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Factors affecting the nodulation of *Pongamia pinnata* (L.) Pierre inoculated with *Rhizobium mesoamericanum* (Lopez-Lopez et al., 2012)

Phoebe S. Nemenzo[1]*, Marianita B. Santos[1], Melanie M. Garcia[2], and Jhonnel P. Villegas[3]

[1]Biology Program, Arts and Sciences Department, San Pedro College, Davao City
[2]Ph.D in Environmental Science-Graduate Student, School of Environmental Science and Management (SESAM) and Professional School for Agriculture and the Environment (PSAE) University of the Philippines-Los Baños, 4031, Philippines
[3]Institute of Education and Teacher Training, Davao Oriental State University, Mati City, 8200, Philippines

ABSTRACT

Pongamia pinnata or "bani" in the Philippines is a non-food crop that can grow on marginal land where food crops do not grow. Its seeds are used for biofuel production such as biodiesel and aviation fuel. It is resilient against abiotic stresses such as drought, salinity and acidity. It can also grow in nitrogen-limited soils. It is capable of nitrogen-fixation activity through its root nodules developed by symbiosis with rhizobia. Rhizobium mesoamericanum (Lopez-Lopez et al., 2012) promotes the nodulation of Pongamia as confirmed by strain symbiotic effectiveness testing where shoot length, number of nodes, number of leaflet ts, nodule number, and plant dry weights were analysed. Variability test was carried out to determine any variability of nodulation in the seeds coming from one mother tree. A time course of nodulation revealed that there was an increasing pattern of nodule number, leghemoglobin concentration, area of zone of infection and dry weights. Nodulation increased when nitrate concentration was increased from 2 to 5 mM, but was inhibited in 10 mM nitrate. Nodulation decreased as salinity increased from 1% to 3%. R. mesomaericanum improved the growth and nodulation in Pongamia, whether as a group or as individual inoculants, provided that a sufficient concentration of the inoculant was achieved.

Keywords: Nodulation, symbiotic nitrogen fixation, Pongamia pinnata, Rhizobium mesoamericanum, legume tree

INTRODUCTION

Pongamia pinnata (L.) belongs to family Leguminosae, subfamily Papilionoidae and Millettieae tribe. It is a medium-sized arboreal legume tree indigenous to the Indian subcontinent, Northern Australia and Southeast Asia including the Philippines. Pongamia is considered a tropical plant and can be found in coastal areas or close to marin environments use as inlets, river mouths and sea fronts, along limestone and rock coral outcrops, edges of mangrove forests and tidal streams and rivers (Calica, 2017; Gresshoff et al., 2017). In the Philippines, Pongamia is commonly called "bani" especially in Pangasinan, Zambales, Pampanga, Bataan and Cotabato. It is locally known as "balikbalik" in Tagalog, "balobalo" in Zambales and Basilan; "balukbaluk", "balutbalut" or "magit" in Cotabato, "baobao" in Agusan, "kudet" in Tayabas, "m arokbarok" in Camarines, and, "salingkugi" in Zamboanga (NFTP-EP, 2021). It can grow up to 15 meters and a diameter of about 0.5 meter. It has alternate and compound leaves with three to seven leaflets, which are smooth, pointed at the apex, rounded at the base, and seven to 10 cm in length. The flowers are pink to purplish, 1.5 cm in length, and borne in raceme. The somewhat flattened pods have oval outline or shape with a single seed. Pongamia is distributed from northern Luzon to southern Mindanao. It is used in the Philippines in crafts and furniture making. The bark and bast are used for making strings and ropes (NFTP-EP, 2021).

Pongamia form association with bacteria present in soil which are generally called rhizobia resulting in root nodulation and symbiotic nitrogen fixation. To optimize the nitrogen fixation activity in Pongamia, the best or superior rhizobia must first be isolated for Pongamia symbiosis. Baiting technique is an indirect technique to isolate rhizobia from soil by using a host plant as bait and then later on isolate from the surface-sterilised nodule using one-nodule-one-drop technique (Nemenzo-Calica et al., 2016). This uses the nodulation process as effective trap for selective enrichment and allows the isolation of superior strain of rhizobia. Once the best rhizobia is isolated, it can be inoculated to Pongamia for efficient nodulation and nitrogen fixation. Nemenzo-Calica et al (2016) previously established that Rhizobium mesoamericanum previously isolated from Queensland, Australia, is a superior rhizobial strain for Pongamia.

Pongamia nodulation can be affected by several factors which can further influence the efficiency of biological nitrogen fixation. The symbiosis between Pongamia and R. mesoamericanum is maximized when niche requirements are met for both host and rhizobia species. Factors that limit plant health and photosynthetic

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Phoebe S. Nemenzo
Email Address: f.phoebe.nemenzo@cmu.edu.ph
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capacity will likewise limit nitrogen fixation potential. However, even when optimal conditions are met for Pongamia growth and development, the establishment of R. mesoamericanum can be independently inhibited by factors including nutrient excess or deficiency, moisture, acidity, element toxicity, native microbial competitors and problems with inoculation (Kasper et al., 2019). Pongamia nodulation studies were previously done using different rhizobia strains including Bradyrhizobium species (Samuel et al., 2013). Biswas et al (2013) reported that nitrate inhibited the nodulation and autoregulation of nodulation in Pongamia when inoculated with Bradyrhizobium japonicum CB1809 and nodulation was decreased with increasing salinity. Gresshoff et al (2017) also reported that low NaCl concentration stimulates growth of Pongamia seedlings compared with no NaCl. However, there has been no published study on the factors affecting the nodulation of Pongamia when inoculated with R. mesoamericanum. Hence, this study was conducted to determine the factors affecting the nodulation of Pongamia inoculated with R. *mesoamericanum*.

