Investigation of the symbiotic associations of *Acacia ligulata* Benth. and *Acacia tetragonophylla* F.Muell: the potential for use in the rehabilitation of excavated sites at Shark Bay Salt Pty. Ltd.

by

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DECLARATION

I hereby declare that, unless otherwise stated, the work presented in this Thesis is my own and has not been submitted for a degree at any other institution.

Yvette Joan Hill

For Jason and Sophia, you are the smile
in my heart and sunshine in my soul.

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PUBLICATIONS ARISING FROM THESIS

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Hill, Y., Watkin, E., Dixon, K. W. & O'Hara, G. W. (2008). Root-nodule bacteria of arid-zone legumes for use in rehabilitation in the Shark Bay World Heritage Area. In *Biological Nitrogen Fixation: Towards Poverty Alleviation through Sustainable Agriculture*, pp. 97-98. Edited by F. D. Dakora, S. B. M. Chimphango, A. J. Valentine, C. Elmerich & W. E. Newton. Netherlands: Springer.

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ABBREVIATIONS

Acacia s.l.	*Acacia* sensu lato
Acacia s.s.	*Acacia* sensu stricto
ANOVA	analysis of variance
ATR	acid tolerance response
AWC	available water capacity
BSC	biological soil crust
c.	currently
CRS-M1	Centre for Rhizobium Studies-Media 1
dpi	days post inoculation
EC	electrical conductivity
ECM	ectomycorrhizal fungi
Hsps	heat shock proteins
ITS	internal transcribed spacer
LSD	least significant difference
MPN	most probable number
MLSA	multilocus sequence analysis
MYA	million years ago
NCBI	National centre for biotechnology information
NF	Nod factors
OD	optical density
PCR	polymerase chain reaction
PD	provenance distance
RFLP	restriction fragment length polymorphism
RGR	relative growth rate
RNB	root-nodule bacteria
RPM	revolutions per minute
SBS	Shark Bay Salt
SBSLA	Shark Bay Salt Lease Area
SBWHP	Shark Bay World Heritage Property
SIC	soil inorganic carbon

sp.	species
spp.	species (multiple)
SOC	soil organic carbon
T-RFLP	terminal restriction fragment length polymorphism
UPGMA	unweighted pair group with arithmetic mean
VAM	vesicular–arbuscular mycorrhizal fungi
WARMS	Western Australian rangeland monitoring system
WHC	water holding capacity
WGD	whole-genome duplication
YMA	yeast mannitol agar

SUMMARY

The integration of plant available nitrogen (N) into the nutrient cycles of dryland ecosystems is integral to the establishment and persistence of the flora in these regions. Much of this available N is due to the conversion of atmospheric dinitrogen (N_2) by legumes and their bacterial microsymbionts, root nodule bacteria (RNB). There are numerous environmental constraints in dryland areas that impede the growth and interactions of both symbiotic partners. At Shark Bay Salt Pty. Ltd., a solar salt facility in Western Australia, the associations between provenant RNB and the key over-story species *Acacia ligulata* Benth. and *Acacia tetragonophylla* F.Muell. were investigated *in situ* and in glasshouse conditions. This was done to determine whether the selection of provenant RNB that effectively fix nitrogen, and their inoculation onto these two species, could improve plant establishment at degraded pit sites within the Shark Bay Salt lease area (SBSLA).

The effect that mining processes has had on the biological, chemical and physical characteristics of the remaining substrate of selected borrow pit soils was evaluated. The removal of the soil, subsoil and regolith had altered the chemical characteristics of these sites in comparison to adjacent undisturbed areas. This activity had been deleterious to the biota, with no established floral community and reduced populations of RNB that nodulate *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. in the pit areas. There was reduced organic carbon, nitrate and phosphorus concentrations in the pit soils in comparison to the adjacent undisturbed soils and at one pit site, soil salinity was at toxic levels. There were marked differences in the floristic structure and diversity between the different undisturbed sites, with *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. identified at all the selected sites. The RNB in the soils was assessed in 2007 and 2008, years with contrasting annual rainfalls of 79.3 mm and 513.6 mm and it was found that the RNB population increased with the higher rainfall in all pit and undisturbed site soils, with the exception of the toxic saline pit soil where RNB were not detected. In both years, the most probable number (MPN) of RNB that nodulated *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. were reduced in the pit soils compared to the adjacent undisturbed soils.

Provenant isolates of RNB from the soils of SBSLA were collected and assessed for the effectiveness of these RNB isolates as well as Wattle Grow™ in promoting the growth of selected host species in glasshouse conditions for 56 day post inoculation (dpi). Many of the RNB isolated from *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. readily cross-infected these two species and a number of strains also nodulated with *Acacia rostellifera* Benth. and *Templetonia retusa* (Vent.)R.Br.. There was a significant growth response of *A. ligulata* Benth., *A. rostellifera* Benth. and *A. tetragonophylla* F.Muell. to inoculation with a number of the RNB in comparison to uninoculated plants, with some producing foliage weights greater than 100% of the nitrogen-fed control. *A. ligulata* Benth. and *A. rostellifera* Benth. produced significantly increased growth when inoculated with Wattle Grow™ (containing *Bradyrhizobium* spp.). The nitrogen concentrations of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. foliage of selected treatments showed a weakly positive, non-significant relationship when correlated to the plant dry foliage weights of these treatments. While only nine RNB isolates were obtained from nodules collected from *A. ligulata* Benth. plants growing within the SBSLA, 78% produced a significant growth response. In contrast, only 22% of 32 *A. ligulata* Benth. isolates trapped from soil collected from SBSLA produced a significant growth response in comparison to the uninoculated control. This indicates a possible selection pressure and bias when trapping RNB from soils in glasshouse conditions opposed to collecting RNB directly from nodules formed on legumes at the field site.

No RNB symbionts of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. have previously been described and the phenotypic characteristics, phylogenetic relationships and the genetic diversity of 25 SBSLA RNB isolates of these *Acacia* spp. were assessed. The RNB showed tolerance of alkaline, saline and high temperature conditions. All grew at pH 11.0 and the majority tolerated up to 750 mM NaCl. With the exception of two isolates, all grew at 37°C and five isolates were able to grow at 42°C. Based on RPO1-PCR fingerprints, there were indications of considerable genetic diversity among the RNB isolates. The 16s rDNA restriction patterns produced by *Alu*I, *Msp*I and *Sau*3*A*I digestions grouped the isolates into one of six RFLP type groups. On determining the phylogeny of ten of the isolates, the 16s rDNA sequences aligned

within the *Ensifer, Rhizobium* and *Neorhizobium* genera. Eight of the isolates aligned within *Ensifer,* six of which formed a distinct cluster. A multi-locus approach of conserved gene regions would need to be examined to more confidently assess the phylogeny of these RNB.

Based on the effectiveness results, a number of RNB were selected to be re-introduced into selected pit sites in seeding and inoculation trials. Coupled with these trials, different carriers for the RNB were also evaluated to determine their efficacy in relation to the nodulation and growth response of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. in the field conditions. There was increased nodulation of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. plants that had been inoculated. The number of germinated plants and the inoculant treatment indicated no significant relationship. However, seeds inoculated with the peat treatment did generally have a greater number of plants that were growing at the assessment periods compared to the other carriers and uninoculated treatments. This itself is noteworthy as reducing seed loss is one of the major impediments to successful rehabilitation of dryland areas. The nodules on the *Acacia* spp. grown in the pits were occupied by RNB whose RPO1-PCR fingerprints were identical to selected RNB and an additional novel isolate. It was found that inoculation of RNB into the pit soils increased and stabilised the RNB population, with MPN comparable to the population in the surrounding undisturbed soils at 4 months post inoculation.

So as to maintain the provenance of the RNB population in the SBSLA soils and avoid introducing genetic material that could transfer into the resident RNB, Wattle Grow™ could not be included in the seeding and inoculation trials. In a glasshouse experiment, growth tanks containing pit soil were used to compare the nitrogen fixation efficacy and competitive ability of Wattle Grow™ to nodulate *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. against the background RNB in the pit soils and with selected SBSLA isolates over successive sowing periods. No *Bradyrhizobium* spp. were isolated from the nodules of the *Acacia* spp. from any of the treatments over the different sowing periods. The majority of the RPO1-PCR fingerprints of the nodule occupants corresponded to SBSLA isolates and an additional three unique fingerprints were

identified. The occupancy of the nodules of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. subtly changed with each successive sowing. A number of RNB occurred with greater frequency at the different sowing periods, however, there was a trend towards increased diversity of nodule occupants with each successive sowing, particularly of the RNB nodulating *A. tetragonophylla* F.Muell.. There was a difference in the response of the two *Acacia* spp. to the treatments and conditions of the growth tanks. The plant foliage nitrogen concentrations and foliage mass of *A. ligulata* Benth. were negatively correlated. In contrast, the *A. tetragonophylla* F.Muell. foliage nitrogen concentrations were positively correlated to the foliage production of these plants.

The use of provenant RNB shows potential in improving the germination and establishment of selected legume species in the degraded areas within SBSLA. However, it was shown that different growth conditions for *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. alters the symbiotic relationships, nitrogen fixation and growth response of these plants. This illustrates the caution to be exercised when screening for effective symbionts of legumes for the purpose of rehabilitation.

Chapter 1 Literature review

From Little Things Big Things Grow

-Paul Kelly & The Messengers “Comedy” 1991

There are currently 1007 properties listed as World Heritage Properties and include places such as the Great Barrier Reef (Australia), Ancient Thebes (Egypt), Hiroshima Peace Memorial (Japan) and the Cape Floral Region (South Africa) (UNESCO, 2015). A site must be considered of outstanding universal cultural and/or natural value to be included on the World Heritage List (UNESCO, 2015). The Shark Bay area was listed in 1991 due to recognition of the great geological, botanical and zoological importance of the area (UNESCO, 2002). The Shark Bay World Heritage Property (SBWHP) in Western Australia covers an area of approximately 2.2 million hectares of land and water and within the SBWHP are several pastoral leases and the two exclusion zones of the township Denham and the Shark Bay Salt lease area (SBSLA) (Figure 1.1). Denham (-25.9247°, 113.53642°) is somewhat central to the SBWHP and approximately 276 km south of the Tropic of Capricorn (-23.43778°).

The Shark Bay area includes rare, endemic, threatened, little known and undescribed plant species in the major transition zone of the South West and Eremaean botanical provinces that are dominated by *Eucalyptus* and *Acacia* species, respectively (CALM, 2005). Many of the perennial species of the area are typically slow growing due largely to rainfall and nutritional constraints (Beadle, 1964). However, many Australian flora are adept at obtaining nutrients through soil microbial associations such as those with mycorrhizal fungi or nitrogen-fixing bacteria (Beadle, 1964; Bell *et al.*, 2003; Brockwell *et al.*, 2005; Brundrett & Abbott, 1991; Jasper *et al.*, 1988; Reddell & Milnes, 1992).

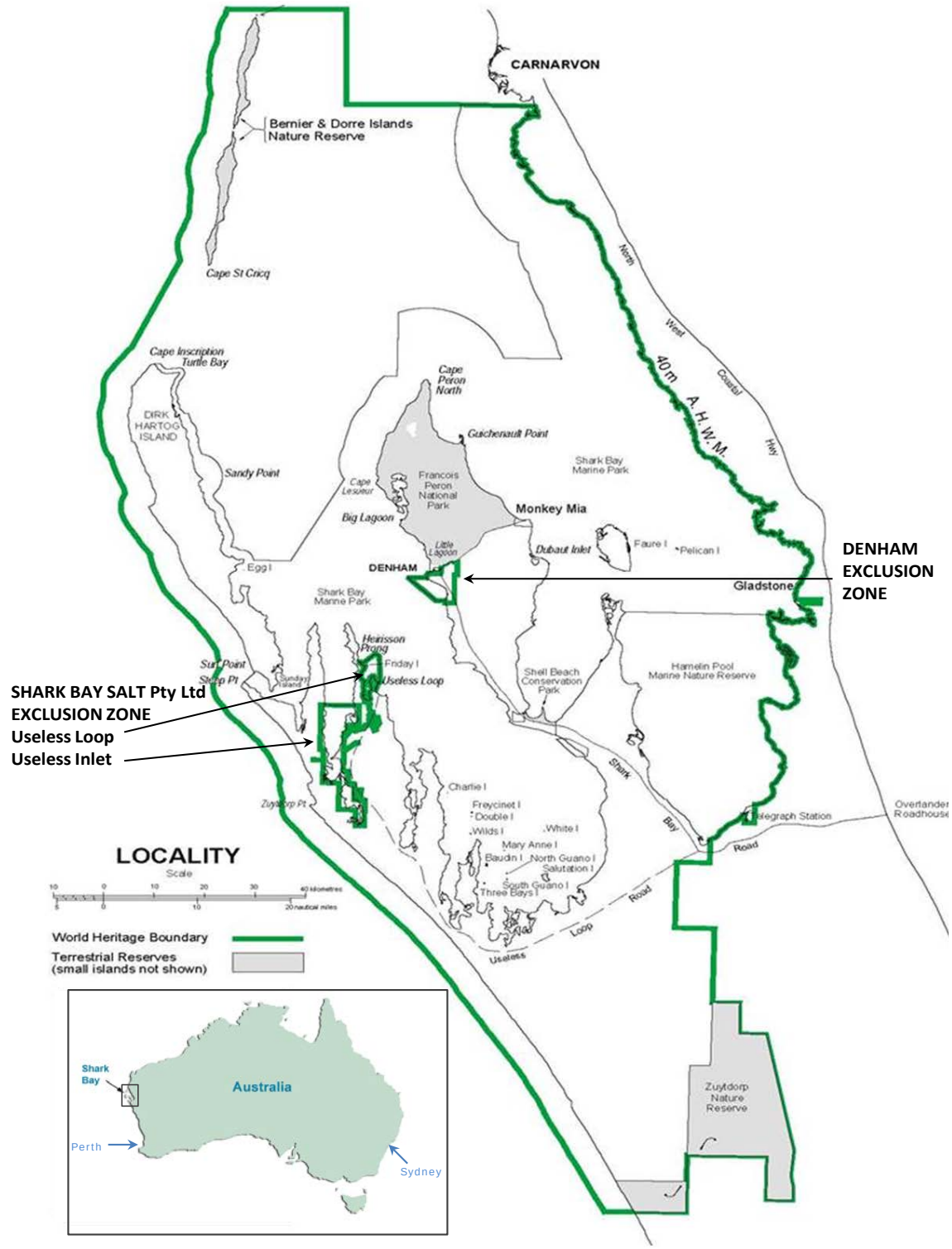


Figure 1.1: The boundary of the Shark Bay World Heritage Property. The two exclusion zones within the property boundary are the township Denham and the Useless Loop and Useless Inlet lease area of Shark Bay Salt Pty Ltd. Pastoral leases within the property boundary are not shown. The insert of Australia shows the locality of the Shark Bay area. Adapted from EPA (1991).

The soils of Australia are ancient and processes over millennia that chemically bind and leach essential nutrients have left the soils depleted (Lambers *et al.*, 2006; Nix, 1981). The SBSLA includes Useless Loop and Useless Inlet which is located within the geological subdivision of the Gascoyne Platform in Western Australia (Flint & Abeysinghe, 2000). This area is composed of Phanerozoic sedimentary rocks of Ordovician to Devonian strata of 450-380 million years ago (MYA) overlain by Mesozoic and younger formations (145-65 MYA) (Flint & Abeysinghe, 2000). More specifically, Useless Loop and Useless Inlet are within the Edel region which is characterised by Tamala limestone over which calcareous dunes have been deposited (Beard, 1976). Outcrops of the limestone are apparent at numerous sites and the soils of this limestone vary from brown and calcareous sands to lithosols and calcareous loams (CALM, 2005).

