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FULL-LENGTH RESEARCH ARTICLE

Nodulation in the Legume Biofuel Feedstock Tree *Pongamia pinnata*

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Abstract The legume tree *Pongamia pinnata* (also called *Millettia pinnata*) is gaining importance as a biofuel feedstock tree because of the abundant annual production of oil-rich seeds, adaptation to a wide range of geoclimatic conditions and significant resistance to abiotic stress, such as water-deficit, salinity and acidity of soils. The major defining benefit of using pongamia as a biofuel feedstock is that it is a legume, enabling biological nitrogen fixation through symbiosis with soil bacteria, collectively called rhizobia, which results in root nodulation. Here, we report preliminary data, (i) indicating the range of rhizobia that can form nodules on pongamia, (ii) demonstrating the measurement of nitrogen fixation activity of pongamia nodules via the classical acetylene reduction assay, (iii) illustrating nodule morphology and development and (iv) demonstrating aspects of nodule regulation by external nitrate as well as internal autoregulation of nodulation. We note that in pongamia most nodulation-related characteristics are similar to those found in other annual crop legumes such as soybean.

Keywords Rhizobia · Legume · Biodiesel · Autoregulation of nodulation · Nitrogen fixation

Introduction

Pongamia pinnata (also called *Millettia pinnata*; hereafter referred to as pongamia) is a medium-sized tree legume native to India, Malaysia, northern Australia and Indonesia [33]. Large diversity exists [17]. The tree is generally considered to be a fast-growing, saline- and drought-tolerant species and is able to grow in a range of suboptimal conditions, including water-logged and alkaline soils [10].

Historically used as a medicinal plant, green manure and a source of heating fuel in India [1], pongamia is a non-edible crop that has recently become of interest to the renewable energy industry for its ability to produce seeds with an oil content of approximately 30–50 % [4, 17]. Pongamia's attributes as a sustainable feedstock for biofuel production stem from its (i) high annual yield potential (up to 90 kg of seed per tree per year), (ii) high oil content per seed (up to 54 % oil comprising approximately 55 % oleic acid; C18:1), (iii) silvicultural adaptability, including growth on so-called marginal land, (iv) absence of human food value and (v) legume biology allowing symbiotic nitrogen fixation [1, 12, 18, 22].

The latter point is important in the evaluation of a feedstock species for biofuel [16], as all plants require reduced nitrogen for protein and nucleotide synthesis, and general metabolism. Most legume species, including many annual food crops of soybean, pea, lentil, bean and peanut, and trees such as *Acacia* spp. have the ability to form root nodules via nitrogen-fixing symbioses with soil bacteria, collectively called rhizobia. In contrast, the common biofuel feedstocks sugarcane, canola, sweet sorghum, maize, and woody trees (e.g. eucalypts and willows) do require nitrogen to be supplied as a reduced form, usually nitrate, urea or ammonia. The production and application of nitrogen fertilisers represents a large economic and energetic burden as costs have increased due to a dependence on fossil fuel and natural gas. Moreover, the application of

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nitrogenous fertiliser to crops results in resident soil bacteria producing N_20, a powerful greenhouse gas (GHG), possessing global warming potential 296 times that for CO_2. These facts make the nitrogen supply to biofuel feedstocks a key issue when considering their sustainability on economic as well as ecological criteria [16, 26].

While much research on descriptive, biochemical and the molecular genetics of pongamia has been reported [17, 19–21, 27, 33], little research has been completed on the nodulation of pongamia and the capability of root nodules to fix nitrogen. The present study was undertaken to examine the components of nitrogen fixation in pongamia including determination of the rhizobia that are capable of forming nodules, the efficiency of nodulation determined via cytological examination of nodules and the regulation of nodule numbers via autoregulation of nodulation (AON).