METHODOLOGY

Isolation, identification and strain symbiotic effectiveness of R. mesoamericanum

Soil samples were collected from different locations across Queensland, Australia. The samples were characterized for their physico-chemical properties such as physical appearance, pH, EC, and total nutrient levels. LECO TruSpec analyzer was used to analyze the total Carbon and Nitrogen level. Total nutrient analysis was done using a Varian Vista Pro ICPOES instrument. Baiting technique and one drop-one nodule technique were done to isolate rhizobia from randomly selected nodules following the protocol of Nemenzo-Calica et al (2016). The isolates were morphologically characterized based on their colony size in diameter, color, shape, margin, elevation and texture. Growth curves in liquid culture of all isolates were also determined. The antibiotic resistance of each isolate was also determined.

Strain symbiotic effectiveness tests were carried out to confirm if the isolates were indeed capable of nodulating Pongamia using uniform growth medium (sterile vermiculite) under uniform growth conditions in the glasshouse. The pure cultures of rhizobia isolated from different soil samples and from Pongamia nodules were inoculated into yeast extract mannitol broth for 48 hours with shaking. Bradyrhizobium strains: USDA110 (U.S. Department of Agriculture, U.S.A.), CB1809 and CB564 (Commonwealth Scientific and Industrial Research Organisation or CSIRO Division of Tropical Agronomy, Brisbane, Australia)were included in this test to compare with the isolates. Meanwhile, several Pongamia seeds were sown into sterile vermiculite in 15cm pots which were thinned out later into one plant per pot. Three to five replicate plants were chosen for each treatment. Plants were maintained for 12 weeks. 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 by refrigeration), and Nodule morphology.

The selected superior isolates previously identified in the strain symbiotic effectiveness test were further characterized and 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).

The selected superior isolated were genetically identified by using nif genes and nod C. The isolates were grown in yeast extract mannitol broth (YMB) in flasks and then transferred into 1.5 ml centrifuge tubes. Tubes were centrifuged at 16,000 g for 10 min. 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. 30 µl of 10% SDS and 3 µl of 20 mg/ml proteinase K were added into the tubes. Tubes were incubated for 1 hour at 37°C. 500 µl of phenol:chloroform:isoamylalcohol (25:24:1) was added. Tubes were centrifuged at 16,000 g for 2 min. 0.1 ml of 3 M Sodium Acetate, pH 5.2 and 0.6 ml of isopropanol was added to precipitate the DNA. Tubes were incubated at least 30 minutes on ice. The tubes were centrifuged for 15 minutes at 16,000 g. The DNA pellet were washed in 500 µl of 70% ethanol. The tubes were centrifuged for 15 minutes. The DNA were dried and resuspended in 100 µl of sterile MilliQ water. Success of DNA extraction was confirmed by loading 5 uL of each rhizobium with 2uL of loading dye into the wells of agarose gel mixed with ethidium bromide. Molecular ladder used was 1kB (brand) and run for 60 minutes at 100 volts. One kb molecular ladder was loaded on the first well. The gel electrophoresis was done using 100 volts in 60 minutes. Identification of the rhizobia up to the species level was done by amplifying the nif genes and nod C (Samuel et al., 2013) using a thermocyler machine (S1000 Bio-Rad Thermal Cycler). The PCR condition was based on (Nemenzo et al., 2016). All amplicons were confirmed using gel electrophoresis and excised using QIAquick Gel Extraction Kit, Qiagen). The amplicons were sent to Australian Genome Research Facility (AGRF) for sequencing. Genetic sequences were run in BLAST for identification.

Variability test

Fifty pots were sterilized and were filled with sterile vermiculite. Fifty sterile Pongamia seedlings were planted to pots and inoculated with R. mesoamericanum. Plants were maintained in the glasshouse and were watered once with 150-ml nitrogen-free nutrient solution, Broughton & Dilworth (B&D solution) and twice weekly with sterile distilled water. After 12 weeks, plants were uprooted and observed. Statistical analysis such as ANOVA and Tukey's Honestly Significant Difference was done to analyse data including leaflets arrangement, number of nodes, number of leaflets, nodule number, nodule morphology, and plant dry weights using R software. An assumed value of variance for each of the parameters: plant structure, nodulation and nitrogen fixation was determined. Hypothetically, if the variance for each parameter will be below 0.5, then it can be concluded that there is no significant variation among the 50 seedlings in terms of the parameters tested. Fifty seedlings were used as sampling size to obtain around 90-95% statistical power to provide reliable inference in supporting the hypothesis.

Time course of nodulation

Time course of nodulation was done with R. mesoamericanum which were incubated with shaking for 48 hours until turbid and inoculated to sterile Pongamia seedlings at 40 ml per plant (108 cells/ml). Plants were watered with B&D solution and tap water for 12 weeks. Plants were randomly harvested per week from week 4 to week 8 with four replicates per harvest. Date gathered include nodule number and fresh weight, leghemoglobin concentration, area of zones of infection, plant dry weights, and N level content in roots and shoots.