In general, the Shark Bay area is subject to temperate and semi-desert climate patterns with rainfall of between 190 and 250 mm per annum, predominately in winter (Pringle *et al.*, 2006). With low rainfall, high evaporation rates and permeable soils, there are very few areas of permanent surface water (CALM, 2005). However, the soils retain moisture due to the presence of fine soil particles (<0.212 mm) (EPA, 2005) and biological soil crusts (Eldridge & Greene, 1994; Eldridge, 2003).

1.2 Disturbance and rehabilitation of Useless Loop

Shark Bay Salt Pty Ltd has been operational as a salt mining company since 1965 and the lease area includes Useless Loop and Useless Inlet which are two of six distinct narrow inlet habitat types only found in the Edel land province of the Shark Bay region (EPA, 1991). The construction of the crystallization ponds and associated salt production infrastructure has irrevocably changed the area of Useless Loop and 34% of Useless inlet has been altered by the construction of the primary concentration ponds (EPA, 1991). The Shark Bay Salt Pty Ltd legal requirements in operation include: i) the *Shark Bay Solar Salt Industry Agreement Act 1983*, in which the project area is defined; ii) the *Environment Protection Act 1986* under Licence 7184/8; iii) the *Wildlife*

Conservation Act 1950; and iv) the federal *Environmental Protection and Biodiversity Act 1999* (SBSJV, 2005).

During the construction of the crystallization ponds and associated infrastructure, small quarries or 'borrow pits' were excavated in the project area. A number of these have been decommissioned for up to 25 years and many remain in a highly degraded state with little or no plant recruitment from the surrounding undisturbed vegetation. There was no requirement of Shark Bay Salt Pty Ltd to rehabilitate the pits prior to the *Shark Bay Solar Salt Industry Agreement Act 1983*, and the company now attempts rehabilitation in both more recent and historically decommissioned pit sites. Since 1983, the standard practice was to immediately restore active pits by blending the pit topography to the surrounding area and the replacement of stored top soil which has resulted in some re-vegetation (SBSJV, 1993). Top soil replacement was not possible at older pit sites and rehabilitation efforts have ranged from mulching with prunings collected from the Useless Loop town site to ripping, fertilising and seeding both with local seed and purchased non-provenance species (SBSJV, 1993). It was recognized that the practice of mulching had led to the introduction of unwanted weed species in the pits and previous attempts at seeding have had low germination and survival rates with a 'plateau vegetation' being reached which is not representative of the surrounding density or diversity of flora (SBSJV, 2005).

1.3 Climate classification of Australian drylands

Defining an area with the terms 'dryland' or 'arid zone' has historically been influenced by anthropogenic utilisation and implies a delineation of climate (Thomas, 2011). These areas are diverse and heterogenous in climate and geology which in turn affects the landscape formations, vegetation and soil characteristics (Dregne, 1976; Thomas, 2011). The *World Atlas of Desertification* (UNEP, 1992) define dryland zones as those with a aridity index (AI) classification of less than 0.65. The AI is determined by the equation: $AI = P/PET$ where P is precipitation and PET is the potential evapotranspiration, calculated from eteorological data. There a four dryland sub-types: hyper-arid deserts (<0.5 AI), arid (0.05–0.20 AI), semi-arid (0.20–0.50 AI) and dry sub-

humid (0.50–0.65 AI) (UN, 2011). On this basis, approximately 75% of Australia's continental area is classified as dryland (Thomas, 2011) , composed of arid, semi-arid and dry-subhumid areas (Figure 1.2).

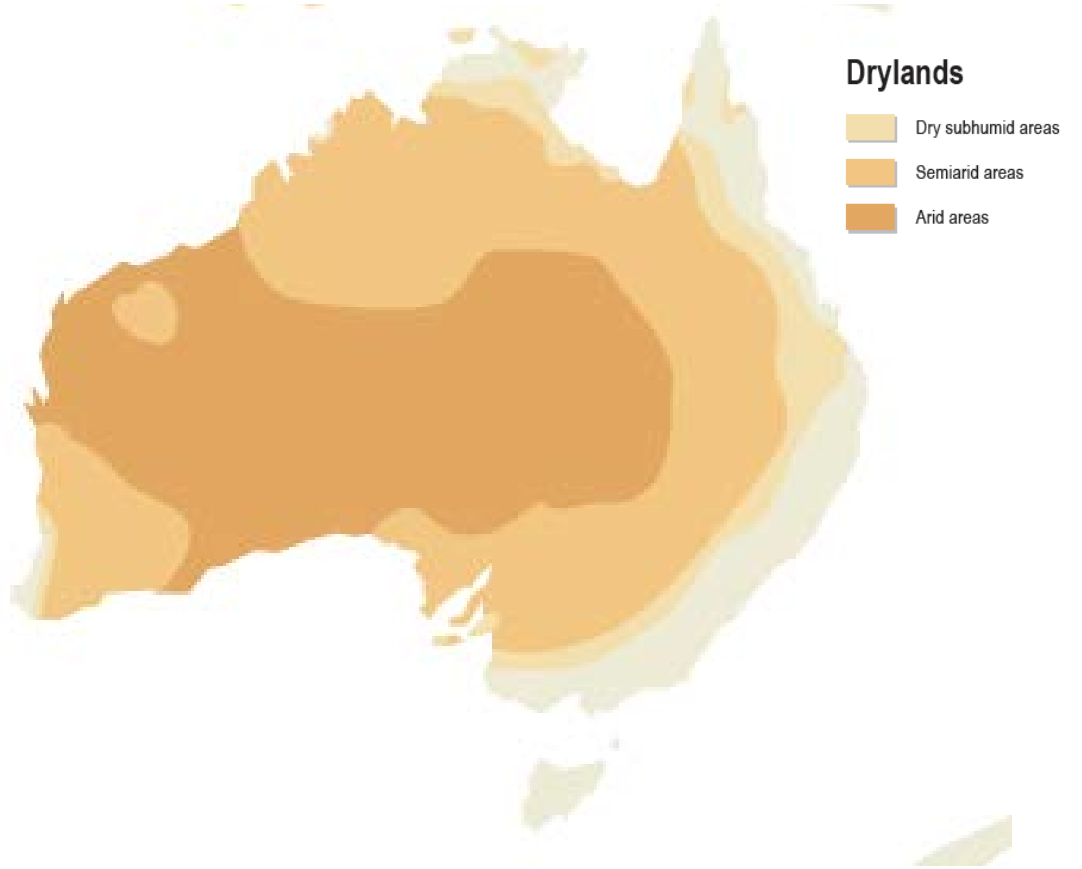


Figure 1.2: Map of Australian drylands and the areas of the dryland sub-types. There are no hyperarid areas within Australia. The pale grey represents other non-dryland climate regions. Adapted from UN (2011).

1.4 Dryland disturbance ecology

A loss or degradation of plant communities are the visible sign of disturbance in dryland areas, often leading to erosion and desertification (Caravaca *et al.*, 2003; Requena *et al.*, 2001). The possible adverse consequences of disturbance to drylands are altered soil characteristics such as chemistry, permeability, nutrient availability and microbial activity that are difficult to ameliorate (Bentham *et al.*, 1992; Hobbs, 1999; Requena *et al.*, 2001). These biotic interactions and abiotic limitations in ecosystems

have the capacity to exist in a number of different states of function and thresholds (Hobbs & Harris, 2001). In order to return to a less degraded state, an ecosystem disturbed to a threshold state requires the input of resources and management, which can be specific to each ecosystem (Hobbs & Cramer, 2008). It is therefore critical to understand the disturbance processes involved and their effects on the soil abiotic and biotic characteristics to assess restoration efforts (Harris, 2003; Murray *et al.*, 2001; Thrall *et al.*, 2001). To merely measure the return of vegetative cover may present a misleading indicator when evaluating the success or failure of dryland restoration (Bentham *et al.*, 1992; EPA, 2006; Harris, 2003). As well as the diversity, abundance and distribution of vegetation, other dryland restoration indicators should include assessing the landscape patterns (i.e. water retention and topography) and comparing indicators (i.e. presence of animals for seed dispersal and the soil microbial community) to a self-sustaining representative ecosystem (Aronson *et al.*, 1993; Ludwig *et al.*, 1997; Pringle *et al.*, 2006).

Typically, the objectives are to restore a disturbed site as close as possible to the original condition along with intact environmental, heritage and conservation values (Gardner, 2001; Mitchell & Wilcox, 1994). This often requires implementation of management strategies and intercession techniques to obtain an ecosystem with original or similar ecological functioning (Figure 1.3). In dryland restoration, it is rarely possible to achieve these same objectives and rates of successful dryland restoration have remained low (Carrick & Kruger, 2007; James *et al.*, 2013; Mitchell & Wilcox, 1994). Often the tendency is to complete only the initial intercession stages, with the expectation that the ecosystem will then equilibrate with time (Herrera *et al.*, 1993).

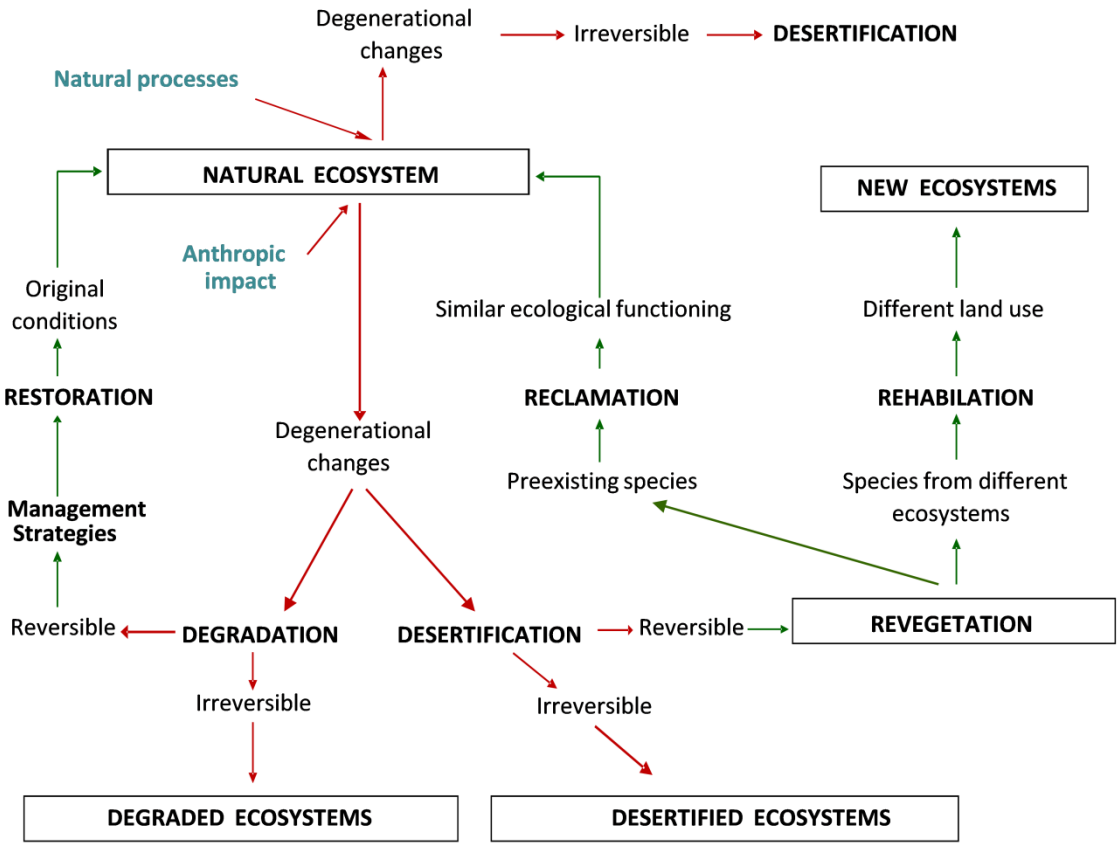


Figure 1.3: Effects of anthropogenic activities on the degradation or desertification of an ecosystem and the different management strategies leading to restoration, reclamation or rehabilitation. Adapted from Herrera *et al.* (1993).

'Standard Practice' rehabilitation techniques in Australia include top soil replacement, deep ripping and sowing seed of provenant species (ALCOA, 2003; Gardner, 2001; Mulligan *et al.*, 2006). However, where no successional recovery of the vegetation has occurred the success of these techniques is often limited (Carrick & Kruger, 2007; DMEWA, 1996). The construction of windbreaks and the establishment of nitrogen-fixing legumes for the improvement of soil fertility and creating a microclimate for the establishment of other herbaceous species are some of the measures that have been beneficial in dryland restoration (Caravaca *et al.*, 2003; Carrick & Kruger, 2007; Requena *et al.*, 2001; Zhao *et al.*, 2007). In some Australian restoration projects, the reintroduction of legumes, particularly *Acacia* spp. and inoculation with beneficial microbes such as root nodule bacteria (RNB) has had demonstrated ecological benefits (Barnet *et al.*, 1985; Brockwell *et al.*, 2005; Thrall *et al.*, 2001). However, these restoration projects and other reports of mine rehabilitation focus largely on areas in

temperate Australia (ALCOA, 2003; Bell, 2001; Gardner & Bell, 2007). There is some reporting of restoration strategies in the semi-arid and arid zones of Australia, which have met with variable success (Bell *et al.*, 2003; Bell, 2001; Gwenzi *et al.*, 2014; Li *et al.*, 2014; Ludwig & Tongway, 1996). Many highlight the need for new dryland restoration practices dependant on the disturbance processes and other site-specific factors. The impediments to rehabilitate disturbed dryland sites are not restricted to Shark Bay Salt Pty Ltd, with 202 mines operational in Western Australia as of January 2014 (GSA, 2014), located predominately in the dryland areas of the state (Figure 1.4). Each of these mines operate under legislation and caveats requiring rehabilitation of the lease areas during and/or at mine closure (DMP, 2014). The status of rehabilitation success or failure or the procedures adopted by many of these mines operating in the northern areas of Western Australia is largely unknown.