Here, we present data illustrating that the nodulation of pongamia resembles the general properties seen in annual legumes such as soybean. Pongamia nodules were initially observed as determinate and were induced by inoculation with *Bradyrhizobium japonicum*. Nitrogenase activity was observed by the acetylene reduction assay, and nodule numbers were regulated by AON and nitrate, all properties similar to that seen in soybean and other annual crop legumes. Our understanding of nodulation biology will benefit the establishment of a more sustainable biofuel feedstock based on *P. pinnata*.

Materials and Methods

Plant Material, Germination and Growth

Pongamia seeds were obtained from trees in Brisbane, Australia. Seeds were soaked in warm tap water (30–35 °C) for 48 h before being sterilised in 5 % domestic bleach for 1 min. The seeds were then rinsed twice in 70 % (v/v) ethanol, rinsed five times in sterile tap water before being planted in sterile pots filled with sterilised grade 3 vermiculite (Australian Perlite Company, Bandsmeadow, Australia). Seeds were germinated in a growth cabinet for 1–2 weeks before being transferred to a glasshouse (28 °C, 16 h day) for a period of 3 months or until harvested. Pots were watered with tap water every day, and supplemented with nitrogen-free B&D medium [5] once a week.

Isolation, Collection, Growth and Analysis of Rhizobia

Bradyrhizobium japonicum strains USDA110, CB1809, CB564 and NGR234 and eight strains, (IC59, IC76, IC2002, IC4059, IC4060, IC4061, IC7001 and IC7017), isolated from pongamia nodules by colleagues from ICRISAT (India) were maintained on Yeast Mannitol Agar (YMA) [17]. For inoculation of pongamia, strains were grown in Yeast Mannitol Broth (YMB) for a period of 48 h before direct application onto the plant growth medium.

The presence of *NodC* (essential for Nod factor biosynthesis in rhizobial species) was determined by PCR using universal primers [24] on all strains of rhizobia. Based on the results of PCR, strains of rhizobia were used in pot trails to determine the capacity for nodulation of pongamia. For these trials, seeds were germinated and grown for 4 weeks before an actively growing culture of the appropriate strain of inoculant, or mock treatments were applied. The inoculum for each application contained 10^9 cells in a volume of 20 ml. Nodulation was assessed after a further 4–6 weeks.

Microscopy

Selected nodules from both inoculated and field-grown pongamia were first removed from the respective root systems and fixed in ethanol:acetic acid (3:1) for 30 min at room temperature. After fixing, nodules were stored in 70 % ethanol at 4 °C until sectioned for microscopy. Sections ranging from 30 to 60 μm were cut from nodules imbedded in 3 % agar using a Leica VT1200S dissecting microtome (Leica Microsystems, Nussloch, Germany). Once cut, these sections were observed under a Nikon Eclipse E660 light microscope (Nikon Corporation, Tokyo, Japan).

Acetylene Reduction Assay

Acetylene reduction assays (ARA) were carried out following a modification of the method of Cathey et al. [9]. Four seeds were germinated using the before mentioned method; two of these plants were designated test plants and inoculated with *B. japonicum* strain CB1809, and the remaining two saplings were used as negative (uninoculated) controls. Plants were grown in a growth cabinet for 6 weeks before being assayed. The roots were washed until they were free of vermiculite before being placed into room temperature tap water. For the ARA, moistened whole plants were totally transferred into flasks and capped with a gas-tight Suba-Seal (Sigma-Aldrich, Australia). Ten percent of the flask's gaseous contents was removed and replaced with 100 % acetylene gas (10 % final concentration). Immediately, a 1-ml sample of the flask's gaseous contents was removed and 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. Flasks were incubated at room temperature and samples removed and injected into the gas chromatograph every 5 min for the first 15 min and subsequently every 15 min.