Leghemoglobin concentration was quantified following the method by Keilin and Wang (1945). Nodules from individual plants were weighed (fresh weight) and homogenised in 5 ml of 0.1 N KOH using a mortar and pestle. Maximum of 1g of homogenized plant nodule was used as sample. The suspension was centrifuged for 10 min at 10000 g. A 1.5 ml aliquot of the supernatant was mixed with 1 ml distilled water and 0.5 ml 5 N KOH. After reduction with 0.1 g sodium sulfate, the optical density or absorbance was determined using spectrophotometer at 600 nm. The leghemoglobin concentration of the seedlings was derived from the standard curve using prepared leghemoglobin of various concentrations as standards (0.01 mg/ml, 0.1 mg/ml, 0.5 mg/ml, 1 mg/ml, and 1.5 mg/ml).

The area of the zones of infection were determined by cutting the largest nodule per week of harvest into half and observed using a compound microscope with attached camera. NIS-element software (4.20.00 64- bit program) was used to automatically highlight and measure the area of infection.

Effect of nitrate and salinity in nodulation of Pongamia

Four replicate Pongamia seedlings previously sterilised as seeds were inoculated with R. mesoamericanum. Treatments include 2 mM KNO_3, 5 mM KNO_3, 10 mM KNO_3, 1% NaCl, 2% NaCl, 3% NaCl, positive control (inoculated) and negative control (uninoculated and not treated). The seedlings were watered with the treatments only once at 250 ml per plant. Plants were maintained under uniform glasshouse conditions and watered with sterile distilled water for 12 weeks and data gathered included shoot length, number of nodes, number of leaflets and plant dry weights.

Synergistic effect of rhizobia in nodulation

Pongamia seedlings were inoculated with either R. mesoamericanum strain PR-UQ-03, PR-UQ-05 only or combination of PR-UQ-03 and PR-UQ-05 strains to demonstrate any synergistic effect of strains to nodulation of Pongamia. There were five replicates per treatment. Plants were maintained for eight weeks under glasshouse conditions and nodule number, shoot length, number of nodes and leaflets and plant dry weights were gathered and analysed statistically using ANOVA and Tukey's HSD test.

RESULTS AND DISCUSSIONS

Strain symbiotic effectiveness and identification of R. mesoamericanum

There were 21 different locations that were sampled. A total of 42 samples were characterized (2 replicates per sample). The sampling was done in December to February where the weather is sunny and dry. In the baiting experiment using saline soils, it was observed that there were different zones of nodulation in the baited seedlings using the 5 different soil samples from Darbalara. The soil samples used corresponded to the gradient in the field (S2 least saline, S5, S8, S11 and S13 as highly saline). S2 soil sample contained rhizobia that nodulated the Pongamia seedlings at the early stage as evident in the nodules located close to the base of the roots, whereas, the S5, S8, S11 and S13 nodules tend to form farther from the base of the roots.

In several studies, strain symbiotic effectiveness testing is also referred to as the authentication test for rhizobia. The uninoculated control plants did not produce nodules, which confirmed that there was no contamination in the glasshouse set-up. The highest shoot length was observed in plants inoculated with isolate PR-UQ-03, with a mean value of 42 cm. The highest number of nodules was observed in plants inoculated with PR-UQ-05 with a mean value of 143 per plant and with a range of 70-240 per plant. All nodules were lateral with some distinct clustered nodules found in PR-UQ-05 inoculated plants. The shape of the nodules was globular to coralloid, having a range in size of 1-5 mm, and light to dark brown in color. Using Tukey's HSD test, the treatments had significant effects (at 0.01 and 0.05 levels) on biomass (shoot dry weight), number of nodules and other tested parameters.

Based from the results of the strain symbiotic effectiveness test, two superior rhizobia isolates were identified: PR-UQ-03 and PR-UQ-05. A single molecular band was produced from all the PCR products. Restriction enzyme digestion showed that the pattern of band fragments differed among the selected two superior rhizobia isolates, which means that they belong to different strains. BLAST results (e values=0) revealed that PR-UQ-03 and PR-UQ-05 are R. mesoamericanum (at 99% certainty). When, PR-UQ-05 was aligned with PR-UQ-03, the BLAST alignment revealed that these two isolates were highly similar (>99%), although the restriction nuclease digestion pattern, antibiotic profile and symbiotic tests results differ between them. Hence, they could be the same species but different strains.

Variability test

The nodulation studies are dependent on the differences in growth patterns among the different treated plants, aside from the nodule number and morphology. The leaflet arrangement, number of nodes, number of leaflets, shoot length, nodule number and plant dry weights of all 50 sterilised seeds from a single mother tree were not significantly different. Figure 1-3 show the results of the variability test for the 50 Pongamia from a single mother tree. There was no variation in the data for all parameters tested among all the 50 seedlings. All of the 50 Pongamia seedlings had a pinnately trifoliate leaflets arrangement,