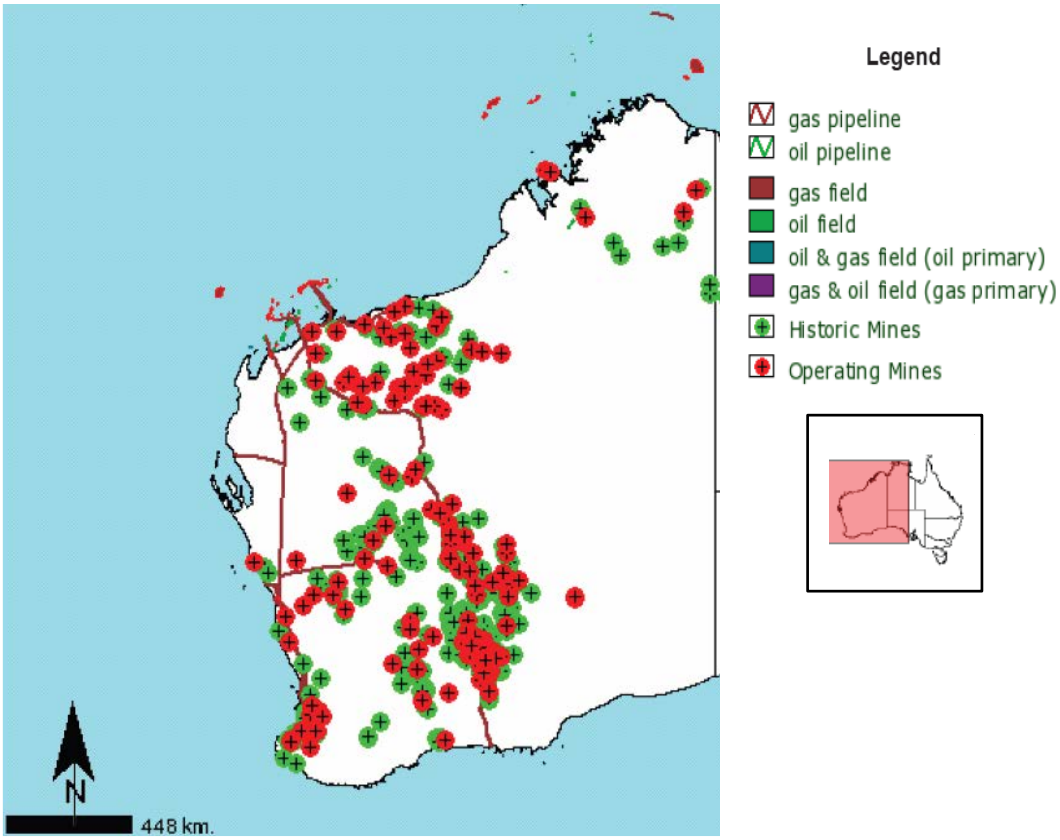


Figure 1.4: The operating and historic mine locations in Western Australia (including gas and oil operations) as of January 2014. Map adapted from Geoscience Australia (GSA, 2014).

1.4.1 Impacts of climate change for Australian drylands

1.4.1.1 Rainfall and temperature: trends and predictions

Globally, the average surface temperature has increased by 0.6-0.7°C between 1951 to 2010 and there are further projected increases of between 0.3-0.7°C by 2035 with heat waves of higher frequency and longer duration likely (IPCC, 2013). Variables such as topography affect the degree of climate response in many global regions. However, Australia's climate is strongly influenced by the surrounding oceans rather than topography and the temperatures of the oceans of south-western and south-eastern Australia have risen up to 0.7°C since 1910 (IPCC, 2013). Some of the expected climate changes are of altered storm and rainfall regimes with increased intensity of existing climatic events and significant decreases in rainfall over large areas of the Australian continent (IPCC, 2013).

While the annual rainfall across Australia has increased, this is largely due to a change in seasonality and intensity (Hughes, 2003) and restricted to certain regions (Figure 1.5). Over recent decades, there has been decreased winter rainfall over the southern parts of Australia, with long term reductions experienced by the South West of Western Australia (CSIRO, 2012; Hughes, 2003). In the South West of Western Australia the winter rainfall has decreased by greater than 25% and there have been significant decreases in summer rainfall events (Hennessy *et al.*, 1999). This trend extends from the South West up the coastal area into the Gascoyne region of Western Australia, which includes Shark Bay and the surrounding area (Figure 1.5). The annual temperatures across the majority of Australia have increased between 0.05 to 0.30°C from 1970 to 2013 (Figure 1.6). Temperatures are expected to continue to rise by 1 to 5°C by 2070 (CSIRO, 2012), with the Australian interior projected to increase by 4.6 to 5.2°C (Washington & Thomas, 2011).

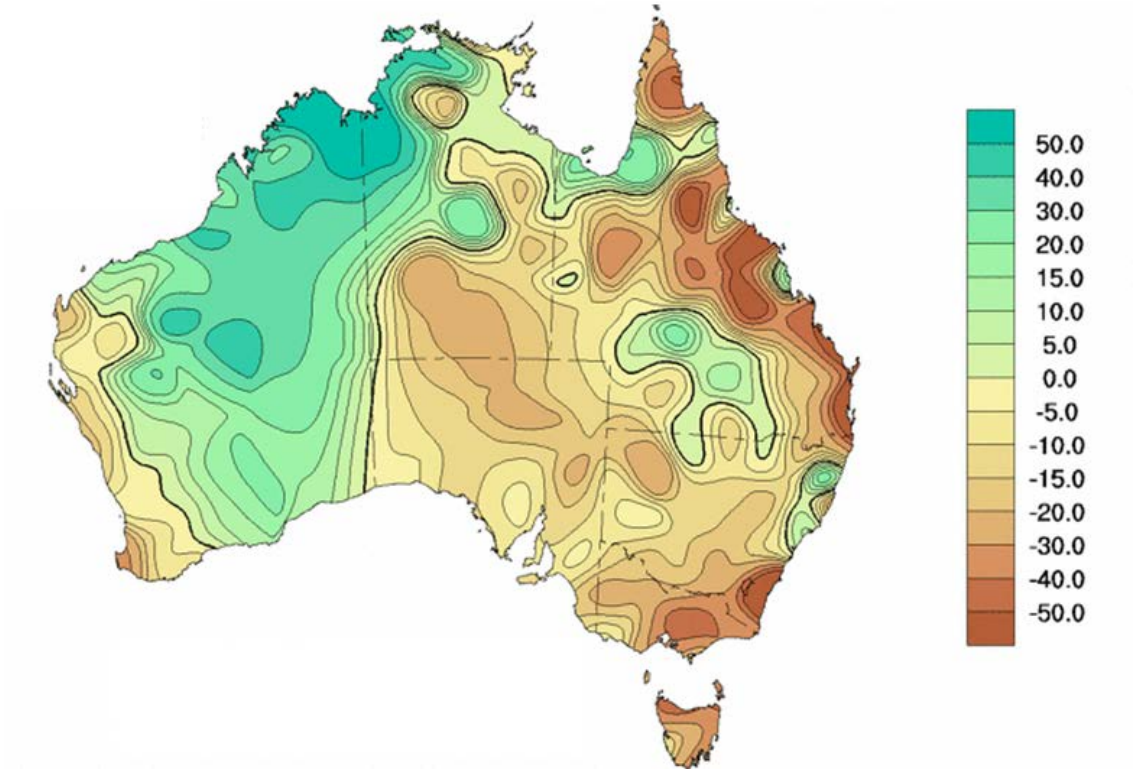


Figure 1.5: Changing annual rainfall in Australia, the trend from 1970 to 2013 (mm/10year) (ABM, 2014b) .

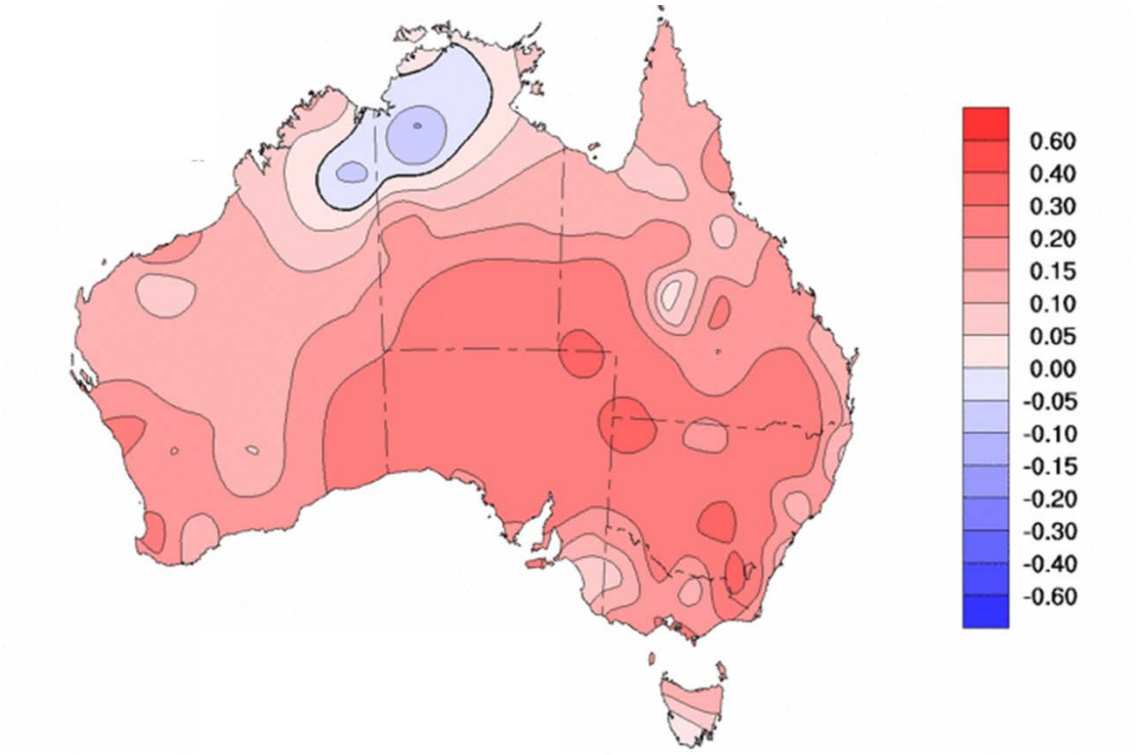


Figure 1.6: Changing **a**nnual temperature in Australia, the trend from 1970 to 2013 (°C/10year) (ABM, 2014a).

1.4.1.2 Vegetation

The IPCC (2001) reports the potential impacts of climate change include 'alteration in soil characteristics, water and nutrient cycling, plant productivity, species interactions, and ecosystem composition and function'. While many of Australia's ecosystems tolerate climatic variability, this is usually on short time scales of up to a few years and this tolerance may not extend into the long term (Walther *et al.*, 2002). It is likely that the changes in temperature and rainfall across the different biomes will alter fluvial systems (Washington & Thomas, 2011) and the borders of vegetation associations between different ecosystems (Lavee *et al.*, 1998).

Anthropogenic activities have led to increased atmospheric carbon dioxide (CO_2), which enhances plant growth in optimal conditions. In recent decades, there has been an 11% increase in vegetation globally which is supported by predictions made based on gas exchange theory and the 14% increase in atmospheric CO_2 during 1982 to 2010 (Donohue *et al.*, 2013). Superficially, this global trend in increased vegetation may appear to be a positive change. However, indications are that many arid and semi-arid regions are subject to changes which in the longer term may be adverse, such as short periods of greening (of the vegetation) followed by lengthening periods of browning (de Jong *et al.*, 2012). This trend of abrupt changes in the greening and browning of the vegetation in recent decades is particularly evident in the southern hemisphere arid and semi-arid regions of South America, South Africa and the majority of dryland Australia (Figure 1.7).

The response of vegetation to elevated CO_2 is dependent on whether plants operate C_3 or C_4 photosynthetic pathways, this combined with the abrupt changes in periods of greening and browning in arid and semi-arid regions is a likely cause for the composition of vegetation being modified in these regions. Globally, dryland regions have experienced increases in woody C_3 vegetation compared to herbaceous C_4 vegetation (Andela *et al.*, 2013; Donohue *et al.*, 2013), with legumes and C_4 plants particularly sensitive to the combination of heat stress and elevated CO_2 which results in significantly reduced below ground and plant biomass (Wang *et al.*, 2012). Along with heat stress, the impact of increased CO_2 on vegetation growth is also dependent

on factors such as water and nutrient availability and changing fire regimes (Andela *et al.*, 2013; Hughes, 2003).

Factors such as coastline, fragmentation or landscape isocline contribute to a lack of migration opportunities and Australian ecosystems may suffer high levels of biodiversity loss as a result (IPCC, 2001). Climate change modelling with 100 *Banksia* spp. of Western Australia show indications that the distribution of 66% of these species will decline (Fitzpatrick *et al.*, 2008). With *Acacia* spp. dominant across much of Australia's drylands, BIOCLIM modelling predicted distribution to be reduced by greater than 75% for the 27 species assessed (Hughes, 2003; Pouliquen-Young & Newman, 2000). These climate change impacts of elevated CO_2, temperature and precipitation are potentially devastating and are likely to contribute significant additional pressure to future dryland restoration efforts.

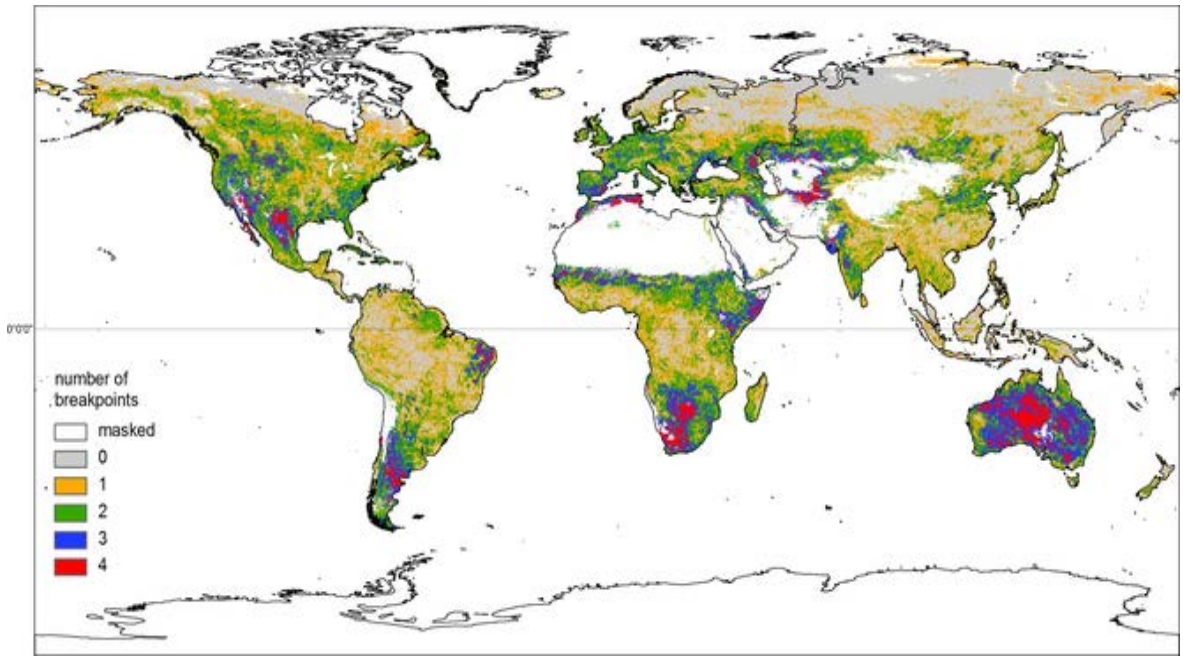


Figure 1.7: Trend changes in global greening and browning. The colours indicate the number of detected abrupt changes, irrespective of the magnitude of the changes. Areas with a yearly mean normalized difference vegetation index < 0.10 were masked out (de Jong *et al.*, 2012).

1.4.1.3 Soil microbial communities

With the observed and expected changes in vegetation (Figure 1.7), climate change factors such as increased CO_2, altered precipitation (Figure 1.5) and elevated temperatures (Figure 1.5) already seen over much of dryland Australia, it is highly likely that the soil microbial communities have altered in response. The studies of the microbial ecosystems of Australian soils have largely focused on those related to cropping or pastoral lands (Date, 1983; Martin, 1983), either broadly (Pankhurst *et al.*, 1996; Wakelin *et al.*, 2008), or for specific beneficial microbes such as rhizobia (Bowen, 1956; Brockwell, 2001; Howieson & O'Hara, 2008; Lange, 1961) and mycorrizal fungi (Abbott & Robson, 1977; Brundrett *et al.*, 1996).

However, to date there has been little focus of climate change effects on microbial diversity or abundance when compared to plant, animal and edaphic characteristics in different ecosystems. Climate change factors can contribute to soil aridity, which in the long term would lead to decreased total Carbon (C), Nitrogen (N) and microbial biomass in the soil (Graaff *et al.*, 2014; Smith *et al.*, 2002). The structures of both plant and microbial communities are likely to be altered by such changes to C and N cycling in different ecosystems.

