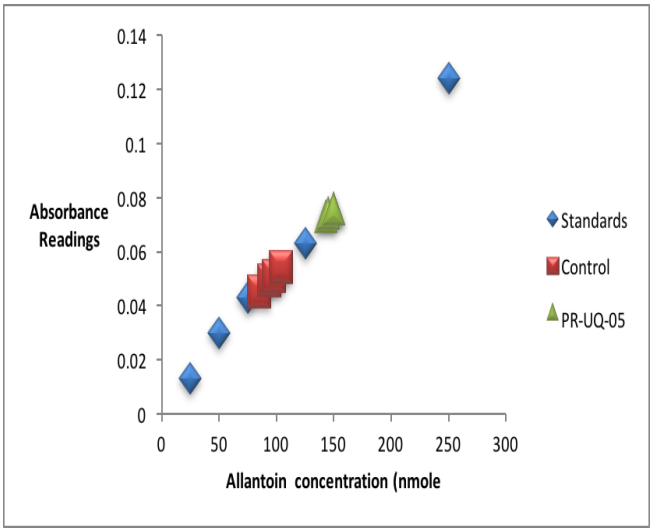

Fig. 1. Standard curve of allantoin standards based on their absorbance readings.

There is theoretical and empirical support for a conversion ratio of C_2H_2 reduced to fixed N of 3–4 to 1, and 1 mole of acetylene yields 1 mole of ethylene (Hardy et al. 1973). Using this ratio, the volume of ethylene produced by PR-UQ-05 plants is equivalent to 1.16 x 10^{-4} mole of ethylene/plant. Therefore, the fixed nitrogen is estimated to be 1.19 x 10^{-6} mole/plant.

Nitrogen Difference Method

The N difference method further confirmed that the inoculants PR-UQ-03 and PR-UQ-05 aid in the nitrogen fixation of Pongamia, as reflected in the increase of N level content in the shoots and roots of Pongamia seedlings in a 16-wk growth period. Inoculated plants had higher N level content in their shoots and root compared with the uninoculated control. Consistently, Pongamia plants inoculated with PR-UQ-05 had higher nodule number, nodule fresh weight and nitrogen fixation activity.

*N fixed (g) = N yield by inoculated plant (g) – N yield by uninoculated plants (g)

The total N level content of PR-UQ-05 plants in g/plant is equivalent to 0.17 while its fixed nitrogen is 0.10 g/plant (100,000 μg/plant).

Ureide Analysis

Pongamia seedlings, both inoculated and uninoculated, were found to have ureides in the form of allantoin in their xylem. Table 1 shows the colorimetric results of ureide analysis using allantoin standards (25 nmole, 50 nmole, 75 nmole, 125 nmole and 250 nmole) compared with the uninoculated control and inoculated xylem sap analysis. The color of the standards was different shades of pink-red, while the water blank remained yellow after the analysis. Figure 1 shows the absorbance readings of the allantoin standards, uninoculated control and that

Table 1. Ureide analysis results for uninoculated control versus inoculated control and absorbance readings of allantoin standards.

Treatments	Nodule No.	Total Nodule Fresh Weight (g)	Absorbance Readings	Color	Allantoin Concentration (nmole)
CONTROL-1	0	0	0.054	Light pink	104
CONTROL-2	0	0	0.051	Light pink	98
CONTROL-3	0	0	0.049	Light pink	94
CONTROL-4	0	0	0.045	Light pink	85
PR-UQ-05-1	65	1.13	0.076	Pink	150
PR-UQ-05-2	60	1.01	0.076	Pink	150
PR-UQ-05-3	61	1.20	0.074	Pink	145
PR-UQ-05-4	61	1.02	0.073	Pink	143
Water blank	-	-	0.013	Yellow	25
Standard-1	-	-	0.030	Very light pink	50
Standard-2	-	-	0.043	Light pink	75
Standard-3	-	-	0.063	Pink	125
Standard-4	-	-	0.124	Dark pink	250

inoculated with PR-UQ-05 plotted against the allantoin concentrations. The absorbance readings and the computed allantoin concentrations of the inoculated versus the uninoculated samples were also shown in Table 1. The allantoin concentration of PR-UQ-05 plants is 147 nmole/plant.