Split Root Analysis and Nodulation Response to Nitrate

Split root experiments were set up following the method of Li et al. [25], to determine if pongamia nodule number is under the control of AON [11, 13]. Under AON, the initiation of late nodulation events is developmentally suppressed by the first formed nodules. Thus, delayed inoculation of split roots allowed the verification of AON, as such roots are severely depressed in nodule number [29]. The process of AON is common in annual legumes, but needed to be verified in the tree legume pongamia.

Briefly, pongamia seeds were germinated and grown for a period of 1–2 weeks before having their root apical region (1–2 cm) removed. These plants were then transferred into a split root apparatus (Fig. 1), and watered every day until profuse root growth developed. One of the split root systems was inoculated with *B. japonicum* strain CB1809, and then the second of the split root systems was inoculated a further 14 days later.

Tests to determine if nitrate would have an effect on pongamia nodulation was also completed. This was done by applying KNO_3 (0, 2, 5 and 10 mM) to plants grown in growth cabinets (16 h days, 28 °C). All plants were inoculated with 2×10^9 cells/ml of *B. japonicum* strain USDA110 and grown for 6 weeks before being harvested and checked for nodule number per plant.

Results

Nodulation Trials

Pongamia pinnata seedlings were shown to form functional nodules with *B. japonicum* strains CB1809 and USDA110, both of which are known to also form nodules on soybean. Nodulated plants displayed more green foliage than plants inoculated with the *B. japonicum* USDA110 nodC$^-$ mutant or un-inoculated plants fed nitrogen-free growth medium. However, contrary to previous studies, *B. japonicum* strains CB564 and NGR234 were not able to nodulate pongamia. Though all ICRISAT-derived strains tested positive for *nodC* by PCR, only strains IC4061, IC7001 and IC7017 were able to nodulate pongamia.

Pongamia Nodule Ontogeny

Pongamia nodules were mainly spherical or oblate, yet several nodules on more mature plants exhibited a coralloid appearance, suggesting that the original 'determinate-like' nodule structure was modified towards 'indeterminate-like' structure through activation of new cell divisions in the nodule. Nodule sections were observed by light microscopy to have an internal structure that was characterised as typical of legume nodules due to the presence of a central infected zone surrounded by a vascularised cortex [6]. It is noteworthy that the proportion of nodule cells infected with bacteroids, as judged by dark staining, was relatively low as evidenced by the large number of unstained, uninfected cells infiltrating the infected zone. The overall internal structure of nodules appeared to differ with respect to the inoculum, with the commercial soybean strains (CB1809 and USDA110) producing larger, more uniformly filled infected zones (Fig. 2), while the undefined inocula from ICRISAT produced nodules with several lobed infection zones with variable bacterial concentration (Fig. 3).

Nitrogen Fixation Activity

Acetylene (C_2H_2) serves as a substrate for the bacterial encoded nitrogenase enzyme complex, and its reduction to ethylene (C_2H_4) can be quantified by gas chromatography as a relative measure of bacterial nitrogen