Microbial communities appear to be more sensitive to temperature and precipitation changes leading to altered communities in arid and semi-arid shrubland ecosystems of North America and the Mediterranean oak forests of California (Graaff *et al.*, 2014; Waldrop & Firestone, 2006). Both Graaff *et al.* (2014) and Waldrop & Firestone (2006) found that the composition and function of undercanopy microbial communities rapidly altered with increased temperature and reduced soil moisture. At the National Ecological Research Park, Oak Ridge, Tennessee where a range of C_3 and C_4 grasses, shrubs and herbs were grown, both Castro *et al.* (2010) and Gray (2011) reported increased abundance and diversity of the soil microbial community with increased temperature and reduced soil moisture. While this may seem positive superficially, analysis revealed that gram negative bacteria and some fungal groups such as arbuscular mycorrhiza and saprophytes were significantly decreased leading to an

altered composition and function of their respective microbial ecosystems (Castro *et al.*, 2010; Gray *et al.*, 2011).

There was a similar effect of altered composition and microbial ecosystem function in a ten-year study of a grassland ecosystem at BioCON experimental site in Minnesota, with the availability of N and the complexity of plant species (1, 4, 9 or 16) strongly influencing the microbial response to elevated CO_2 (He *et al.*, 2012). In contrast however, He *et al.* (2012) reported significantly reduced soil microbial abundance and taxonomic diversity, when exposed to elevated CO_2 at 560 ppm in comparison to the ambient level of the time at 368 ppm. It may be that the response of soil microbial systems is also dependant on the floristic complexity and biome.

1.4.2 Australian dryland soils: edaphic characteristics and mineral nutrition

An organism's nutritional requirements may differ depending on the ecological niche occupied (Ehrlich *et al.*, 1977). Broadly, chemical requirements of life are carbon, oxygen (O), hydrogen (H), primary mineral nutrients of nitrogen, phosphorus (P), potassium (K), secondary nutrients of calcium (Ca), sulphur (S), magnesium (Mg) and micro nutrients of boron (B), chloride (Cl^-), manganese (Mn), iron (Fe), zinc (Zn), copper (Cu), molybdenum (Mo), cobolt (Co) and nickel (Ni) (Ehrlich *et al.*, 1977). The absence, presence and availability of these chemicals is largely determined by the environmental conditions of a region and the edaphic characteristics of the soil (Dregne, 1976).

While the parent rock type affects the production and the nutrient content of soil, in drylands it is secondary to the affects of climatic weathering processes, topography, vegetation and time (Dregne, 1976; Tiller, 1983). The majority of Australia's dryland soils are classified as Tenosols, Rudosols and Kandosols with large tracts of Calcarosols, Vertosols and Sodosols in dryland Southern, North Eastern and Western Australia respectively (Figure 1.8). With Australia's ancient landscapes and weathering, the deep

sands of numerous dryland areas are prone to excessive drainage, resulting in the gross deficiencies of many mineral nutrient elements (Hubble *et al.*, 1983).

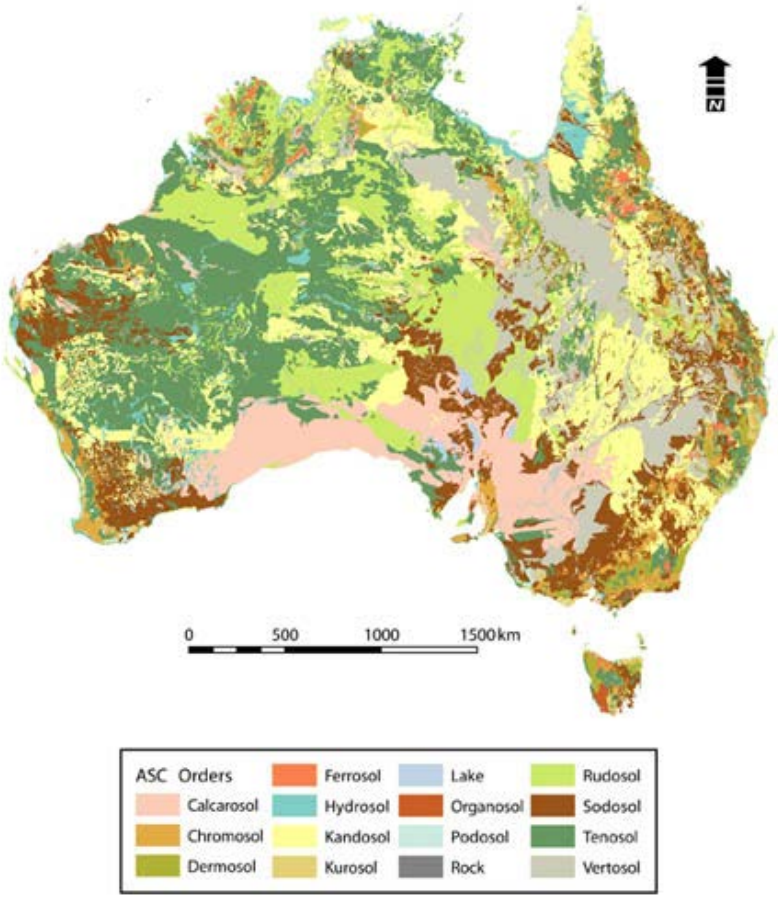


Figure 1.8: Atlas of Australian Soils based on the Australian Soil Classification (Ashton & McKenzie, 2001).

In the majority of arid soils, N availability is limited (Dregne, 1976; Pointing & Belnap, 2012) and most Australian soils are characteristically low in N and P (Nix, 1981) with a correlation between soil organic carbon (SOC) and N (Spain *et al.*, 1983). The N levels in Australia's dryland surface soils are predominately less than 0.1% (w/w) (Spain *et al.*, 1983) and the most abundant form of soluble N in many drylands is nitrate due to mineralization and nitrification at the high temperatures of these regions (Lambers *et al.*, 1998; Specht & Specht, 1999). It is estimated that 75% of Australia's surface soils contain less than 1% (w/w) SOC (Spain *et al.*, 1983). Soil C in dryland areas is heterogeneous and often in low levels (Pointing & Belnap, 2012). Dryland SOC concentrations are usually less than 0.5% (w/w) and soil inorganic carbon (SIC) is in the form of carbonates (Lal, 2004). The distribution of SOC is largely due to the patchiness

of the vegetation, soil texture, topography and by soil deposition or erosion of landforms (Lal, 2004). In dryland Australia, the low soil P levels are directly related to the P levels of the parent rock or due to formation from previously leached material (Beadle, 1962). Many dryland soils have a high limestone content and being alkaline, the P anion may be found in the form of various calcium phosphates (Kooijman *et al.*, 1998) or bound with common cations (Ca^{2+}, Al^{3+} and Fe^{3+}) to form insoluble salts (Davet, 2004; Richardson, 2001).

Globally, the soils of dryland regions with less than 250 mm annual rainfall are generally neutral to alkaline (Dregne, 1976). In the sandy soils of dryland Australia, many mineral nutrients are associated with organic and oxide surfaces and form complexes with a range of ligands (Tiller, 1983). Soil pH principally determines the availability of nutrients with iron, manganese, copper and zinc ions being significantly reduced with increased alkalinity (Lambers *et al.*, 1998). From a national Australian survey of 1186 sites by de Caritat (2011) of surface (0-0.10 m) soils of floodplains, the median pH was 6.5 with the majority of the dryland sites between pH 6 and 10 (Figure 1.9).

Dryland salinity is a great threat to agricultural areas, particularly in arid and semi-arid areas (Raza *et al.*, 2001; Zahran, 1999). Abundant amounts of carbonates, sulphates and chlorides are found in the soils of many dryland regions (Hubble *et al.*, 1983). The soils of much of southern Australia are derived from parent material with high concentrations of salt, which can remain in the surface and subsurface soils where the annual rainfall is insufficient to leach the soils of the accumulated salts (Hubble *et al.*, 1983). Reduced or loss of vegetative cover, increases groundwater recharge, flushing dissolved salts into waterways or low lying areas (Bennett *et al.*, 2003). This can lead to detrimental effects on agricultural ecosystems (Bennett *et al.*, 2003; Zahran, 1999) and likely to negatively affect any remnant native vegetation. In the exploration of mineral resources, drill line disturbance and sump holes have led to salinity contamination. Osborne & Brearley (1999) reported electrical conductivity (EC) levels of 7.88 to 32.97 dS/m at exploration sites compared to 0.82 dS/m at uncontaminated sites in the Kalgoorlie region of Western Australia. Restoration of these exploration sites

decreased salinity as the percentage of plant cover increased (Osborne & Brearley, 1999). Maintaining native and deep-rooted perennial plant cover in an area contributes to reduced groundwater recharge and sustains the hydrology of the region.

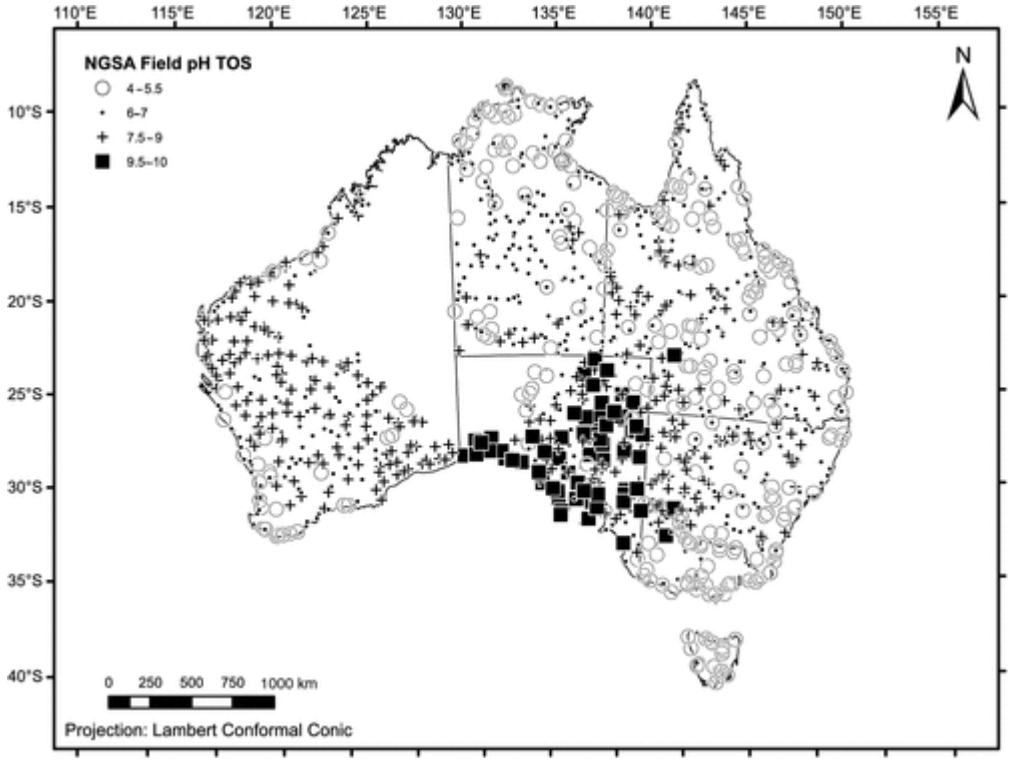


Figure 1.9: The field pH of topsoil (0–0.10 m depth) measured from floodplain soils (de Caritat *et al.*, 2011).

1.4.3 Provenance of flora species

The depletion or loss of a seed bank resource is one of the major limiting factors to restoration of long term degraded areas such as mine sites or pastoral lands where extensive clearing or grazing has occurred (Waters *et al.*, 1997). The use of provenance seed is considered the best practice approach to native plant rehabilitation (Bussell *et al.*, 2006) and is specified by Australian state and commonwealth legislation such as the *Environmental Protection Act 1986, Environment Protection and Biodiversity Conservation Act 1999*, *Mining Act 1978* and the *Conservation and Land Management Act 1984* (EPA, 2006). In order to apply the use of provenance, there must be some understanding of what is 'local' seed and the genetic variability amongst the populations of species in an area (Broadhurst & Young, 2007; Bussell *et al.*, 2006).

Basic guidelines in seed collection delineate regions of provenance, which include biological, ecological or climatic criteria and geographical proximity (Mortlock, 2000). These guidelines are often difficult to adhere to (Krauss & Koch, 2004) and may not reflect the genetic composition for the populations of individual species (Krauss *et al.*, 2013; Vander Mijnsbrugge *et al.*, 2010).

Plants are evolved to suit environmental conditions, which in conjunction with other flora and fauna, form a genetic and ecological sustainable system. The use of provenance seed for restoration seeks to avoid outbreeding depression by the introduction of genes that may result in the loss of the locally evolved genotypes (Lesica & Allendorf, 1999; Mortlock, 2000; Vander Mijnsbrugge *et al.*, 2010). Species or populations that are adapted to local conditions exhibit genetic fitness and resilience, enabling a response to stressful or changing environments (SERISPWG, 2004). In conjunction with the traditional determinates of provenance, genetic markers are becoming a necessary tool in the delineation of plant provenance (Bussell *et al.*, 2006; Krauss & Koch, 2004). Genetic markers are also useful for determining the threat to remnant populations by hybridisation which occurs readily among important plant groups of Australian ecosystems, such as *Eucalyptus* (Broadhurst & Young, 2007) and *Acacia* (Chapman & Maslin, 1992).

Provenance distance (PD) is the delineation of an area from which genetic exchange can occur and the genetic distinctiveness of the species population is maintained (Krauss *et al.*, 2013). A number of genetic studies of Australian native flora have shown a large variation of the PD of different species. Krauss & Koch (2004) determined the multi-locus DNA genetic profiles of *Dryandra lindleyana, Lomandra hermaphrodita* and *Bossiaea ornate* and showed that the minimum PD was a radius of 10 km, 6.5 km and 7.5 km. Whereas, for *Banksia menziesii,* a PD of 30 km was suggested by the genetic profile analysis of 24 widely distributed populations of the species (Krauss *et al.*, 2013), the seed collection boundaries of 20 km adhered to by Alcoa World Alumina Australia and Worsley Alumina Pty Ltd for each revegetation site in Western Australia may be too large for some species (Krauss *et al.*, 2005). Species with broad geographic distribution may have significant differences in genetic diversity, such as taxa within

the complex of *Acacia acuminata* Benth. (Broadhurst & Coates, 2002). The implications of PD of different species is another obstacle to restoring landscapes, particularly those whose disturbed soils are markedly different to those in which the natives pre-existed (Lesica & Allendorf, 1999) or where 'local' seed is unavailable.

1.5 Leguminosae

The Leguminosae is one of the largest families of flowering plants with cosmopolitan distribution throughout all continents with the exception of Antarctica, and is divided into three sub-families, the Caesalpinioideae (tropical or subtropical trees and shrubs), Mimosoideae (tropical, subtropical or arid trees and shrubs), and Papilionoideae (herbs, trees and shrubs with widespread distribution) (Allen & Allen, 1981; ILDIS, 2007). Currently, there is approximatley 19,400 species of legumes classified into 751 genera (KRBP, 2014).