Isotope Techniques

Four Pongamia plants inoculated with PR-UQ-05 were analyzed for ^{15}N natural abundance and the results were then compared with non-nodulated Pongamia plants. The nodulated Pongamia plants represent the nitrogen-fixing plant. The non-nodulated Pongamia plants represent the non-N_2-fixing reference plant which was totally dependent on seed N for growth. With the increasing N_2 fixation, the abundance of ^{15}N in the nodulated plant declined, as nitrogen assimilated from the roots/seeds was diluted by atmospheric N_2 of lower ^{15}N abundance fixed in the root nodules. Therefore, the atom % ^{15}N of the test plants for natural abundance were the expected values (non-nodulated > nodulated Pongamia). The non-nodulated Pongamia has a mean ^{15}N abundance of 0.37014 atoms % ^{15}N and a delta value of 10.5, while nodulated Pongamia has 0.36882 atoms % ^{15}N with a delta value of 6.9.

The percentage of Pongamia N fixed from atmospheric N_2 (P) was obtained using the following equation (Peoples et al. 1989):

$$P = 100 \times \frac{\delta^{15}N \text{ (control N)} - \delta^{15}N \text{ (inoculated N)}}{\delta^{15}N \text{ (control N)} - B}$$

where $\delta^{15}N$ was obtained from the formula:

$$\delta^{15}N = \frac{1000(\text{atom\% } ^{5}N \text{ sample}) - (\text{atom\% } ^{15}N \text{ standard})}{\text{atom \% } ^{15}N \text{ standard}}$$

where atom % ^{15}N standard = 0.3663.

The "B value" should ideally be prepared for each new legume species studied. It is the $\delta^{15}N$ of the total N of effectively nodulated legume grown on media free of combined N. But without the B value of Pongamia available, it was suggested to use the B values used to report the results of biological nitrogen fixation determination based on the ^{15}N natural abundance method for Acacia species, also a legume tree just like Pongamia (Hosseini-Bai et al. 2012). Potential B values reported for acacia species could be from -0.3‰ to 1‰.

With B value = -0.3‰,

$$\text{33 Pongamia N fixed from atmospheric } N_2 \text{ (P)} = 100 \times \frac{(10.48\text{-}6.88) = \%}{10.48\text{-}(\text{-}0.3)‰}$$

With B value = 0.0‰,

$$\text{Pongamia N fixed from atmospheric } N_2 \text{ (P)} = 100 \times \frac{(10.48\text{-}6.88)}{10.48\text{-}0.0‰} = 34\%$$

Table 2. Natural abundance of ^{15}N in shoots of nodulated and non-nodulated pongamia and the calculation of the proportion of plant N derived from N_2 fixation.

Treatments	Atom % ^{15}N	$\delta^{15}N$	N Amount (µg)	Fixed Nitrogen (P)	Fixed Nitrogen (µg per 10 mg sample)
Nodulated Pongamia	0.36882	6.88	**177.2**	38%	68
Non-nodulated Pongamia	0.37014	10.48			

Fixed nitrogen (P) = Percentage of Pongamia N fixed from atmospheric N_2.
B value = 1‰

Table 3. Content of ^{15}N in shoots of nodulated and non-nodulated Pongamia from ^{15}N enriched pots, and the calculation of the proportion of plant N derived from N_2 fixation (P).

Treatments	Atom % ^{15}N	Atom % ^{15}N Excess	N Amount (µg)	Fixed Nitrogen (P)	Fixed Nitrogen (µg per 10 mg sample)
Nodulated Pongamia	6.45671	6.09041	**294.59**	53.6%	158
Non-nodulated Pongamia	13.49219	13.12589			

Fixed nitrogen (P) = percentage of Pongamia N fixed from atmospheric N_2

With B value = 1‰,

$$\text{Pongamia N fixed from atmospheric } N_2 \text{ (P)} = 100 \times \frac{(10.48-6.88)}{10.48-1‰} = 38\%$$

For the enrichment technique, the percentage of fixed nitrogen of Pongamia (P) is estimated from the following equations (Peoples et al. 1989):

$$P = 1 - (\text{atoms \% } ^{15}N \text{ nodulated Pongamia} - \text{atoms \% } ^{15}N\ N_2) / (\text{atoms \% } ^{15}N \text{ non-nodulated Pongamia} - \text{atoms \% } ^{15}N\ N_2) \times 100$$

$$P = 1 - \frac{(6.45671 - 0.3663)}{(13.49219 - 0.3663)} \times 100$$

$$P = 1 - \frac{(6.0904)}{(13.1259)} \times 100$$

$$P = 53.6\%$$

For natural abundance (Table 2), the estimated fixed N of Pongamia inoculated with PR-UQ-05 was 68 µg/10 mg of plant sample (dried leaves) while, for the enrichment method (Table 3), the estimated fixed N was 158 µg/10 mg of dried plant sample (leaves).