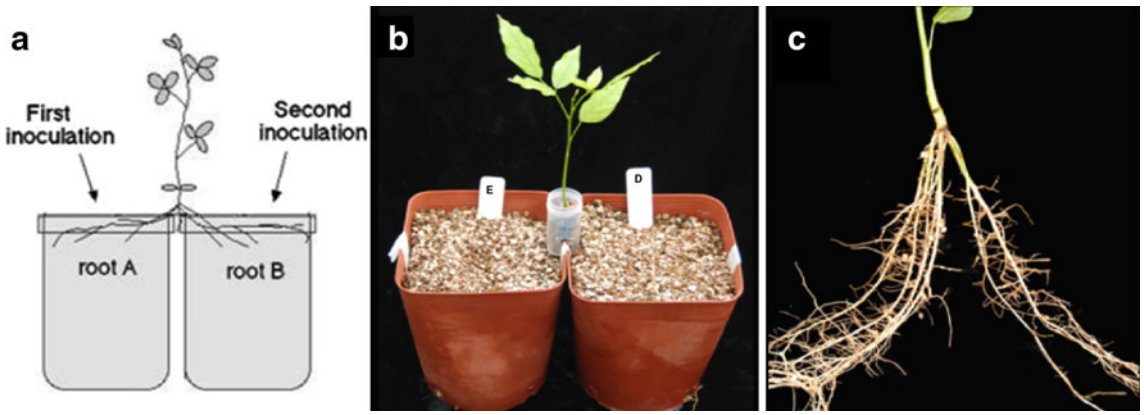

Fig. 1 Split root system to evaluate inter-root communication of AON signals: **a** diagrammatic image of a split root system with root systems labelled as A and B. **b** Shows an example of a split root setup for pongamia, root A with the first inoculation is termed 'E' for early, while root B is termed 'D' for delay. **c** Shows the root system once the vermiculite has been removed