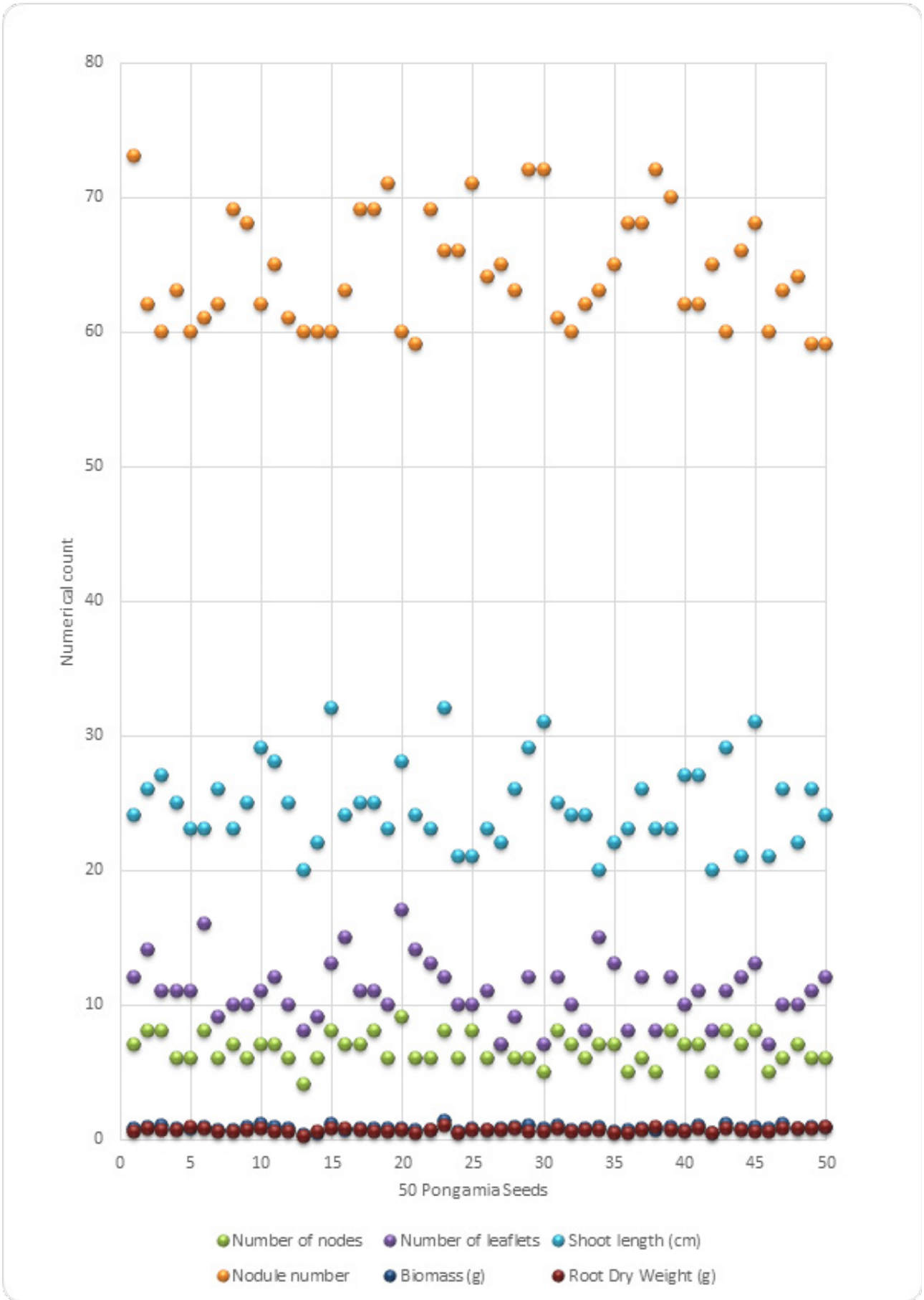

Figure 1. Scatter plot for the 50 *Pongamia* seedlings across all parameters.