DISCUSSION

The symbiotic nitrogen fixation in Pongamia was assessed and quantified. The summary of the results for each method was shown in Table 4. Nitrogenase activity in nodulated roots confirmed active nitrogen fixation of Pongamia which exhibited an increase in the volume of ethylene produced in acetylene reduction assay, while soybeans (control plants) produced low ethylene levels and the uninoculated control had negligible levels. Among the treatments, Pongamia inoculated with PR-UQ -05, produced 2.76 mL of ethylene per plant from 86 nodules with 0.868 g total fresh nodule weight. In the study by Samuel et al. (2013), ARA was done using *Bradyrhizobium* CB1809 strain to demonstrate nitrogen fixation by Pongamia but the results showed that Pongamia roots produced around 0.80–1.20 µM of ethylene per plant which is relatively lower than the ethylene produced by Pongamia when inoculated with PR-UQ-05. Meanwhile, ARA of soybeans inoculated with CB1809 produced higher amount of ethylene compared with the soybean results of this study by Qu et al. (2014). Therefore, PR-UQ-05 is suitable for Pongamia while CB1809 is suitable for soybeans based on ethylene production. The ethylene production implied that nitrogenase was actively present in the nodules and with the increasing levels of ethylene. It was therefore proven that nodulated Pongamia roots were fixing nitrogen. However, according to Samuel et al. (2013), quantification of nitrogen gain based on acetylene reduction values is

Table 4. Estimates of biological nitrogen fixation of 16-wk-old pongamia seedlings inoculated with rhizobia (PR-UQ-05) under glasshouse conditions obtained from the different methods employed.

Methods	Nitrogen Fixation Rates (nmol N per g of Plant Material)
Acetylene reduction assay (ARA)	1,190 (total nitrogen)
Ureide analysis	147 (allantoin)
Method	mg N per g of plant material
N-difference method	100.0
^{15}N natural abundance	20.4
^{15}N enrichment technique	47.4

difficult as physiological and diurnal factors vary the relative efficiency. Hence, it is significant that other methods such as in the succeeding paragraphs were used in the assessment of Pongamia nitrogen fixation which require more in depth quantification of nitrogen fixation activity and its contribution to the growth of Pongamia. This is particularly important for Pongamia as a tree legume that is being promoted as a future biofuel feedstock.

The estimated fixed nitrogen of Pongamia based on the N-difference method inoculated with PR-UQ-05 was 3.73 g. Plants inoculated with PR-UQ-05 had the highest estimate of fixed N which was 100 mg/plant, with a total plant dry weight of 2.78 g/plant. The N difference method further confirmed that the inoculant PR-UQ-05 aids in the nitrogen fixation of Pongamia, as reflected in the increase of N level content in the shoots and roots of Pongamia seedlings over a 16-wk growth period. This method is appropriate for pot experiments under glasshouse conditions and the calculation for fixed nitrogen is easier (Unkovich et al. 2008). A similar pot experiment was done by Viera-Vargas et al. (1995) using common bean versus non-nodulating common bean as reference plant. Fixed nitrogen for common bean was 112 mg/plant, which is almost the same with Pongamia inoculated with PR-UQ-05. The results of N difference method can be affected by growth rates and rooting depth (Unkovich et al. 2008). Even if non-nodulating legumes are used as reference plant, the nitrogen-fixing plant will acquire more nitrogen, nutrients and water due to its higher plant vigor and larger root system (Ruschel et al. 1979).