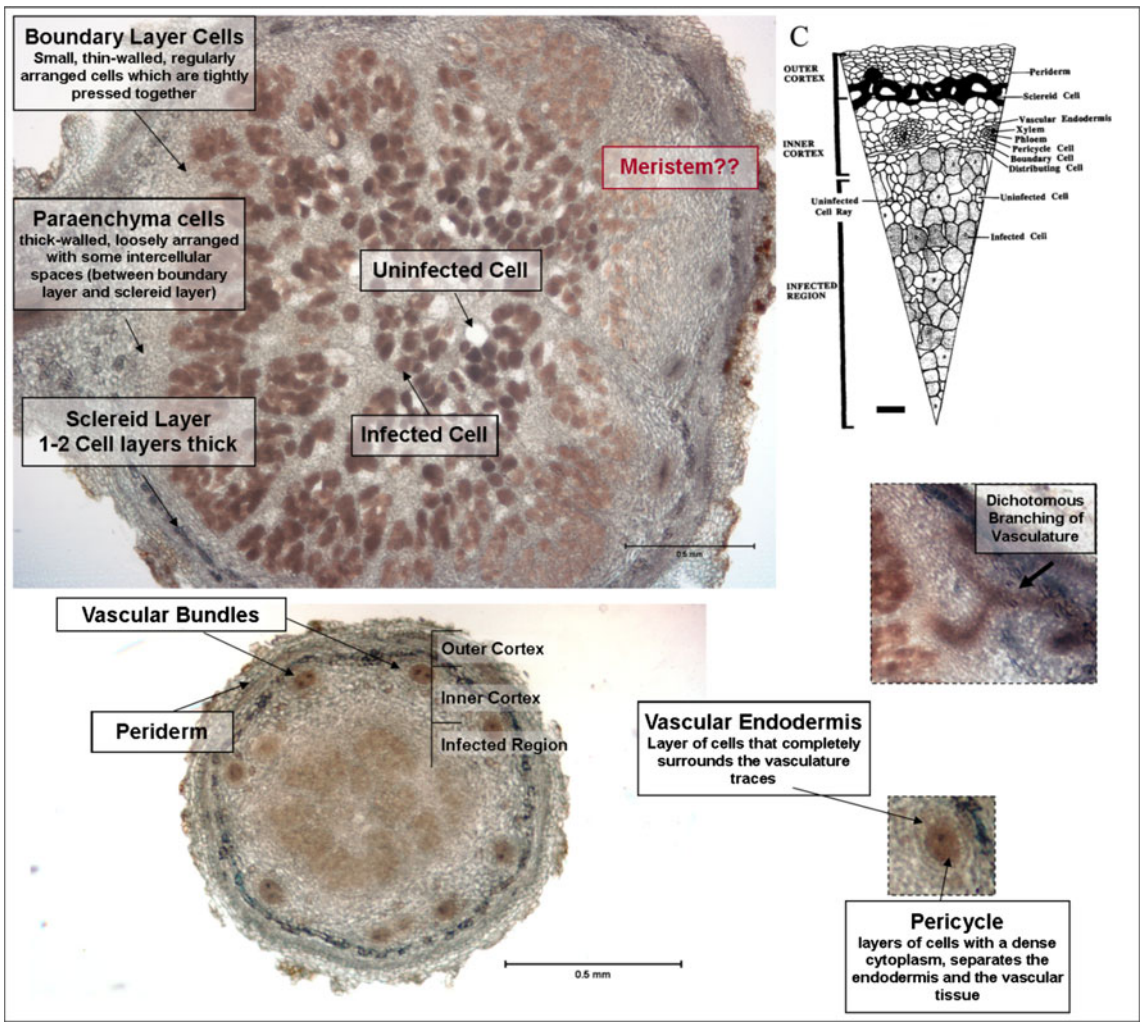

Fig. 2 Pongamia nodule morphology: pongamia nodule cross-sections shown are the result of inoculation with *B. japonicum* strain CB1809. Pongamia nodules have infection zones surrounding several boundary layers. Vascular bundles are located close to the infected zone within the cortex and are dichotomous, branching several times to fully encapsulate the nodule. Diagram adapted from [14]

fixation. Initial experiments where just the root system was introduced into the assay flask saw ethylene production in the absence of nodules, indicating the evolution of ethylene resulting from the stress of plant decapitation. Subsequently, assays were done with whole plants. The reduction of acetylene by pongamia nodules was demonstrated via increase in the amount of ethylene detected in both the test samples (inoculated with strain CB1809; see Fig. 4). The two non-nodulated plants showed no increase in detectable ethylene nor did the control flask of 10 % acetylene with no added plant tissue sample. The rate of ethylene evolution was linear over the 60-min assay period, suggesting that the enzyme activity was not limiting in these preliminary assays. We avoided such limitations by using fresh material, avoiding plant stress and keeping the nodules and roots attached.

Autoregulation of Nodulation (AON) in Pongamia

To demonstrate the presence of the AON control circuit in pongamia, five plants were tested for the suppression of nodulation in a split root system where the inoculation of one root system was delayed by 14 days with respect to the other root system. In all the five plants, suppression of nodulation was marked with two roots systems showing complete suppression (Fig. 5). Nodulation, as judged by nodule number per plant, was variable as commonly seen in this highly heterogeneous and outcrossing species. Suppression was calculated by the formula; (number of nodules in the first inoculated root system minus number of nodules in the second inoculated root system)/number of nodules in the first inoculated root system × 100. All five replicates indicated an AON control circuit, as the delayed inoculated root system always had lower numbers of nodules.

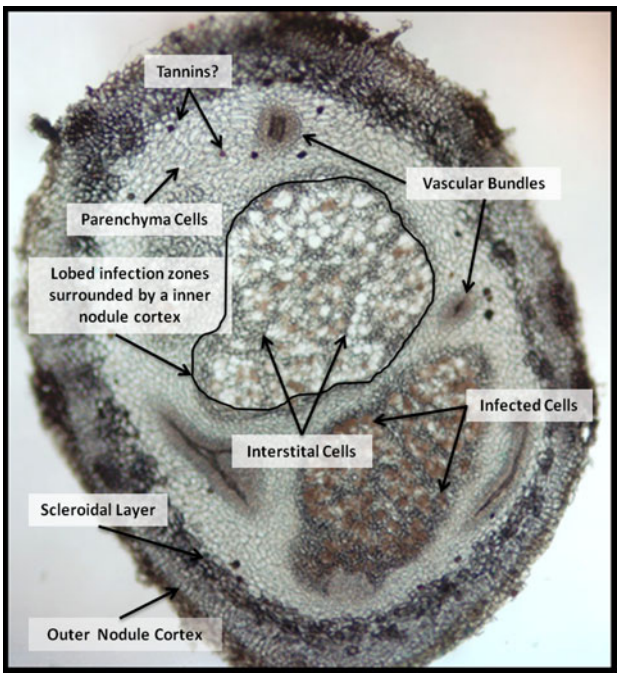

Fig. 3 Pongamia nodule section with variable nodule occupancy: pongamia nodule from a young sapling. Here, we can see two major infection zones with variable bacteroid concentration engulfed by an inner nodule cortex. Parenchyma cells surround this central infection zone infiltrated by vascular tissue and tannin cells. The whole nodule is bordered by an outer nodule cortex and scleroidal layer