Legumes date back approximately 60 MYA, with supporting legume fossils dating to 56 MYA in the Late Paleocene (Lavin *et al.*, 2005). Diversification of the sub-families occurred shortly after with the Papilionoid clade dating from 56-45 MYA, followed by the Caesalpinoid clade at 54-30 MYA and the Mimosoids more recently at 40 MYA (Lavin *et al.*, 2005). The legumes evolved and diversified alongside N_2-fixing or diazotrophic soil bacteria with root nodulation evident at approximately 58 MYA (Sprent, 2007). Theories regarding the evolution and distribution of legumes have ranged from a Gondwana or Laurasian origin to current distribution being related more to factors such as rainfall and temperature (Sprent, 2001).

Nodulation is restricted to families within the Eurosid 1 clade, though it is predominant in the Leguminosae (Doyle & Luckow, 2003; Lavin *et al.*, 2005; Sprent, 2009b). The development of nodulation appears to have been from a parasitic state of bacteroids retained in infection threads to a more symbiotic relationship with the diazotrophic bacteria released from threads and nodule differentiation (Provorov *et al.*, 2002). To date, only a fraction of the legume species have been assessed for nodulation, which is found to commonly occur within the Papilionoideae and Mimosoideae and markedly

less within the Caesalpinioideae (Sprent, 2001). Symbiotic N_2 fixation has been estimated to account for at least 70 million metric tons of nitrogen fixed per year globally (Brockwell *et al.*, 2005) and the symbiosis of legumes with root-nodule bacteria (RNB) confers a significant ecological advantage (Allen & Allen, 1981; Gualtieri & Bisseling, 2000; Sprent, 2001) which is vital to sustainable agricultural and ecological systems.

Many legume species have been exploited for timber, gum, fibre and tannin, (Allen & Allen, 1981; Brockwell *et al.*, 2005) and some members of the Papilionoideae are of significant agricultural importance (Allen & Allen, 1981). Legumes represent some of the major staple foods such as *Pisum* (peas), *Lens* (lentils) and *Glycine* (soya) and green manures and forages such *Medicago* (alfalfa) and *Trifolium* (clover) (Makri *et al.*, 2005; O'Hara *et al.*, 2003; Zahran, 2001). Notwithstanding the economic value of legumes, the importance of the association of native legumes with RNB in natural systems is considerable. In the nutrient poor soils such as those in Australia, legumes can be an integral component of the floristic structure and are significant providers to the nitrogen cycle (Barnet & Catt, 1991; Beadle, 1964) which would also be of benefit to the non-leguminous plants of the area (Requena *et al.*, 2001).

1.6 The genus *Acacia* sens. lat.

The *Acacia* genus was first described by Philip Miller in 1754 (Ladiges *et al.*, 2006). The botanical name is believed to have derived from the Greek 'akis' meaning point or barb, a feature of African spp. (Brockwell *et al.*, 2005). George Bentham from 1840 to 1845, more clearly defined *Acacia* (Maslin *et al.*, 2003). Further classification and nomenclature changes proposed by Pedley (1986) have not been widely adopted. The tribe *Acacieae* (Dumort 1829) is mongeneric with the genus *Acacia* sensu lato (*Acacia* s.l.) containing the largest number of species [currently (c.) more than 1380 spp] compared to other genera in the Mimosoideae and occurs in all continents except Europe and Antarctica (Maslin *et al.*, 2003; Simmons, 1988). The current classification of *Acacia* s.l (Leguminosae) divides the genus into five subgenera and one other subgenus not presently recognised. At the 2005 XVII International Botanical Congress

in Vienna it was decided to retain the name *Acacia* s.s for *Phyllodineae,* thereby giving recognition of *Vachellia* (syn. *Acacia*), *Senegalia* (syn. *Acileiferum*), *Acaciella* (syn. *Acileiferum* sect. *Filicineae*) and *Mariosousa* (syn. *'A. coulteri'* group) (Miller & Seigler, 2012; Seigler *et al.*, 2006). The retypification had not received widespread acceptance but was upheld at the XVIII International Botanical Congress in 2011 in Melbourne (McNeill & Turland, 2011). Further resolution of the phylogenetic relationships within the *Acacia s.l* genus requires additional molecular and morphological evidence (Bala *et al.*, 2003; Ladiges *et al.*, 2006; Maslin *et al.*, 2003).

Acacia s.s (c. 960 sp.) is found mainly in Australia with some species extending from Madagascar, the Mascarenes, Mauritius, New Guinea, Taiwan and some Pacific islands to Hawaii (Figure 1.10a). *Vachellia* (c. 161 sp.), occurs mainly in Africa but also in Asia, South America and northern Australia (Figure 1.10b). *Senegalia* (c. 235 sp.) is found throughout the tropics with one species in Australia (Figure 1.10c). *Acaciella* (c. 16 sp.) occurs in the Americas (Figure 1.10d). *Mariosousa* (c. 13 sp.) is distributed in central America (Figure 1.10e). The subgenus not currently recognised is the '*S. skleroxyla*' group, with an unknown number of species from the Americas which are currently subscribed to *Senegalia* (Miller & Seigler, 2012).

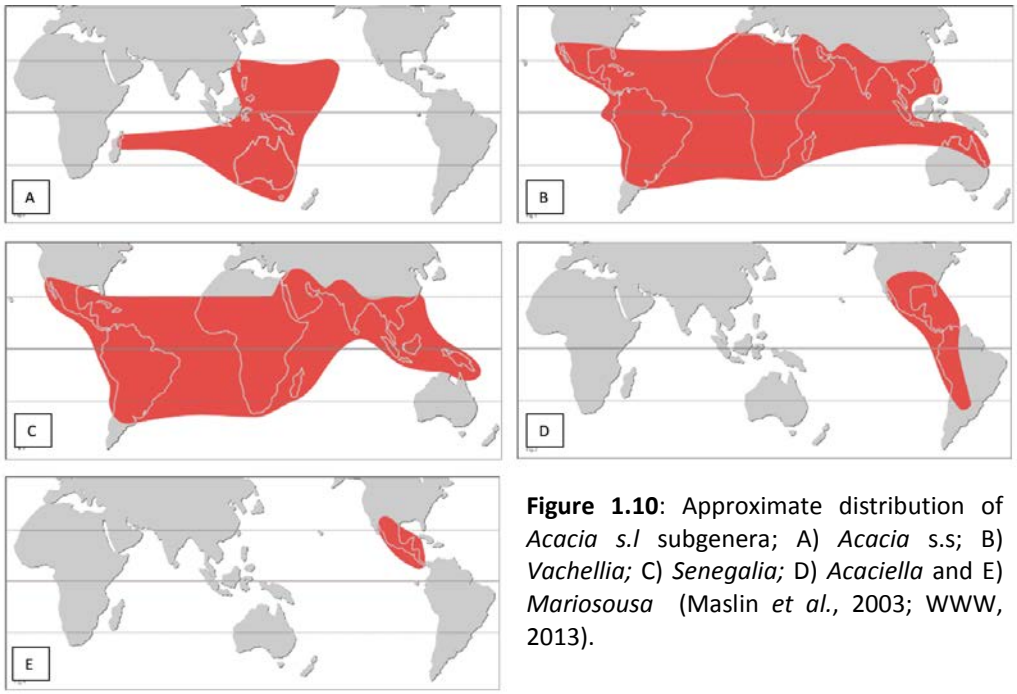


Figure 1.10: Approximate distribution of *Acacia s.l* subgenera; A) *Acacia* s.s; B) *Vachellia;* C) *Senegalia;* D) *Acaciella* and E) *Mariosousa* (Maslin *et al.*, 2003; WWW, 2013).

1.6.1 The subgenera *Acacia* s.s.

The 'Australian group', *Acacia* s.s*,* appears to be monophyletic and with approximately 960 species, is the largest genus of higher plants in the region (Bala *et al.*, 2003; Ladiges *et al.*, 2006; Maslin *et al.*, 2003). There are seven sections within the *Acacia* s.s*,* three with large and widespread distribution (*Phyllodineae, Plurinerves* and *Juliflores*) and four of smaller and more restricted distribution (*Botrycephalae, Pulchellae, Alatae* and *Lycopodiifoliae*) (Table 1.1). The current distribution of Australian flora is believed to be a result of aridity developing in the Miocene, 15 MYA (Beard, 1976). The arid zone or Eremean zone in Australia is composed of endemic and cosmopolitan taxa believed to have derived by invasions from adjacent peripheral regions or from areas of marine incursions and ancient coastlines, as arid environments became more widespread (see Ladiges *et al.* (2006) and references therein).

Table 1.1: Sections within subgenus *Phyllodineae*: total number of species and major geographic regions. Adapted from Murphy (2003).

Sections within *Acacia* s.s	**Distribution in Australia**	**Number of species**
Botrycephalae	Temperate eastern—south-eastern Australia	42
Pulchellae	Temperate south-western Australia	27
Alatae	Temperate south-western Australia	21
Lycopodiifoliae	Tropical and subtropical Australia	17
Phyllodineae	Temperate southern Australia (W&E)	408
Plurinerves	South-western and eastern Australia	212
Juliflores	Tropical, subtropical and south western Australia; few eastern and southern	235

Murphy (2003) sequenced the internal transcribed spacer region (ITS) of 51 *Acacia* spp. and demonstrated a low resolution of relationships within the *Acacia* s.s. with a small amount of divergence. This may be an indication of a large, recent morphological radiation (Maslin *et al.*, 2003) which is further supported by the high frequency of hybridization among the taxa (Broadhurst & Coates, 2002; Maslin, 2001). It has been proposed that speciation occurred as populations, particularly those on the peripheral areas between deserts and temperate areas, became fragmented during the

Quaternary (Maslin & Hopper, 1982). The molecular phylogeny work of Ladiges *et al*. (2006) focused on 21 spp. which included widespread and narrow endemics in the *Acacia victoriae, A. pyrifolia* and *A. murrayana* groups. The data suggests that the earliest differentiation was in the semi-arid regions of the North-West and the Arnhem region of the Northern Territory. Topography, soil weathering, changing fire frequency and climate are likely to have separated this ancient area of endemism. Furthermore, the geographic ranges of the *Acacia* spp. in this study, indicate aridity was first experienced in the north, as species of Central and MacDonnell Ranges of central Australia were closely related to those of the South-West interzone (Coolgardie bioregion) and the species of the Eastern Desert, Western Desert and Pilbara are associated as a group (Ladiges *et al.*, 2006). The diversity of taxa complexes is well recognized within *Acacia* s.s and may represent series of hybridisation events, with numerous species known to form hybrids with other closely related species growing in the same community (Maslin, 2001).

Members of the *Acacia* s.s. may be trees or shrubs (prickles absent) with stipules normally present and have bipinnate leaves that are commonly modified to phyllodinous adult foliage (some taxa retain bipinnate leaves at maturity) (Maslin *et al.*, 2003). *Acacia* spp. are abundant understorey species in temperate climates, and often dominate the landscape in arid or semi-arid regions (Simmons, 1988) with phyllodinous *Acacia* spp. particularly dominant in the vegetation of arid Australia . The sclerophyllic features that phyllodinous *Acacia* spp. have attained in the xeric environments of Australia is thought to have developed as a response to the deeply weathered and leached nutrient impoverished soils and pre-adapted the flora to increasing aridity (Beard, 1990). Of the *Acacia* s.s. studied, most have been reported to nodulate with RNB (Beadle, 1964; Sprent, 2001), and to be colonized by etcomycorrhizal fungi (ECM) and/or arbuscular mycorrhizas (VAM) (Brundrett, 2008; Brundrett & Abbott, 1991; Jasper *et al.*, 1988). These microbial interactions are important associations in the nutrient poor soils of Australia.

The importance of nodulation to the plant varies between species. In arid and semi-arid regions, nitrogen fixation by RNB in nodules is believed to be more important in the early stages of establishment of *Acacia* s.s. as opposed to nitrogen fixation in maturing populations (Schortemeyer *et al.*, 2002). However, populations of mature *Acacia* spp. do take advantage of increased soil moisture. Brockwell *et al.* (2005) have observed *Acacia tetragonophylla* F.Muell. with new root growth and nodulation following rainfall with this being a common phenomenon of legume species following rain in Australian arid and semi-arid regions (Beadle, 1964). Dryland *Acacia* spp. have adapted root systems to take advantage of soil moisture where root systems are extended prior to developing new photosynthetic tissue and two month old seedlings of *Acacia senegal* have been reported as having tap roots almost 2 m long (Sprent, 2007). Periods of sustained water stress can result in altered floral communities with the loss of dwarf shrubs or perennial grasses which are often replaced by scrub *Acacia* spp. such as *A. tetragonophylla* F.Muell. (Pringle *et al.*, 2006) which are able to utilize infrequent periods of soil moisture regardless of season. There are between 0 to 39 *Acacia* s.s spp. distributed across the arid and semi-arid dryland zones of Australia (Hnatiuk & Maslin, 1988). A few *Acacia* species have widespread distribution throughout dryland Australia and include *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. (Figure 1.11). These two species are important overstorey plants in the vegetation associations of the SBSLA (SBSJV, 1998).

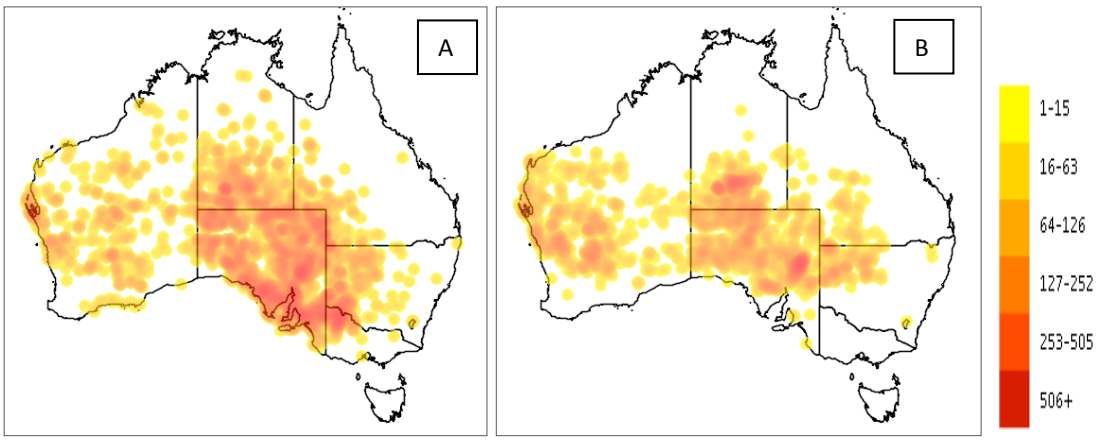


Figure 1.11: The records of occurrence and distribution map of A) *A. ligulata* Benth. and B) *A. tetragonophylla* F.Muell. across Australia. Adapted from The Atlas of Living Australia web site, licensed under Creative Commons Attribution 3.0 .

1.6.1.1 *Acacia ligulata* Benth.

George Bentham described *A. ligulata* Benth. in 1842 from specimens collected by Allan Cunningham from the diverse coastal locations of north west Australia to eastern Australia (IBIS, 2012; Maslin, 2001). The species has a widespread distribution throughout all the mainland states of Australia in the sandy soils of the coast or inland waterways of central and southern regions (Figure 1.11a). The phenotypic features such as phyllode and inflorescence characters can vary depending on the location of *A. ligulata* Benth. and also within populations (Chapman & Maslin, 1992). Many species of *Acacia* s.s. overlap in their distribution throughout Australia and as in the case of the '*Acacia bivenosa*' group in the Pilbara region of Western Australia, hybridity appears to be common (Chapman & Maslin, 1992). There are 12 spp. in the '*A. bivenosa* group', including *A. ligulata* Benth. and the known hybrids or putative hybrids with *A. ligulata* Benth. are *A. bivenosa* DC. x *A. ligulata* Benth., *A. ligulata* Benth. x *A. sclerosperma* F.Muell. subsp. *sclerosperma* and *A. ligulata* Benth. x *A. tysonii* Luehm. (Chapman & Maslin, 1992). Identification of these species can be further confused by their relatedness and by ecotype morphology such as with *A. ligulata* Benth. and *A. rostellifera* Benth. where the distinguishing features are ambiguous and in the case of Shark Bay, *A. ligulata* Benth. may be an intermediate between inland populations and the more southerly distributed *A. rostellifera* Benth. (Chapman & Maslin, 1992).