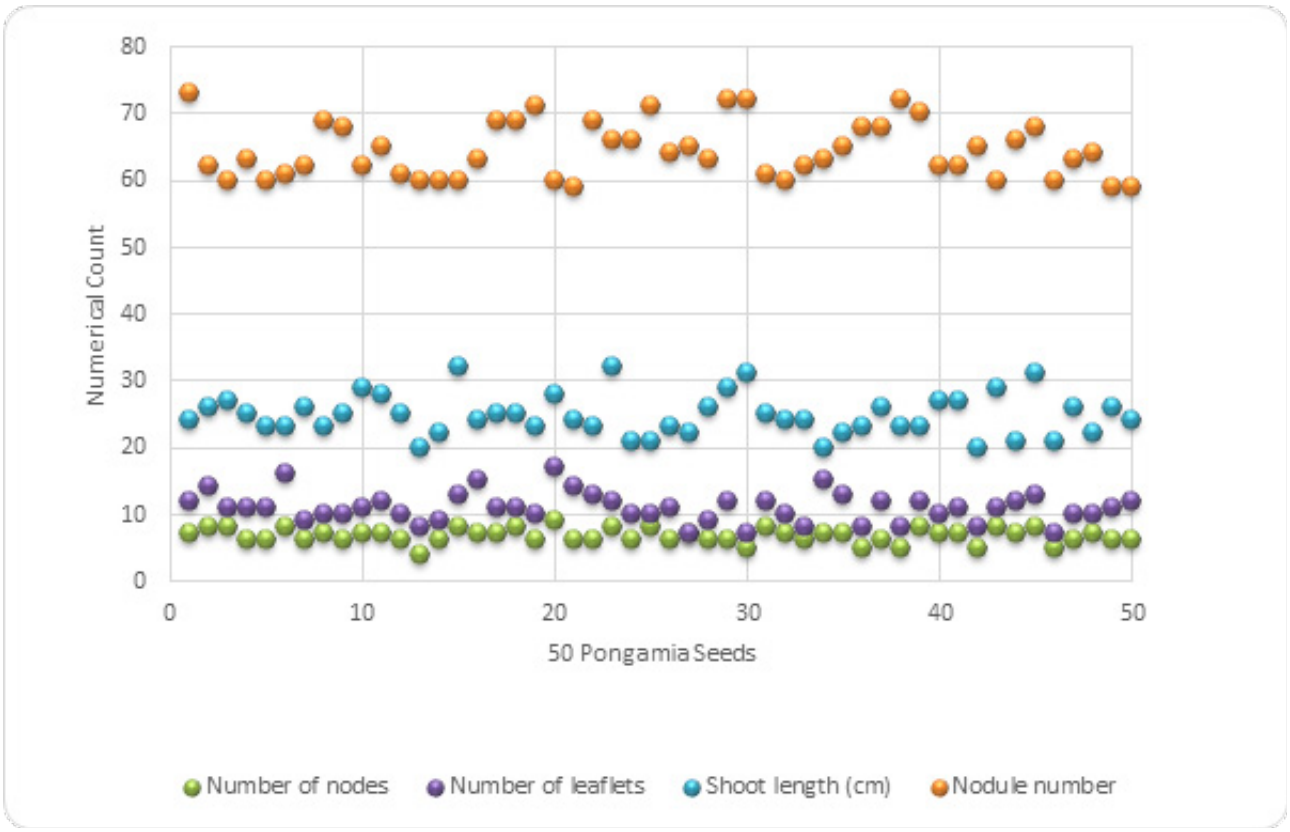

Figure 2. Scatter plot for number of nodes, number of leaflets, shoot length (cm) and nodule number.

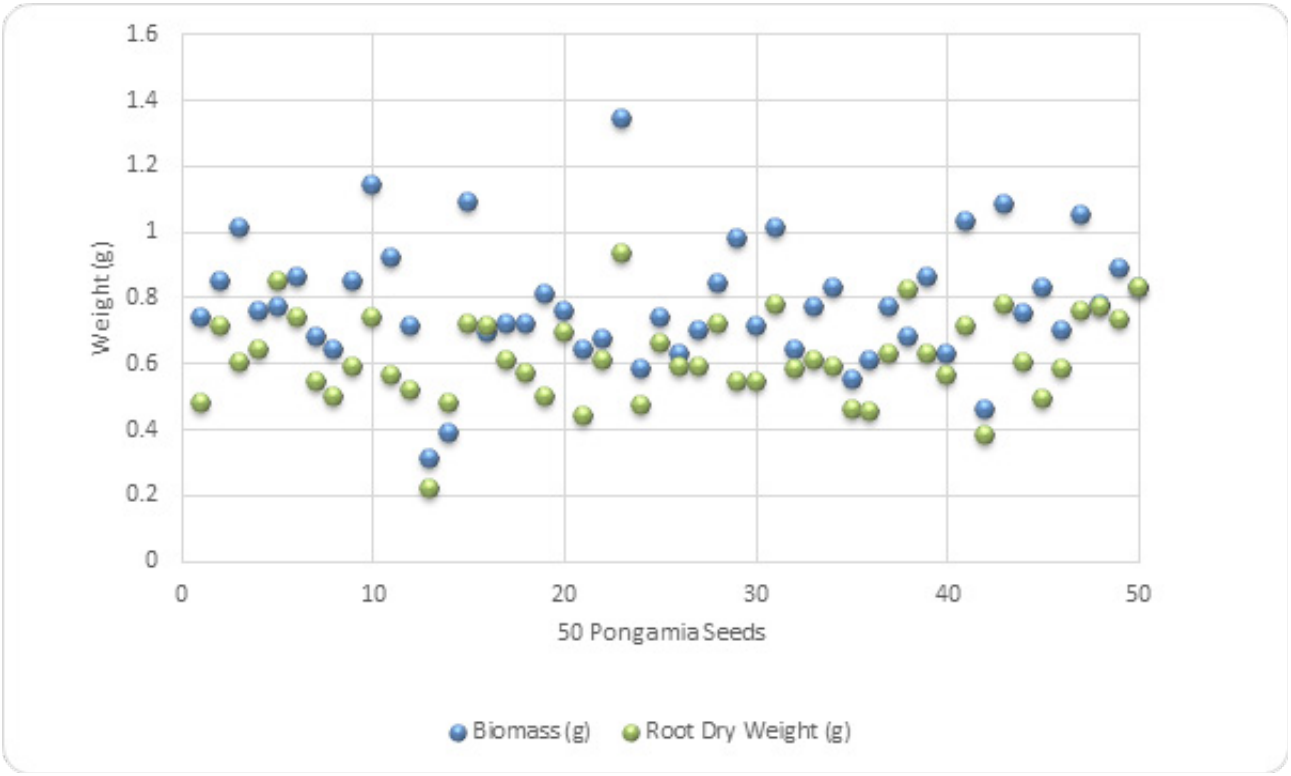

Figure 3. Scatter plot for biomass (g) and root dry weight (g).

spherical to coralloid and brown nodules.

Time course of nodulation

For the time course of nodulation using R. mesoamericanum strains, all uninoculated control plants had no nodules from weeks 4 to 8. Total plant dry weight increased from 1.23 g in week 4 up to 2.11 g in week 8. The total N level in shoots and roots increased from 5.12% Wt in week 4 to 6.22% Wt in week 7 but decreased to 5.89% Wt in week 8. Strain 1 (PR-UQ-03) plants nodulated in week 4 with 38 nodules per plant and continued 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 there were almost no changes in weeks 7 and 8. Leghemoglobin concentration at week 4 was 0.132 mg/ml and increased to 0.640 mg/ml in week 8. The area of the zone of infection of the biggest active nodule was 1.58 mm2 in week 4 and this continued to increase up to 3.89 mm2 at week 8. This is measured as the cross-section of the nodule. The total N level in shoots and roots was 6.56% Wt in week 4, with no significant change until week 8. The Strain 2 (PR-UQ-05) plants produced 56 nodules per plant in week 4, which continued to increase until week 8 with 78 nodules per plant. The total plant dry weight also doubled from 1.139 g in week 4 to 2.283 g in week 8. Leghemoglobin concentration increased from 0.235 mg/ml in week 4 to 0.663 mg/ml in week 8. The area of the zone of infection of the biggest nodule started at 1.86 mm2 in week 4 and increased to 3.08 mm2 in week 8. The total N level in shoots and roots was 4.30% Wt in week 4, increasing to 5.12% Wt in week 8.