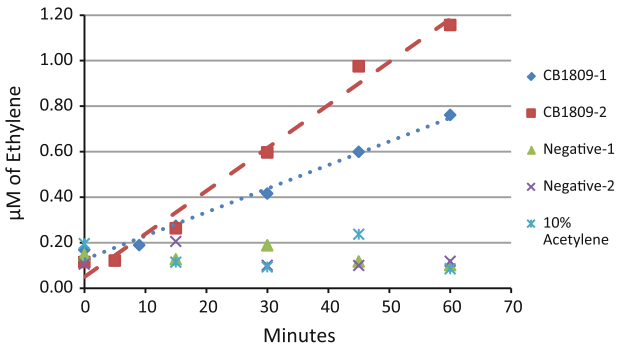

Fig. 4 Acetylene reduction assay of *Pongamia pinnata*: Test plants were inoculated with *B. japonicum* strain CB1809. Single plants with different nodule number, but nearly matching nodule mass were chosen. CB1809-1 formed 428 nodules while CB1809-2 formed 152 nodules. Samples were placed in a gas-tight container before a 10 % acetylene atmosphere was applied. The containers were incubated at room temperature and ethylene production was determined at 0, 5, 10, 15, 30, 45 and 60 min. *Negative*-1 and -2 are 8-week-old uninoculated pongamia; 10 % acetylene is a plant-free 10 % C_2H_2 control, also with no spontaneous ethylene evolution

In separate experiments, KNO_3 was supplied to pongamia seedlings inoculated with *B. japonicum* strain CB1809. Plants were examined after 8 weeks cultivation and significant reduction (90 %) in nodule number per plant was observed with 10 mM KNO_3 supplied on

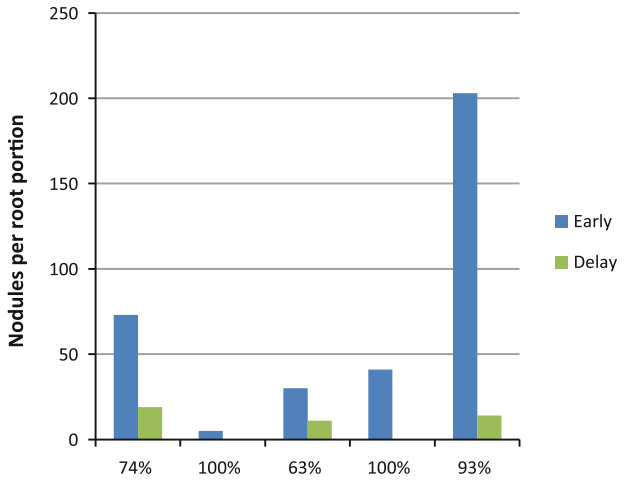

Fig. 5 Systemic inhibition of nodulation in *Pongamia pinnata* Split root experimentation of pongamia seedlings with a 2-week inoculation delay. 'Early' represents the root system that was inoculated initially with *B. japonicum* CB1809. 'Delay' represents the root system that was inoculated 2 weeks later. Percentage data below the columns indicate the degree of nodule number suppression

alternate days. In addition, with 10 mM KNO_3 nodules were smaller and failed to indicate rhizobial occupation of nodule cells. With 5 mM KNO_3 bacteroid development, as indicated by pink pigmentation of nodules from expression of rhizobia-associated leghaemoglobin, was severely inhibited or absent. Control treatments with up to 25 mM KCl had no effect on nodule numbers and development. These observations put the pongamia–rhizobium symbiosis in the same nitrate tolerance range as other annual legumes like soybean [8, 15].