There is surprisingly little known about the soil microbial interactions of this widespread species. Currently, there are no mycorrhizal associations recorded for *A. ligulata* Benth. and although no RNB have been described for *A. ligulata* Benth. in Western Australia or other states of Australia, it is reported as being nodulated by RNB phylotypes of *Bradyrhizobium*, *Burkholderia*, *Rhizobium* and *Ensifer* (2013) isolated from other native legume species of South-eastern Australia (Thrall *et al.*, 2011). The properties of *A. ligulata* Benth. could make it an amenable species for the rehabilitation of degraded sites, particularly for erosion control at sandy sites.

1.6.1.2 *Acacia tetragonophylla* F. Muell.

Baron Sir Ferdinand Jacob Heinrich von Mueller described *A. tetragonophylla* F.Muell. in 1863 (WAH, 1998) from collections sent to him from other botanists. The species is widely distributed across central and southern areas of all mainland states of Australia with the exception of Victoria (Figure 1.11b). *A. tetragonophylla* F.Muell. grows in loam and well drained alluvial soils of floodplains and watercourses (WWW, 2010). It is a shrub or small tree with a phyllode arrangement uncommon to *Acacia* s.s. spp. but which is common in the species of *Vachellia* (Maslin, 2001). *A. tetragonophylla* F.Muell. has been observed to increase its population on degraded lands and has been used in the rehabilitation of areas in the goldfields of Western Australia (Barrett & Jennings, 1994; Osborne *et al.*, 1994) with potential for soil stabilization and shelter belt plantings. However, Rusbridge *et al.* (1996) had noted that there had been difficulties establishing *A. tetragonophylla* F.Muell. in these areas.

As with *A. ligulata* Benth., the current status of the mycorrhizal associations of *A. tetragonophylla* F.Muell. is unknown. *A. tetragonophylla* F.Muell. has been reported as capable of nodulating with a mix of *Bradyrhizobium* spp. isolated from several *Acacia* spp. of northern Australia (Bowen *et al.*, 1997; Schortemeyer *et al.*, 2002). To date no RNB have been described as specific symbionts of *A. tetragonophylla* F.Muell. from any location in Australia, including Western Australia.

1.7 Root nodule bacteria

Nitrogen is abundant in the air (78% N_2), however, its availability to plants is often limiting in the soil (Krebs, 2001). Nitrogen in the form of inorganic and organic nitrogenous compounds, are important sources of this nutrient for many plants (Ehrlich *et al.*, 1977). A major process for life on earth, is the biological fixing of atmospheric nitrogen to ammonia by symbiotic or free-living diazotrophs in the soil that assimilate nitrogen into terrestrial nitrogen cycling (Ehrlich *et al.*, 1977; Hartwig, 1998).

RNB are gram-negative bacteria which are capable of associating with legumes and non-legumes as symbionts and endophytes (Gualtieri & Bisseling, 2000; Slattery *et al.*, 2001). The RNB are a taxonomically diverse group with genera capable of nodulating hosts found in both the Alphaproteobacteria and Betaproteobacteria. The number of species is ever increasing, with novel strains being described and taxonomic studies resolving the phylogenies of the current genera. There are currently 15 genera with recognised RNB species in the Alphaproteobacteria, these are *Agrobacterium, Allorhizobium, Aminobacter, Azorhizobium, Bradyrhizobium, Devosia*, *Ensifer* (syn. *Sinorhizobium*), *Mesorhizobium,* Methylobacterium, *Microvirga, Neorhizobium* (syn. '*R. galegae'* group), *Ochrobactrum*, *Phyllobacterium, Rhizobium* and *Shinella* (Ardley *et al.*, 2012; Chen *et al.*, 1988; Lin *et al.*, 2008; Martínez-Romero & Caballero-Mellado, 1996; Maynaud *et al.*, 2012; Mousavi *et al.*, 2014; Rivas *et al.*, 2002; Sy *et al.*, 2001; Trujillo *et al.*, 2005; Valverde *et al.*, 2005; Young & Haukka, 1996). The genera in the Betaproteobacteria with RNB are the *Burkholderia* and *Cupriavidus* (syn. *Ralstonia*) (Chen *et al.*, 2001; Vandamme *et al.*, 2002).

The study of the genome of *Medicago truncatula* has shown that a whole-genome duplication (WGD) approximately 58 MYA had contributed to the evolution of endosymbiotic nitrogen fixation (Young *et al.*, 2011). Evidence suggests that there have been several events in the evolution of nodulation during the radiation of legumes, approximately 60 MYA (Doyle, 2011). The transfer of genetic material and adaptation to various soil conditions of temperature, pH and salinity and host-rhizobial associations may be contributing factors to the current global distribution of RNB (Sprent, 1994). Parker (2012) found little diversity of symbiotic genes in the *Bradyrhizobium* isolates of 14 legume genera of eastern North America compared to the nonsymbiotic genes, supporting the hypothesis of the transfer of genes at multiple events. The genetic composition of RNB populations in the soil can alter rapidly. Recent work with *Mesorhizobium* has shown that the resident RNB which did not nodulate *Biserrula pelecinus,* a pasture legume used in Australia, were then able to nodulate this host after the horizontal transfer of a genomic island containing symbiotic genes subsequent to the introduction of a *Mesorhizobium* commercial

inoculant for this species (Nandasena *et al.*, 2006). This resulted in RNB which were not as effective as the selected inoculant for N_2 fixation.

1.7.1 Australian RNB

The historical study of the diversity of symbiotic microflora of Australian native legumes has relied on growth characteristics only allowing differentiation between fast (*Rhizobium*) and slow-growing (*Bradyrhizobium*) RNB and N_2-fixing efficiency (Barnet & Catt, 1991; Beadle, 1964; Lawrie, 1983). In Australia, it is supposed that *Bradyrhizobium* are the most abundant naturally occurring RNB, with whom the majority of native legumes are able to nodulate (Lafay & Burdon, 2001; Lafay & Burdon, 2007; Thrall *et al.*, 2000). More recently, molecular studies have identified the presence of *Azorhizobium, Bradyrhizobium, Burkholderia, Devosia, Ensifer, Mesorhizobium, Phyllobacterium* and *Rhizobium* strains associated with Australian native legumes (Hoque *et al.*, 2011; Lafay & Burdon, 1998; Lafay & Burdon, 2007; Marsudi *et al.*, 1999; Stępkowski *et al.*, 2012; Walker *et al.*, 2013).

The distribution of the RNB genera associated with Australian legumes may differ across the continent. There appears to be a predominance of *Bradyrhizobium* strains in the soils of temperate South-Western Australia (Marsudi *et al.*, 1999), South-Eastern Australia (Lafay & Burdon, 1998) and tropical Northern Australia (Lafay & Burdon, 2007) that nodulate native *Acacia.* However, only fast growing isolates were reported on *Acacia* spp. in the arid regions of North-Western Australia (Yates *et al.*, 2004) and New South Wales (Barnet & Catt, 1991). The distribution of Australian *Bradyrhizobium* also is distinctly different across the continent with isolates from Western Australia and the Northern Territory grouping separately based on multilocus sequence analysis (MLSA) and Western Australian isolates in a clade which includes temperate eastern Australian isolates (Stępkowski *et al.*, 2012).

1.8 Legume-RNB symbioses

RNB enter a plant symbiont and result in the formation of a nodule in which the bacteria have differentiated to form bacteroids. The RNB in nodules can be symbiotically functioning or present as saprophytes in disintegrating nodules (Gualtieri & Bisseling, 2000). The symbiotic relationship between legumes and RNB is determined by the ability of a legume and bacterium to form a symbiotic relationship known as specificity (Allen & Allen, 1981; Kiers *et al.*, 2002) and the ability of RNB in nodules to fix nitrogen for which the host benefits (effectiveness). Three factors that affect the capacity of a particular bacterium to nodulate a host legume are the genome of the plant, the genome of the RNB and the environment (Dowling & Broughton, 1986).

It is generally regarded that legume-RNB associations are highly specific (Perez-Fernandez & Lamont, 2003; Perret *et al.*, 2000; Pueppke & Broughton, 1999). However, some plants and RNB are promiscuous in their symbiotic associations (Marsudi *et al.*, 1999; Perez-Fernandez & Lamont, 2003; Perret *et al.*, 2000; Zahran, 2001). Specificity may significantly contribute to the distribution of a species either in a natural context or for the successful establishment of introduced legume species (Emms *et al.*, 2005; Howieson & Ballard, 2004). Additionally, different floral ecosystems can result in the dominance of certain RNB communities such as *Bradyrhizobium* in eucalypt ecosystems (Lafay & Burdon, 1998) or *Mesorhizobium* in Kenyan *Acacia* ecosystems (Odee *et al.*, 2002). Work by Barnet & Catt (1991) indicated that populations of *Acacia* RNB differed in composition and demonstrated geographic localization. However, specialization in symbiotic associations does not seem to be related to whether the *Acacia* spp. have widespread or restricted distribution as was demonstrated by Murray *et al.* (2001) and Thrall *et al.* (2000) in their respective studies of numerous *Acacia* s.s spp. with variable distribution in the Australian states of Queensland, New South Wales, Victoria and Tasmania. *Acacia* have generally been regarded as promiscuous, opting to nodulate with a range of rhizobia (Sprent, 2007) and while this may indeed be the case, there are significant variations in the ability of strains from each *Acacia* host species to achieve effective plant growth across *Acacia*

host species (Murray *et al.*, 2001; Thrall *et al.*, 2000). This specificity of the symbiotic interactions is a result of an exchange of special signals released by both partners. There are two co-ordinated but separate processes required for the development of functional root nodules; these are bacterial infection and nodule organogenesis.

1.8.1 Bacterial infection

There are three modes of host root infection that lead to the formation of nodules, these are root hair, crack entry and epidermal entry (Sprent, 2009a). The process of root hair infection is common to the majority of legumes (Geurts & Bisseling, 2002) and requires a complex exchange of molecular signals between the symbionts to initiate the physiological processes of infection.

1.8.1.1 Signalling pathways

Numerous compounds from both the legume and RNB are involved in a coordinated exchange of molecular signals for the establishment of a symbiosis between the partners (Figure 1.12). The seed and roots of legumes contain flavonoids, that when released into the rhizosphere enhance the growth rate of RNB and promote their migration to the developing roots (Hartwig *et al.*, 1991; Hassan & Mathesius, 2012; Ndakidemi & Dakora, 2003). Flavonoids and other plant compounds have been shown to induce nodulation genes (*nod* genes) which are involved in the production of lipochitooligosaccharide Nod factors (NF) as well as acting as transcriptional regulators (Banfalvi & Kondorosi, 1989; Firmin *et al.*, 1986; Lerouge *et al.*, 1990).

A signalling pathway is induced in legumes by RNB through the production of NFs and complex surface polysaccharides to promote root attachment and biofilm formation, induce root hair curling, the development of infection threads, intracellular division with localized cortical cell divisions and nodule primordial development (Downie, 2010; Janczarek *et al.*, 2015; Kobayashi *et al.*, 2004; Long, 1996; Mathesius *et al.*, 1998; Perret *et al.*, 2000). The pathway includes the induction of various plant proteins with binding domains that activate Ca^{2+} oscillations which prime the legume root for

infection and the induction of phytohormones which promote cortical cell division (Oldroyd *et al.*, 2011; Sprent, 2001; Udvardi & Poole, 2013). The NFs produced by the RNB are the primary determinates by which they are able to nodulate a broad or a narrow range of legume hosts (Perret *et al.*, 2000; Sprent, 2009a). However, it is the host plant that determines the manner of infection and the number, anatomy and localization of the nodules (Haag *et al.*, 2013)

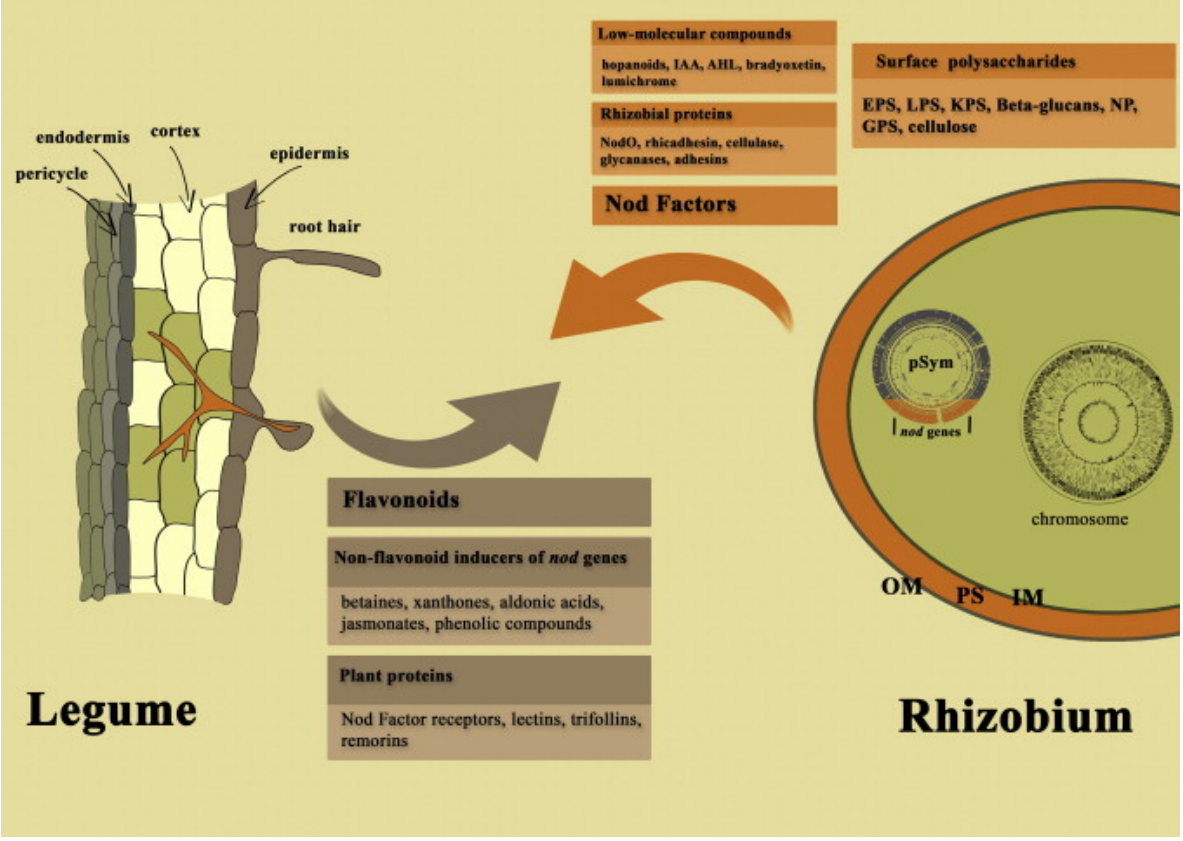


Figure 1.12: Signals and other plant-derived and bacterial compounds involved in legume–rhizobium symbiosis (Janczarek *et al.*, 2015).