The inoculation of PR-UQ-05 showed a 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, but there was no significant increase in total plant dry weight and total N level in shoots and roots between weeks 4 and 8.

Effects of nitrate and salinity on Pongamia nodulation

R. mesoamericanum was inoculated to Pongamia seedlings which were then subjected to different nitrate concentrations (2 mM KNO3, 5 mM KNO3 and 10 mM KNO3) and NaCl concentrations (1% NaCl, 2% NaCl, and 3% NaCl). Negative control (uninoculated) and positive control (inoculated but without added nitrate and NaCl) were included. The results showed that treatment plants inoculated with R. mesoamericanum but without nitrate and NaCl added had 65+1.58 nodules and a biomass of 0.85+0.09 g. Plants grown with 2 mM KNO3 has higher number of nodules (72+-1.29) which further increased to 83+-1.44 for plants with 5mM KNO3. However, nodule count decreased significantly (33+-1.03) at 10mM KNO3. Nodules significantly decreased at 10 mM KNO3 with a mean value of 33+1.03. The addition of 1% NaCl had no significant effect relative to the positive control but decreased significantly when increased to 2% (NaCl) (from 63+0.71 nodules to 27+3.5). Addition of 3% NaCl significantly inhibited nodulation with only 1 replicate having three nodule while the rest has none. Biomass had no significant difference among all the treatments. The uninoculated plants (negative control) had no nodules and had significantly the lowest shoot and biomass measurements among all the treatments.

Synergistic effect of rhizobia on nodulation

Table 2 shows that there was no significant difference in all the parameters tested except for the nodule number and nodule dry weight in which the combined strains had significantly lower mean values at 0.1 level than either R. mesoamericanum strains PR-UQ-03 or PR-UQ-05. There was no synergistic effect of combined strains to Pongamia nodulation.

RESULTS AND DISCUSSIONS

Rhizobia nodulating Pongamia were found to

Table 1

Nodulation results of Pongamia seedlings inoculated with R. mesoamericanum subjected to different nitrate and NaCl concentrations.

Treatment	Shoot Length	Number of Nodules	Number of Nodes	Number of Leaflets	Biomass (g)	Root Dry Weight (g)
Positive Control (inoculated)	26.50^{a}	65.00^{bc}	6.75a	12.00a	0.85a	0.64a
Negative Control (uninoculated)	23.25^{a}	0.00^{e}	5.75a	8.00a	0.54a	0.45a
2 mM KNO3	26.25^{a}	72.00^{b}	6.00a	8.75a	0.85a	0.66a
695 mM KNO3	27.75^{a}	82.75^{a}	7.00a	12.25a	0.94a	0.62a
10 mM KNO3	27.00a	32.50^{d}	7.25a	10.50a	0.83a	0.50a
1% NaCl	25.25^{a}	63.00^{c}	7.25a	10.25a	0.80a	0.63a
2% NaCl	25.75^{a}	26.75^{d}	6.75a	10.00a	0.74a	0.52a
3% NaCl	24.75^{a}	0.75^{e}	7.00a	8.75a	0.87a	0.53a
Treatment Mean	25.81^{ns}	42.84**	6.72^{ns}	10.06^{ns}	0.80^{ns}	0.57^{ns}

Means having the same letter superscripts are not significantly different at .05 level using Tukey's HSD test
*** Mean is highly significant at .01 level*
ns = not significant at .05 level

Table 2.

Nodulation results of single inoculant R. mesoamericanum strains PR-UQ-03 and PR-UQ-05 versus multiple/ combined inoculants to determine synergistic effect of rhizobia.

Treatments	Shoot Length	Number of Nodes	Number Leaflets	Nodule Number	Biomass (g)	Root Dry Weight (g)	Nodule Dry Weight (g)	Total Dry Weight (g)
Combined Strains	29.00[a]	7[a]	16[a]	51[b]	1.41[a]	0.70[a]	0.04[b]	2.15[a]
PR-UQ-03	42.20[a]	6a	13[a]	84a[b]	2.02[a]	0.94[a]	0.16[a]	3.13[a]
PR-UQ-05	35.60[a]	5a	11[a]	143[a]	1.33a	0.76[a]	0.15[a]	2.24[a]
Treatment Mean	36.07[ns]	6ns	13[ns]	96*	1.60[ns]	0.81[ns]	0.12*	2.53[ns]

Means having the same letter superscripts are not significantly different at .05 level using Tukey's HSD test
** Mean is significant at .01 level*
ns = not significant at .05 level

be present in any soil at different sites in Queensland. These unique bacteria established symbiotic nitrogen fixation with Pongamia to different degrees, from very efficient nodules formed in the baiting techniques to less efficient ones. The superior rhizobia were identified as R. mesoamericanum. The establishment of effective Pongamia-rhizobia symbiosis is crucial in the optimization of nitrogen fixation in Pongamia, which greatly depends on the rhizobia's adaptability and ability to form efficient nodulation, leading to an efficient nitrogen-fixation process. This study revealed that there were 95 potential rhizobia in the soil that nodulated Pongamia. This helps prove that Pongamia is a promiscuous legume, based on the wide range of rhizobia that formed nodules during the baiting experiments. The presence of rhizobia nodulating Pongamia is shown in this study to be widely distributed geographically. Across Queensland, rhizobia were found to be present even in those areas where Pongamia was not previously grown. The geographical distribution of rhizobia nodulating Pongamia shown in this study provides hope for people or institutions who may want to grow Pongamia in their agricultural or marginal lands as source of biofuel. Moreover, it was established in the study that these rhizobia are able to grow in soils which are low in nutrients, acidic to basic pH values, and even in high saline environments.