Discussion

Effective Bradyrhizobial Inoculum for *Pongamia pinnata*

A key aspect of symbiotic nitrogen fixation is the efficacy of the rhizobia to nodulate the host legume. Most species of rhizobia interact with only a select few legumes, but some (e.g. *Rhizobium sp.* strain NGR234) have been shown to have a broad host range. Pongamia is known to be a promiscuous legume and is able to establish efficient symbioses with species of both *Bradyrhizobium* and *Rhizobium* [2, 32]. Our experience suggests that caution needs to be taken in nodulation trials. We found previous to this study that some uninoculated, but seemingly sterilised plants formed nodules, in which one needs to assume incomplete sterilisation. The procedures outlined in this study were an advance on previous sterilisation regimes, as background nodulation was not observed.

Microsymbionts can promote the growth of a crop species by allowing them to uptake nutrients that would normally be unavailable to the plant. Therefore, the selection of suitable rhizobial strains is of upmost importance as it may help to promote the growth of pongamia and increase potential yields of oil-rich seeds.

Previous studies in our laboratory showed that pongamia was able to nodulate with *B. japonicum* strain CB1809, *Bradyrhizobium* sp. strain CB564 and *Rhizobium* sp. strain NGR234, although this present study failed to confirm the nodulation with the latter two strains. Similarly, Pueppke and Broughton [30] were unable to demonstrate the nodulation of pongamia with *Rhizobium* sp. strain NGR234. The quality of the seed sterilisation in previous experiments was difficult to verify, as we noted that often seemingly surface-sterile pongamia seed still nodulated despite being uninoculated. Contaminating bacteria hidden in the crypts of the seed surface may present a problem in nodulation studies. However, this problem was solved when using the here-described seed sterilisation method.

Testing of rhizobia and the isolation of both highly infective and effective nitrogen-fixing strains has been attempted by several research groups. For example, Rasul et al. [32] isolated 29 nodule-occupying strains from sterilised pongamia seedlings planted into soils collected from Andhra Pradesh, Maharashtra and Karnataka in southern India. Even though the capacity for nodulation and phylogenetic analysis of the isolated strains was conducted, the ability to fix nitrogen was not assessed and no superior isolate was found.

In a similar project, Arpiwi et al. [2] recently reported the extraction of 40 isolates from soil samples where pongamia was grown in Kununurra, northern Western Australia. This study compared the strains 'relative effectiveness' (shoot dry weight of inoculated over shoot dry weight of N-fed plants) and was able to name *Bradyrhizobium yuanmingense* as the most dominant microsymbiont and a potential superior inoculum. All strains collected in both these studies were described as 'creamy or white opaque' having close phylogenetic relationships with known *Bradyrhizobium* and *Rhizobium* strains.

Characterisation of the Pongamia Nodule

There are two distinct structural types of nodules, determinate (e.g. soybean, *Lotus japonicus*) and indeterminate (e.g. pea, *Medicago truncatula*). Determinate nodules are characterised by the lack of a persistent meristem, making them more or less spherical in appearance, while indeterminate nodules maintain an active apical meristem for some time. Bacteroids of determinate nodulators tend to retain the size of the vegetative bacterial cell, while indeterminate types enlarge, becoming amorphous and pleiotropic. Indeterminate nodule meristems produce new cells over the life of the nodule resulting in the resulting nodule having a generally cylindrical shape with different developmental zones (meristematic, infection, fixation and senescence). In addition to the spherical and cylindrical types, there are other diverse nodule types, such as collar nodules in lupin and coral-shaped nodules in many tree species.