1.8.1.2 Process of infection

The RNB that are localized in the rhizosphere receive nutrients from the roots which enable them to adhere to the epidermal cells of the root hair and to form a surrounding biofilm, leading to the deformation and curling of the root hair, entrapping the RNB which form a colony (Downie, 2010; Vijn *et al.*, 1993). The RNB digest the epidermal cell wall and the RNB are transported into the cell within a tube-like structure called an infection thread which is formed by the invagination of the root hair plasma membrane (Janczarek *et al.*, 2015). During this process the RNB also

initiates a response in both the cortical and pericycle cells. The pericycle undergo a limited number of cell divisions and normal lateral root primordium formation occurs in the pericycle opposite the protoxylem poles (Callaham & Torrey, 1977). The cells of the cortex enter the cell cycle, dividing to become the nodule primordium where the infection thread is directed and branches to the individual cells which become infected with the RNB contained in a plant plasma membrane, the symbiosome membrane (Geurts & Bisseling, 2002; Hartwig, 1998; Monahan-Giovanelli *et al.*, 2006). The RNB and membrane divide in conjunction with each other with the resulting structure called a symbiosome (Downie, 2010; Geurts & Bisseling, 2002; Sharma *et al.*, 1993).

1.8.2 Nodule organogenesis

There are two broad groups of nodulating plants, those that form determinate or indeterminate nodule types (Figure 1.13). While there are similar molecular signals and pathways for nodule organogenesis and N_2 fixation, the development of these nodule types differs. Determinate nodules are distinguished by the lack of a persistent meristem and N_2 fixation occurring in all the infected cells simultaneously (Maunoury *et al.*, 2008). Indeterminate nodules are divided into several zones where the plant and bacterial cells are at different physiological states with a persistent meristem (Vasse *et al.*, 1990). All the nodules studied from species within the subfamily Mimosoideae, including those in genus *Acacia* sens. lat., form indeterminate nodules (Sprent, 2007).

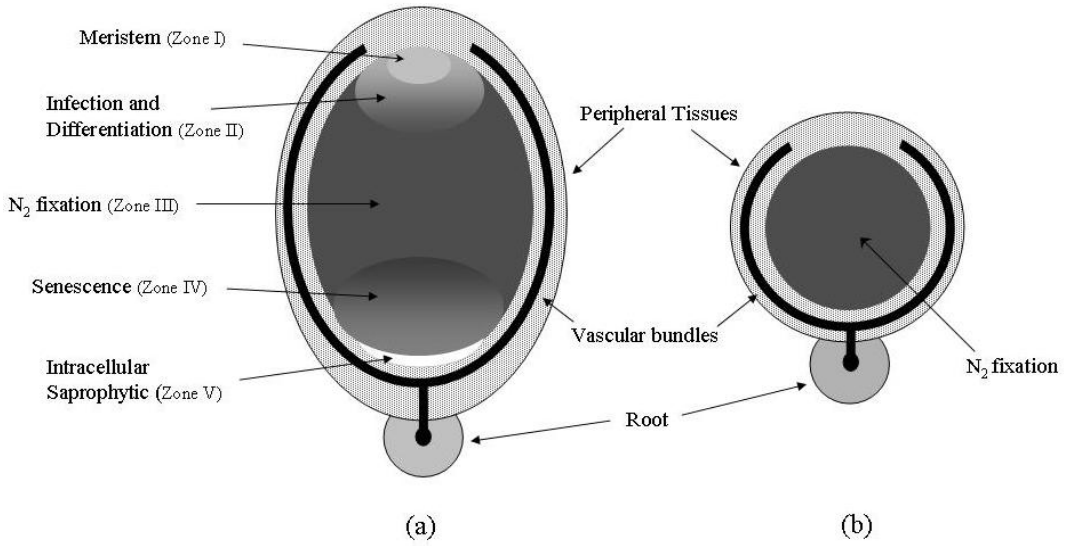


Figure 1.13 - Schematic representation of (a) indeterminate and (b) determinate nodule types. Image from Terpolilli (2009) as adapted from Maunoury *et al.*(2008) and Brewin (1998).

1.8.2.1 Nodule development

The nodule structure begins to form with the continued growth and division of the RNB along with the symbiosomal membrane leading to thousands of symbiosomes within the infected plant tissue (Mergaert *et al.*, 2006; Udvardi & Poole, 2013). The RNB within the symbiosomes differentiate into the form known as bacteroids which is their N_2-fixing physiological state (Sprent, 2009a). Symbiosomes may contain one or many bacteroids with the shape of the bacteroids varying from rod to pleomorphic shapes (Dart & Mercer, 1966).

The morphology of determinate nodules is due to outer cortical cell division for the nodule primordium and subsequent occupation by the RNB occurring before these cells become meristematic. The infected tissue is composed of infected cells derived from the root cortex and uninfected cells that are derived from the root pericycle resulting in the characteristic spherical form of determinate nodules (Brewin, 1991). The indeterminate nodule morphology derives from the nodule primordium developing from inner cortical cells, adjacent to the pericyle. As cells become occupied with RNB, the cells proximal to the primordium and the adjacent pericycle cells continue to divide and together comprise the apical meristem of the developing nodule, growing outward from the root and occasionally branching resulting in the varied indeterminate nodule forms (Brewin, 1991; Sprent, 2001). The presence of persistent meristem in the indeterminate nodule results in different zones throughout infected tissue (Figure 1.13). The cells adjacent to the meristem are comprised of differentiating cells undergoing infection, distal to this is the zone where N_2 fixation is taking place and proximal to the root is senescence zone (Brewin, 1991; Vasse *et al.*, 1990). Not all RNB are released into the cells, with a number being retained in the infection threads that are ramified throughout the developing nodule (Monahan-Giovanelli *et al.*, 2006), these remain viable and are released into the rhizosphere when the entire nodule senesces.

These processes described are for the development of functioning nodules and are dependent on continued molecular signalling between the symbionts. The legume and RNB genes for symbiosis are functionally diverse (Giraud *et al.*, 2007; Janczarek *et al.*, 2015) and at numerous phases of nodule initiation and organogenesis, the processes can be halted or impaired. In a number of legume species, nodule-like structures may be induced to form but be un-infected by RNB (Brewin, 1991; Hirsch *et al.*, 1989).

1.8.2.2 Nitrogen fixation

Nitrogen fixation occurs in the bacteroids, where atmospheric N_2 is reduced to ammonium (NH_3) in the following reaction: $N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i$ using the enzymatic complex of nitrogenase (Lodwig & Poole, 2003). This reaction requires the induction of a number of genes and is only possible when a low O_2 tension is maintained in the nodule (Terpolilli *et al.*, 2012). Subsequently, there is an exchange of nutrients between the bacteroids and plant, primarily in the form of NH_3 from the bacteroids and C_4-dicarboxylic acids from the plant (Haag *et al.*, 2013; Lodwig & Poole, 2003; Terpolilli *et al.*, 2012).

The O_2 tension is maintained by co-ordinated processes of limiting the rate of gas diffusion in the outer cell layers of the nodule, the consummation of O_2 by the respiration of bacteroid and plant mitochondria and leghemoglobins which bind and transport the O_2 to the infected cells (Udvardi & Poole, 2013). For an effective symbiosis, the expression of a suite of *nif* (N_2 fixation) and *fix* (associated processes) genes during bacteroid development is required as well as the induction of specific plant proteins known as nodulins (Bisseling *et al.*, 1983; Udvardi & Poole, 2013). Low oxygen is a key signal in the induction of *nif* and *fix* gene expression and poor N_2 fixation is linked to a reduction in late induced nodulins and reduced levels of leghemoglobin (Bisseling *et al.*, 1983; de Lajudie & Huguet, 1988; Hirsch *et al.*, 1989; Legocki & Verma, 1980).

The benefit of the symbiosis to the legume is the supply of nitrogen and the formation of a nodule may also benefit the RNB as a refuge from environmental stress (Sprent,

2007). Symbiotic N_2 fixation is not just dependant on the complex exchange of molecular signals between the symbionts, it is also sensitive to numerous environmental variables that affect both the RNB and the legume host.

1.9 Dryland environmental constraints on N_2 fixation

The formation of effective nodules and N_2 fixation is subject to the adaptation of both the RNB and the legume host to a particular environment. Aridity, extremes of temperature, nutrient availability, salinity and soil pH contribute to inhibit RNB and legume survival, thereby limiting N_2 fixation (Brockwell *et al.*, 2005; Howieson & Ballard, 2004; Walsh, 1995). Section 1.4.2 discussed the nutrient status of the ancient nutrient poor soils of dryland Australia. Many Australian plant species have adapted to these conditions and utilize interactions with soil microflora such as mycorrhizal fungi to scavenge for nutrients such as P (Brundrett & Abbott, 1991; Lambers *et al.*, 2008). The constraints on RNB-legume symbioses in dryland areas discussed here are temperature, water availability, pH and salinity.

1.9.1 Temperature and water availability

High temperature affects numerous processes of nodulation such as RNB infection, nodule differentiation and the structure and function of the nodule (Bordeleau & Prévost, 1994; Graham, 1992; Hartel & Alexander, 1984). Nodules formed under temperature stress are markedly smaller and have reduced nitrogenase activity (Hartwig, 1998; Zahran, 1999). For many RNB, the optimum temperature for growth in culture is between 28 and 31°C (Graham, 1992), though some populations of RNB are found to persist at much greater temperatures (Kulkarni *et al.*, 2000; Shamseldin & Werner, 2005; Wilkins, 1967; Zahran, 1999). Often in soils exposed to high surface temperatures, nodulation is restricted to the roots in the subsurface region (Johnson & Mayeux, 1990; Zahran, 1999). Populations of RNB have been detected at great depths. In the Sahelian area of West Africa the rhizosphere of *Acacia albida* contained a

population of *Bradyrhizobium* spp. at $1.3x10^3$ cells g^{-1} at a depth of 34 m (Dupuy & Dreyfus, 1992).

The cellular responses of RNB exposed to high temperatures show that growth and survival is less impaired when the RNB are subjected to heat shock rather than exposure to sustained heat stress (Alexandre & Oliveira, 2011; Laranjo & Oliveira, 2011; Münchbach *et al.*, 1999). RNB strains adapted to the heat are able to persist in the soil but may have reduced ability to form a functioning symbiotic association (Zahran, 1999). However, an increase in temperature is known to induce the synthesis of heat shock proteins (Hsps) in a number of organisms (Münchbach *et al.*, 1999). Hsps are involved in subunit exchange and substrate binding and act as chaperones, maintaining the active conformation of proteins such as those required for symbiosis (Lentze *et al.*, 2004). While Hsps usually number no more than two in many bacteria, RNB are exceptional in that *Rhizobium, Ensifer, Bradyrhizobium* and *Mesorhizobium* are found to have multiple copies of Hsps (Alexandre & Oliveira, 2011; Münchbach *et al.*, 1999).

The availability of water is necessary to the survival and persistence of symbionts in arid or dryland environments and desiccation is certainly detrimental to positive legume-RNB interactions. Populations of RNB are reduced during periods of drought or through seasonal changes in rainfall (Chatel & Parker, 1973; Hartwig, 1998; Zahran, 1999). Various adaptations aid in the persistence of RNB at periods of water stress where upon improved conditions, which are often sporadic, nodulation and N_2 fixation can resume. Trehalose biosynthesis appears to have a functional role as an osmo-protectant in the tolerance to desiccation. A number of RNB genera have been reported to accumulate trehalose and where certain trehalose biosynthetic genes have been disrupted, reduced fitness to desiccation is observed (McIntyre *et al.*, 2007; Sugawara *et al.*, 2010). Some RNB have the ability to create microbial biofilms on the root surfaces in large cell aggregates of microcolonies able to survive desiccation stress (Carlier *et al.*, 2015; Russo *et al.*, 2014).

1.9.2 Soil pH and salinity

Soil chemistry and nutrient availability affects the plants, soil microflora and their interactions through root morphology or plant vigour and symbiotic interactions can be affected at root hair infection, nodule formation or just limit the numbers of RNB (Hartwig, 1998). There is evidence that the tolerance of some RNB genera to high temperatures is positively correlated to tolerance of alkaline pH and saline conditions (Kulkarni *et al.*, 2000; Laranjo & Oliveira, 2011; Shamseldin & Werner, 2005; Zahran *et al.*, 1994). Core gene clusters such as *pha2* (Na^+ resistance and alkaline pH) that are involved in alkaline-saline adaptations have been identified in *Ensifer* spp. and *Rhizobium* spp. (Putnoky *et al.*, 1998; Tian *et al.*, 2012).

The effect of pH is highly variable between alkaline and acid soils. In alkaline soils, some RNB genera are not just resistant to alkaline conditions but able to grow and create an environment of competition between various genotypes capable of nodulating a particular host species (Howieson & Ballard, 2004). N_2 fixation is limited more by the adverse effects of alkaline conditions on the survival and growth of the legume hosts rather than the persistence of RNB in these conditions (Zahran, 1999). Under acidic soil conditions and/or salt stress, two of the minerals shown to be deficient are boron and calcium, these are essential to many of the processes of nodulation such as signalling, attachment, *nod* gene activation, infection thread development, cell invasion, differentiation of RNB to bacteroids and nitrogen fixation (El-Hamdaoui *et al.*, 2003; Watkin *et al.*, 2003). There are RNB and legumes adapted to survive and persist in acid soil conditions. Many RNB exhibit an acid tolerance response (ATR) which is coupled to the production of specific proteins in adaptation and exposure to acid stress (Draghi *et al.*, 2010; O'Hara & Glenn, 1994; Rickett *et al.*, 2000).

Many plants are sensitive to saline conditions and struggle to survive at soil salinity levels above 4-8 dS/m (Cook, 2006). Salt tolerance is highly variable among species and in some more salt-sensitive *Acacia* spp, the inoculation of these species with mycorrhizal fungi and RNB improved their growth (Diouf *et al.*, 2005). In soil salinity levels that impede the growth and survival of some legumes, there are many strains of

RNB that persist in the soil (Singleton *et al.*, 1982). However, persistence and survival of the symbionts does not necessarily result in effective nodulation and N_2 fixation in a salt-stressed environment. Saline soils are often deficient in certain nutrients and plants growing in these conditions show detectable imbalances in the shoots and roots (El-Hamdaoui *et al.*, 2003) which may reduce the flavonoids and plant proteins excreted by plant. In salt stress conditions, the addition of certain *nod* gene inducers has been shown to improve nodulation and N_2 fixation (Ghasem *et al.*, 2012). There are a number of enzymes required for either N_2 fixation or for antioxidant defence. Salt stress impedes the functioning of these enzymes leading to elevated concentrations of toxic oxygen compounds which reduces the nodule leghemoglobin content thereby reducing N_2 fixation and leads to nodule senescence (Tejera *et al.*, 2004).

1.10 RNB inoculants for dryland rehabilitation

Commercial inoculant production occurs in many countries, however the quality of the product can be a significant issue, with some inoculant products reported to contain no viable bacteria (Lupwayi *et al.*, 2000). To ensure standards are met by legume inoculant production companies, countries may adopt legislation (Argentina, Canada and France), industry regulation (Australia, Brazil and Thailand) or be controlled by market competition (USA) (Deaker *et al.*, 2004; Herridge *et al.*, 2014; Lupwayi *et al.*, 2000; Woomer, 2013). The standards which vary from country to country, may include a quality threshold number of viable RNB cells and a minimum number of contaminants (Deaker *et al.*, 2004; Woomer, 2013). Major difficulties lie in applying research findings to field conditions, which result in realistic outcomes for both manufacturer and the end user (Herrmann & Lesueur, 2013; Stephens & Rask, 2000). There are two main aspects governing the success of inoculation, the effectiveness of the bacterial isolate and the application technology (Bashan *et al.*, 2014). For the production of a successful legume inoculant, several factors need to be addressed; the physical and chemical characteristics of the carrier medium, the biological limitations of the chosen isolates and the environmental conditions at the time of application (Herridge, 2008; Stephens & Rask, 2000).