However, it was shown that highly saline soil samples inhibited nodule formation of Pongamia, as in the case of the Darbalara soil samples. Darbalara is a farm in the University of Queensland Gatton Campus where the soils are saline. Irrigation-induced and dryland salinity have become major issues for the Lockyer Valley where Darbalara is located. This alluvial plain is associated with excessive clearing of the uplands and an expansion of irrigation after World War II. Dryland salinity expands during and after wet years when rising water tables get close to the surface. By contrast irrigation salinity is exacerbated during drought years, as water levels, depressed by increased irrigation, encourage the migration of saline waters from the overlying sandstone beds into the alluvium (Queensland Water Resources Commission, 1982). Additionally farmers may draw on deeper aquifers, which are closer to the sandstone and invariably higher in salinity. Salinity has been shown to interfere with nodule initiation in chickpea, cowpea and mung bean, and to also cause a reduction in number, weight as well as nitrogen fixing efficiency of nodules (Balasubramanian and Sinha, 1976). Salinity causes a significant decrease in leghemoglobin content up to 125 decasiemens, the effect decreasing with aging of the nodules probably, because of irreversible oxidation of leghaemoglobin. Tu (1981) observed that inhibition of colonization of the root by the rhizobium strain was the main reason for poor nodulation under NaCl stress. Presence of high salt concentration causes root hairs to shrink. Martin and Ruiz-Torres (1992) found that nitrate accumulation was significantly higher in plants using C3 photosynthesis (barley and wheat) than C4 (maize and sorghum).

Salinity showed a significant inhibitory effect on the nodulation of chickpea even at 4.0 dS m-1. NaCl stress decreases shoot and root dry weight, total number of nodules per plant, nodule weight and average nodule weight (Elsheikh and Wood, 1990; Mudgal, 2004). Although, nodules were observed in inoculated plants grown at 6 dS m-1, nitrogen fixation was completely inhibited. These findings indicate that symbiosis is more NaCl sensitive than both the rhizobium and the host plant.

Another important finding in the baiting experiment using saline soils was the observation of the different zones of nodulation in the baited seedlings using the 5 different soil samples from Darbalara. The soil samples used corresponded to the gradient in the field (S2 least saline, S5, S8, S11 and S13 as highly saline). S2 soil sample contained rhizobia that nodulated the Pongamia seedlings at the early stage as evident in the nodules located close to the base of the roots, whereas, the S5, S8, S11 and S13 nodules tend to form farther from the base of the roots. Early nodulation means that the rhizobia infect the roots immediately after inoculation while delayed nodulation means that rhizobia infect the roots few weeks after inoculation. In early nodulation, the nodules are formed near the base of the roots while in delayed nodulation, the nodules formed towards the lateral roots far from the base of the roots or crown.

Rhizobia were shown to be so physiologically versatile that they were isolated even in those soil types in which other bacteria cannot survive, such as in the case of Meandu Mine soil samples. This finding implies that the

rhizobia can be applied in infertile soils, including areas undergoing rehabilitation in mining sites or unproductive areas, to convert them into productive agricultural land.

The nodule morphology of Pongamia was described as being laterally located in the roots, with shapes ranging from spherical, cylindrical or coralloid, their color being dark brown, reddish/pinkish or brown, and a size ranging from 1 mm to 10 mm in diameter. The nodule anatomy of Pongamia in this study was similar to that described by Samuel et al. (2013). Pongamia nodule exhibited several lobed infection zones surrounded by a thicker layer of cells called the inner nodule cortex. The lobed infection zones were filled by non-infected interstitial cells which were located towards the center of the cortex. Large areas of the cortex consisted of parenchyma cells surrounded by a sclerenchyma layer which separated the outer cortex from the rest of the nodule. Within the cortex were several tannin cells. The vascular traces were dichotomous and branched several times to encapsulate the nodule and were located close to the infected zone within the cortex. This close proximity has been reported previously and is most likely present to increase symbiotic efficiency (Walsh, et al., 1992; Guinel, 2009).

R. mesoamericanum, a fast-grower displayed highest nodule number, shoot length and biomass. The variability test shows that there was no significant variation in the nodulation and growth parameters of all the 50 seedlings which came from one source or mother tree. Thus, the results in all the experiments were sufficient to make the conclusion that a particular treatment is most effective when based on different parameters when using seeds coming from same mother tree. There was no genetic effect or variability noted in the seeds from the same source. Therefore, nodulation can be improved when seeds from best performing tree are used for germination. Nodule number of the 50 seedlings was shown to have no variation.

The time course of nodulation was relevant in filling in the gaps of early Pongamia nodulation. Growth pouches enabled the observation of root development and nodule formation at its earliest stage until 6 weeks. Radicles formed at 5 days, while nodules started to become visible at 2-3 weeks after inoculation. Unlike in soybeans where nodules can be counted after 2 weeks, nodulation in Pongamia tends to be delayed or slower than that of soybeans. New nodules continued to be formed even at 8 weeks after inoculation, while old nodules became coralloid. Pot studies were found to be effective for the study of the time course of nodulation for later stages of growth, since growth pouches cannot support the root mass of Pongamia at 6-8 weeks. Also, the roots tend to go outside the pouches, thereby damaging the setup.