In 2008, Rasul et al. [32] published the image of a spherical pongamia nodule stating that the nodule type was determinate; this was echoed in Kazakoff et al. [18]. Though spherical nodules were found to be present on pongamia roots in this study, larger coralloid nodules were also found. These coralloid nodules are hypothesised to be older nodules that have become partially indeterminate and are exhibiting new bacterial colonisation and nodule cell division. It has been previously noted in several studies that most tree legumes tend to have woody indeterminate nodules [3, 28, 34]. We conclude that pongamia nodulation at first leads to determinate-type nodules and often progresses later into an indeterminate state.

Most legumes that form determinate nodules are small annual plants that die at the end of the growing season or when they have flowered and produced seed. These plants do not require a nitrogen supply for extended periods of time, so determinate nodules with a defined lifespan and without a persistent meristem are sufficient. In contrast, as a tree legume pongamia has an ongoing seasonal nitrogen requirement with higher nodulation and nitrogenase activity needed during summer and lower activity during winter [10]. It is therefore somewhat unexpected that pongamia nodules should be determinate. As previously mentioned, indeterminate nodules are to be found on most woody legumes. A survey of wild legumes in China by Ng and Nau [28] observed spherical nodules on all legume species; however, these nodules were thought to be undifferentiated and immature, if nodules of other shapes were also observed. This idea of globular nodules turning into cylindrical indeterminate nodules is not new. *Canjanus canjan* (chickpea) displays determinate nodule growth initially, but is seen as indeterminate growth at a later stage [6]. Qadri et al. [31] also noted that on the tree legume *Pithecellobium dulce* young nodules were globose in all examined species, though mature nodules were elongated, branched and coralloid.

Measurement of Nitrogen Fixation in Pongamia Nodules

Nitrogen fixation of pongamia roots was assumed to occur as we demonstrated acetylene-dependent ethylene production (called acetylene reduction) in inoculated saplings. Non-nodulated plants were used as negative controls. The acetylene reduction assay showed linear kinetics over a

60-min period, making the data useful for comparative studies. However, quantification of nitrogen gain based on acetylene reduction values is difficult as physiological and diurnal factors vary the relative efficiency. Future studies will 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.

AON in Pongamia

Historically, most of the work on regulation of nodulation has been carried out on model legumes through the use of loss-of-function mutants [7]. Since no pongamia nodulation mutants are currently available, the mechanisms of regulation are more difficult to characterise. Following methods from previous studies on model legumes [23, 29, 35], split root systems were initiated and used to test whether nodulation on continually developed root systems is systemically inhibited by the presence of the first formed nodules.

The results in Fig. 5 provided strong preliminary evidence that AON is functioning in pongamia to regulate nodule numbers, and thus the capacity of nitrogen fixation to meet the plant's nitrogen requirements. As a period of 2 weeks separated the initial and second inoculation of rhizobia, this result is expected as previous research had shown that in other legumes AON can be observed in a split root system with as little as 30 h between inoculation. The reported interval in which full induction of AON is displayed varies from species to species. In *Lotus japonicus,* the full effect of AON was seen after 5 days, while in *Glycine max* (soybean) this time was only 4, 2 days for *Trifolium subterraneum* and only 30 h for *Vicia sativa* L. [35] and references therein. Knowing this, a 2-week time between inoculation as used in this demonstration experiment, is likely much longer than needed to see the initiation and effect of AON in pongamia. However, such a long delay ensured the full effect of AON, though variability in the level of suppression was observed. Suppression of nodulation by prior nodule formation was also demonstrated on simple seed-derived plants via the typical 'crown' distribution of nodules (data not shown).

Conclusion

Nodulation and nitrogen fixation were characterised in the biofuel feedstock legume tree pongamia. In particular, the histological organisation of the pongamia nodules was determined following inoculation with rhizobia previously shown to nodulate soybean. Also, the regulation of nodulation demonstrated via nitrate inhibition of nodulation and internal AON. Despite being a long-lived tree, pongamia shares both qualitative and quantitative characteristics found in other more well-characterised annual crop legumes.

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