The ureide analysis of Pongamia seedlings inoculated with PR-UQ-05 confirmed the presence of allantoin in the Pongamia shoots, which was at 143–150 nmole per plant, which came from 61–65 nodules with a total fresh weight of 1.01–1.20 g/plant. In the case of soybean, which is one of the many legumes that transport either ureides or amides in xylem sap as the dominant products of N_2 fixation, ureide present in xylem sap is 0.36–0.82 mM with the values increasing as the growth period or days after sowing increased (Unkovich et al. 2008). With the presence of ureides detected in the xylem of Pongamia, this study suggested the following mechanism for nitrogen fixation in Pongamia. The infected cell, which contains several bacteroids, is positioned adjacent to uninfected cells and close to a xylem pole. Nitrogen from the atmosphere goes inside the infected cell where it is converted to ammonia by the enzyme nitrogenase. Nitrogenase 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 the leghemoglobin. The 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 a 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. These ureides then go to the xylem vessel and are transported from the roots to the shoots of Pongamia.

The isotope techniques further confirmed the nitrogen fixation activity of Pongamia inoculated with PR -UQ-05. The proportion of fixed N was computed based on the atom % ^{15}N results of nodulated and non-nodulated Pongamia seedlings provided by the UC Davis Stable Isotope Facility. For natural abundance, the estimated fixed N of Pongamia inoculated with PR-UQ-05 was 68 μg/10 mg of dried plant sample while, for the enrichment method, the estimated fixed N was 158 μg/10 mg of dried plant sample. The enrichment technique yields more nitrogen in the nodulated plants which resulted in a higher estimate of fixed nitrogen compared

with the natural abundance method. Therefore, the addition of ^{15}N-labeled ammonium nitrate (98% atom) at 10 mM concentration watered once to the plants at 250 mL per plant or pot improved the nitrogen fixation in Pongamia.

In a field experiment using lupin (*Lupinus angustifolius*) as nitrogen-fixing plant and wheat as reference plant, the proportion of plant N from nitrogen fixation based on the ^{15}N enrichment technique was recorded as 78.6% and 84. 5% after 128 and 193 d of sowing, respectively, while the proportion of N from nitrogen fixation based on the ^{15}N natural abundance method was 88.4% and 81.3% after 128 and 193 d of sowing, respectively (Peoples et al. 1989). In this study, pot experiment was done using nodulated Pongamia to represent nitrogen-fixing plant versus non-nodulated Pongamia which represents non-nitrogen fixing plant or the reference plant. The proportion of plant N from nitrogen fixation was 38% for natural abundance and 54% for enrichment technique. These values are lower than those from the lupin experiment given that Pongamia seedlings in this study were grown in pots using vermiculite as medium and only the nitrogen fixation of the nodulated plant was the sole source of nitrogen, whereas, the lupin and wheat assimilated mineral N from the soil.

The different methods or assessment of nitrogen fixation used in this study showed different results based on the amount of fixed nitrogen calculated for each method. However, all of the methods employed in this study demonstrated that Pongamia inoculated with PR-UQ-05 fixed more nitrogen than the uninoculated control.

CONCLUSIONS AND RECOMMENDATION

The different methods employed in this study were all useful in the assessment and quantification of nitrogen fixation of Pongamia. Pongamia inoculated with PR-UQ-05 can produce active nodules, as implied by the increased volume of ethylene produced. Ureide is present in the shoots of Pongamia and was detected at low levels of 143–150 nmole, which means Pongamia utilizes ureides in the form of allantoin to transport its fixed nitrogen to other plant parts to support growth and reproduction. The nitrogen difference method and isotope techniques (natural abundance and enrichment) quantified the amount of fixed nitrogen of Pongamia inoculated with PR-UQ-05, which was estimated to be 100 mg/plant (based on the difference of total N yield between the nodulated and non-nodulated Pongamia seedlings) and from 20.4 mg/plant (natural abundance) to 407.4 mg/plant (enriched).

The symbiotic nitrogen fixation of Pongamia demonstrated in this study is very relevant to the biofuel industries. With the establishment of the capability of Pongamia to fix nitrogen, optimum plant growth and seed production of Pongamia can be achieved in marginal lands without too much investment in fertilizers and land area, as these plants can grow well without the application of expensive, and potentially environmentally harmful chemical nitrogen fertilizers. Ecologically, the nitrogen fixation capability of Pongamia is significant because of its potential impact on climate change adaptation through maintaining a nitrogen balance within the ecosystem. Legumes have been known to facilitate nitrogen transfer through the root exudates or connections between the root systems, thereby providing plant-available nitrogen for other non -nitrogen-fixing plants. Incorporating Pongamia in crop plantations is therefore a potentially good agricultural practice.

Due to the limited number of samples used in the study, it is therefore recommended to include more treatments and samples in future studies.

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