Therefore, growth pouches may be good when observing early root and nodule development in Pongamia but not during its later stages of growth. In addition, nodule number was shown to decrease or be inhibited in the growth pouches setup but not in pots where several nodules were observed. The time course of nodulation in pots showed that there was no significant difference in the data among R. mesoamericanum strains. The size of nodules was less than 1 mm in week 3 and increased up to 5-10 mm in week 8. This is directly proportional to the nodule dry weights.

The leghemoglobin concentration tended to increase from the week that the nodules started to appear but then levelled off in later weeks. This implies that Pongamia nodules started to become less active or less efficient during weeks 3 and 4 as the bacteroids inside the nodules invaded the whole nodule, thereby increasing the leghemoglobin concentration. This finding is consistent with the area of zone of infections computed for the largest nodule found in replicate plants in the week of harvest. Unlike in Bradyrhizobium species cross-sections where it is possible to see many interstitial cells inside the nodules, Pongamia nodules infected with R. mesoamericanum were observed to have been fully occupied by bacteroids (reddish area in cross-sections) and that the area of zone of infections increased over the course of time. The use of a dissecting microscope attached to the computer greatly enhanced the cross-section observations, while with the aid of the software, the area was conveniently obtained based on the highlighted image (the zone of infection inside the nodule can be traced using the mouse cursor and values are generated which can be converted into the actual size of the nodule).

Nitrate and salinity were also found to have effects on nodulation in Pongamia. The addition of nitrate resulted in no significant difference from inoculated plants (without nitrate added), although the mean values of nodule numbers suggested that 2 mM maybe less significant or had no effect on nodulation, as it is close to the mean value of inoculated plants. However, the nodule number at 5 mM nitrate showed an increase, which implies that Pongamia nodulation was not inhibited by the increased nitrate concentration. However, at 10 mM there was a significant decrease in nodule number (to half the values at 2 and 5 mM), which means that the presence of high nitrate concentration of 10 mM can inhibit Pongamia nodulation. In order for Pongamia to have improved nodulation, the nitrate level in the soil or growth medium must be controlled to low amounts such as less than 2 mM – the optimum nitrate level which is best for nodulation. This finding was supported by the study of Eaglesham et al. (1983) which showed that legumes only require low amounts of nitrate for optimum nodulation.

In the case of the salinity test, 1% NaCl did not have significant effect on nodulation but at 2% NaCl nodule number was reduced by 50%, while at 3% NaCl nodulation seemed to be almost completely inhibited. Although these results suggest that Pongamia nodulation is affected by the presence of NaCl, nodule formation, even at 2% NaCl, is proof that rhizobia nodulating Pongamia can still survive at that high saline concentration but not at higher levels (at 3%). Moreover, Pongamia still grew well even at 3% (although with no nodules), which suggests that Pongamia is indeed NaCl tolerant as indicated in the literature.

Nitrate inhibition of nodulation was previously demonstrated by Samuel et al. (2103) in a split-root system but different rhizobia were used (CB1809/USDA110 vs R. mesoamericanum in this study). Also, the results of the former authors showed that at 5 mM nitrate, nodulation

was severely inhibited and at 10 mM nitrate nodules were reduced by 90%. In the current study, 5 mM appears to be a good concentration for Pongamia nodulation, while at 10 mM the nodules were partially inhibited (50%). Therefore, it is apparent that different rhizobia can have different effects on the nodulation of Pongamia at varying nitrate levels.

Lastly, the synergistic effect of combined rhizobia was shown to be not significant or absent in the tested plants. When combined strains was inoculated to baited plants (used to represent synergy), the nodule number and nodule dry weight were significantly lower than that of the single inoculants R. mesoamericanum strains PR-UQ-03 and PR-UQ-05. Therefore, a combination of both fast and slow growing rhizobia has no relevance for Pongamia nodulation. However, the strain symbiotic effectiveness test showed that the fast-grower inoculants to be more effective than slow-growers. The slow-growers replicate at a slower rate, thus they need longer time to reach certain concentration to initiate infection, which is the reason why the fast-growers outcompete them. Also, infected nodules may have lower zones of infection because they tend to invade the nodule at slower rate than the fast-growers. This is the reason why in the strain symbiotic test, plants inoculated with slow-growers tended to have less efficient nodules, while the fast-growers produced better plants more efficiently over the 8-week growth period in the glasshouse.

CONCLUSION AND RECOMMENDATION

Salinity affects nodulation in an inversely proportional relationship, with a decline in nodulation as the NaCl concentration increases. Pongamia is NaCl-tolerant and can still withstand 3% NaCl concentration. R. mesoamericanum, a fast-grower, is considered the superior strain of rhizobia among the 95 isolates from across Queensland. It can survive at 2% NaCl and can still nodulate Pongamia but at a lower rate. Nodules formed within 3-4 weeks after inoculation and continued to increase in size and number over the 8-week growth period, while the area of zone of infection became wider until it fully occupied the whole nodule. Although the leghemoglobin content initially showed an increase over time, it then tended to level off in later weeks. The combination of rhizobia inoculants had no synergistic effect on the nodulation of Pongamia. The results from the study may imply that Pongamia can be grown commercially as biofuel feedstock and its growth can be improved with R. mesoamericanum through the process of biological nitrogen fixation. It is recommended to test legume like Pongamia for NaCl sensitivity on sandy and rich soils and do a soybean comparison. Soil nitrate level must be controlled to a maximum of 5 mM concentration to prevent potential inhibition of nodulation.

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