The first commercialized inoculants were pure agar media preparations (Nobbe & Hiltner, 1896a; Nobbe & Hiltner, 1896b). Historically RNB strain selection and inoculant development has largely focused on agricultural systems where there are high demands for inoculants that are cost-effective, reliable and easy to use (Date, 2001). Today, the most widely accepted inoculant carrier is peat, in use since 1914, against which all other carriers are still compared (Date, 2001). The requirements for inoculant carrier technology today are to improve the number of bacteria per unit of product, extended shelf life and to seek quality alternative carriers as availability of peat is severely limited or not available in a number of countries (Albareda *et al.*, 2008; Bashan, 1998; Lindström *et al.*, 2010). Other inoculant carriers can be liquid, granular or polymer encapsulated cells, each with their own advantages and limitations (Herrmann & Lesueur, 2013). In dryland environments, polymers may have an advantage over peat preparations as the microbial cells are encapsulated, protected from environmental stress such as desiccation and are released gradually into the soil as the polymers degrade (Bashan *et al.*, 2002; Deaker *et al.*, 2007; Herrmann & Lesueur, 2013). The polymer Alginate has been used successfully as an inoculant carrier for various *Acacia* spp. with value for rehabilitation and commercial use in arid and semiarid areas of Africa (Faye *et al.*, 2006; Galiana *et al.*, 1994; Sarr *et al.*, 2005). Polymers of this nature are however, considerably more expensive and labour intensive to produce (Bashan & Gonzalez, 1999) and none are currently used in commercial production (Herrmann & Lesueur, 2013).

The selection of site specific RNB for the inoculation of provenant or local legumes for rehabilitation have been shown to improve plant establishment and survival (Herrera *et al.*, 1993; Murray *et al.*, 2001; Requena *et al.*, 2001; Thrall *et al.*, 2001). It has been demonstrated that changes in the abundance and diversity of RNB can alter the growth response of *Acacia* spp. in a population (Bever *et al.*, 2013). However, large scale production of RNB inoculants for the rehabilitation of legumes native to an area may not be feasible. Although no longer in production, Wattle Grow™, a commercial clay based inoculant that contained several *Bradyrhizobium* strains was developed for eastern Australian *Acacia* spp. based on extensive strain selection research by Thrall *et al.* (2005). The use of Wattle Grow™ in seeding trials improved *Acacia* spp.

establishment by up to 5 times and it was envisaged that its primary use would be for farm management practices addressing dryland salinity (Thrall, 2011). A site-by-site approach for the selection of RNB and an appropriate carrier may be more practicable and cost effective in the long term. Selecting RNB strains from the target environment that demonstrate high N_2 fixation for inocula development may reduce the negative impact of competition from less well adapted non-indigenous strains (Sessitsch *et al.*, 2002). Selecting provenant RNB for rehabilitation retains the integrity of the soil microflora in conserved areas such as National Parks and World Heritage areas where the provenance of soil microflora is overlooked when compared to the floral diversity and distribution.

1.11 Aims of thesis

Factors such as temperature, salinity, rainfall, the slow growing nature of indigenous perennial plants as well as strong and persistent winds are often obstacles to the restoration processes of arid or semi-arid lands (DMEWA, 1996; Mitchell & Wilcox, 1994) and spontaneous revegetation is very unlikely to occur in such conditions (Bastida *et al.*). It is clear from these and other environmental constraints described in section 1.4 that there are many obstacles to the successful rehabilitation of the disturbed pit sites in the SBSLA.

It is a characteristic of the arid shrublands of Australia and elsewhere, to have an area of greater nutrients, organic matter and microbial activity under the canopy of perennial plants compared to the bare interplant areas (Bennett & Adams, 1999; Bolton *et al.*, 1993; He *et al.*, 2011; Zhao *et al.*, 2007). It is reported that utilizing the features of woody shrubland plants, particularly legumes, for revegetation or rehabilitation is beneficial to improving the soil structure, moisture, organic matter and total N and P (Barnet *et al.*, 1985; Jasper *et al.*, 1988; Tlusty *et al.*, 2004; Waters *et al.*, 1997; Zhao *et al.*, 2007). Inoculating legumes with microbial symbionts such as RNB and mycorrhizal fungi in these rehabilitation projects has benefits in improving seedling establishment and survival (Jha *et al.*, 1995; Requena *et al.*, 1997; Requena *et al.*, 2001; Thrall *et al.*, 2005). The loss of beneficial plant-associated microbes in

disturbed dryland areas could be the fundamental issue in the failure to establish native plants (de-Bashan *et al.*, 2012).

While the advantages of using legumes in the rehabilitation of dryland areas is broadly understood, the complex interactions between these plants and their symbionts, particularly in the northern dryland areas of Western Australia is largely unstudied. Understanding the complex interactions of dryland legumes in these areas should be a essential component of the restoration management approaches of the operators responsible for the rehabilitation of these sites as the demand for resources and land use increasingly utilize these areas.

There are multiple factors influencing the soil microbial symbionts of Australian native legumes. The regional effect of RNB distribution is not necessarily related to the associations between the legume-RNB symbionts and varies greatly across both small and large geographic ranges (Barrett *et al.*, 2012; Hoque *et al.*, 2011; Lafay & Burdon, 2001; Stępkowski *et al.*, 2012). Heterogeneity in soil characteristics such pH and salinity can influence the phylogenetic diversity of RNB across a geographic area (Garau *et al.*, 2005; Thrall *et al.*, 2008). However, in a given ecosystem, the diversity of RNB is unclear as there is overlap in the distributions of different legume hosts and the RNB they may more specifically associate with (Lafay & Burdon, 1998).

Given the need to ameliorate the disturbed sites at SBSLA, an important step towards recovering these degraded areas would be to establish a canopy of perennial plants. This may be possible with provenant *Acacia* spp, which are prevalent in the dryland ecosystems of the SBSLA and by providing beneficial microbes such as RNB to improve seedling establishment and survival. Therefore, the aims for this thesis are follows:

1) Determine the biological, chemical and physical differences of selected borrow pit soils and adjacent undisturbed soils, and determine the distribution of vegetation and RNB populations for these sites.

2) Isolate provenant RNB for *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. from the soils of SBSLA and assess the effectiveness of these RNB isolates as well as Wattle Grow™ in promoting plant growth.

3) Describe the phenotypic characteristics, phylogenetic relationships and the genetic diversity of the RNB isolates of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell..

4) Determine the effects of selected RNB isolates in various inoculant preparations on the nodulation and field establishment of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. in disturbed pit sites.

5) Determine the competitive ability and persistence of selected SBSLA RNB isolates and Wattle Grow™ to nodulate *A. ligulata* Benth. and *A. tetragonophylla* F.Muell..

Chapter 2

Disturbance effects on soil characteristics and RNB

No action is without its side effects.
-Barry Commoner (Physicist and Ecologist)

2.1 Introduction

Shark Bay Salt was operational prior to the surrounding area being classed as a world heritage area and the majority of works undertaken predate current environmental legislation (EPA, 1991). The EPA (2005) recognizes there has been limited experience in rehabilitation in the region of the SBWHP and that challenges to effective rehabilitation include the relatively slow growing vegetation, high winds, irregular rainfall and high evaporation. Additional ecological disturbance such as the removal of flora and disruption of the soil profile is likely to negatively impact the physical, chemical and biological properties of soil and be accompanied by a loss of microsymbionts (Bentham *et al.*, 1992; Bolton *et al.*, 1993; Requena *et al.*, 2001). These changes can alter the hydrology, pH and topography of the soil. Vegetation loss can lead to further degradation through reduced soil stabilization and result in the seed bank being irreparably depleted. Similarly, the loss of beneficial soil microbes such as mycorrhizal fungi and root nodule bacteria and the resultant loss of nutrient availability to vegetation can impair the establishment and structure of the floristic community.

Merely measuring a return of vegetative cover may provide a misleading indicator of restoration (Bentham *et al.*, 1992) and successful rehabilitation of an area should aim to enable a return of stable soil microbiological processes and vegetative cover which is sustainable in the long term (Bentham *et al.*, 1992; Hobbs, 1999). Understanding the processes involved in site disturbance and effects on the soil subsystem are an important step towards being able to measure the success or failure of restoration (Harris, 2003; Murray *et al.*, 2001; Thrall *et al.*, 2001) and describing the degree of change disturbance has wrought on soil properties is crucial in order to address possible limiting factors to successful establishment of a sustaining ecosystem.

This chapter reports on biological, chemical and physical characteristics of selected borrow pit soils and adjacent undisturbed soils as well as determining the floral distribution and RNB populations for these areas. Analysis conducted was to test the hypothesis that borrow pit disturbance has negatively affected abiotic and biotic

parameters in comparison to adjacent undisturbed sites. Much of the work in this and subsequent chapters was done either concurrently or separately due to the time restrictions of the project and access to the field site (Appendix 2).

2.2 Materials and Methods

2.2.1 Site assessment of abiotic conditions

2.2.1.1 Site selection

There were four excavated borrow pit areas Pit G south (-26.1355556°, 113.3869444°), Pit P (-26.1572222°, 113.3980556°), Pit Q1 (-26.1658333°, 113.3977778°) and Pit R (-26.1486112°, 113.3981500°) within the Shark Bay Salt lease area selected due to accessibility and inherent differences in their elevation, surrounding vegetation associations and disturbance profiles (Figure 2.1). The co-ordinates for Pit P relate to an area which lays to the west of the excavated pit and has been identified as a 'large scraper cut' in the Shark Bay Salt Joint Venture Environmental Report (1994) but shall be referred hereafter as Pit P due to its proximity. Pit G south refers to an excavated area to the south of an access road and shall hereafter be referred to as Pit G. Some limited information regarding the history of these pits and the efforts made to rehabilitate and monitor recovery are available from environmental reports produced by Shark Bay Salt Joint Venture between the years 1994 to 2005 (Table 2.1). Within each of the pits, a 40 x 10 m area was enclosed with chainmesh fencing to exclude herbivores for *in situ* inoculation trials, to which the following pit site descriptions relate. Pit G south is approximately 17 m above sea level and the elevation of the surrounding profile ranges from 22 to 24 m, outcropping calciferous deposits and sand have been removed and the pit soil consists of heavily compacted sand and limestone debris. The lowest profile of both Pit P and Pit R is 7 m with the surrounding profile elevation of both pits at 11 m above sea level and the soil is composed of sand and limestone debris. Pit Q1 is located on a promontory; dug to approximately 1 m below sea level and comprises a mosaic of sand, limestone debris and clay. The surrounding

profile is 1 to 1.5 m above sea level and both the pit and the remnant vegetation are exposed to salt spray directly from the surrounding evaporation ponds. At each excavated pit site an undisturbed area was surveyed as a comparison that was adjacent to the pit area. The undisturbed areas lay in an westerly direction adjacent to each excavated pit and the co-ordinates were G -26.1344444°, 113.3855556°; P -26.1569444°, 113.3975°; Q1 -26.1663889°, 113. 3961111° and R -26.1482444°, 113.3981456°.

Table 2.1: Shark Bay Salt Environmental Report Pit history of selected sites (SBSJV, 1994; SBSJV, 1997; SBSJV, 2002; SBSJV, 2005)

Pit	Size	Year	Comments from environmental reports
G	4.1 ha	1993	Under rehabilitation. Deep ripped, contoured, mulched, fertilised (Agras1 50 kg/ha) and seeded. Area baited with 1080 by CSIRO for rabbit population control
		1994	Previous year seeding was unsuccessful, a 2500 m^2 area fenced (G south) and seeded with South West seed mix and fertilized
		2002	Pit area reported as undisturbed Continued rehabilitation monitoring
		2004	Fenced area reduced to monitor impact of rabbits
P	1.5 ha	1993	Under rehabilitation
		1994	A weather event in August 1994 reduced the cliff faces of the pit and blended the pit to the topography The area of this project is a large scraper cut to the west of Pit P
		1995	Pit mulched with brush (*Eucalyptus* sp.) from town prunings
		1996	Seeded in June with local seed and infestation of *E. platypus* and *Schinus* sp. noted
		2002	Pit area reported as undisturbed Continued rehabilitation monitoring.
Q1	0.5 ha	1995	Opened as active pit
		2002	Pit deactivated
		2004	Pit area not being actively rehabilitated
R	0.9 ha	1993	Under rehabilitation
		1994	Limited regeneration, high rabbit population in area
		1996	Pit contours blended to the topography and area seeded with low success rate Infestation of *Nicotinia* sp. reported as controlled
		2002	Pit area reported as undisturbed Continued rehabilitation monitoring

Figure 2.1: Arial photo of Useless Loop salt ponds and approximate location of excavated pit sites (SBSJV, 1997). Pit and undisturbed areas G, P, Q1 and R are indicated as the boxed sites.

2.2.1.2 Soil chemical analysis

2.2.1.2.1 Soil sampling

Within each selected pit and undisturbed study area, with the exception of site R and including a fenced rehabilitated area at Pit G, six sub-samples of soil were collected. The sub-samples were profile samples to a depth of 100 mm. At each site the sub-samples were pooled, homogenized and approximately 250-300 g collected into a zip lock bag while the remaining sample was placed into a calico bag.

2.2.1.2.2 Analysis

Soil analysis was conducted by Wesfarmers CSBP Ltd (2 Altona St, Bibra Lake, WA, Australia, 6163). Ammonium and nitrate were determined from the methods of Searle (1984), phosphorus and potassium concentration methods were as for Colwell (1965). The methods for extractable sulfur and organic carbon were as for Blair *et al.* (1991) and Walkley and Black (1934) respectively. Electrical conductivity, pH ($CaCl_2$) and pH (H_2O) methods were as for Rayment & Higginson (1992). Reactive iron was determined by suspending soil with Tamm's reagent (oxalic acid/ammonium oxalate) in a 1:33 soil: solution ratio. The concentration of iron was determined at 248.3 nm on a flame absorption spectrophotometer after the soil suspension had been tumbled for 1 h.

2.2.1.3 Temperature and Rainfall

Meteorological data were provided by Shark Bay Salt. Daily rainfall and temperature were provided for 2007 to 2009 and historical rainfall data provided from 1983. Two Tinytag™ Plus 2 Internal Temp/RH data loggers (Perth Scientific Pty Ltd) were used to measure soil temperatures at 35 min intervals over a 12 month period. On the 17 July 2007, they were placed at 10 cm below the soil surface in Pit P and in an undisturbed area adjacent to Pit P. After the data was retrieved on the 21 June 2008, the two loggers were immediately placed at the same locations on the soil surface and protected from rodent interference with a wire mesh cage pegged into the ground over the loggers; these were collected from site on the 5 February 2009.

2.2.2 Vegetation survey

2.2.2.1 Flora survey and vegetation profile

Flora surveys at pits G, P and Q1 and adjacent undisturbed areas (section 2.2.1.1) were conducted using three randomly placed 18 m transects in each site, along which every 2 m were five 2 x 2 m quadrats. Plants found in quadrats were identified to species where possible. The average total percentage (%) foliage cover of each site was calculated from the recorded percentage foliage cover of each quadrat of the three transects. To determine floristic structure the height of all vegetation intersecting the second transect at each site was measured.

2.2.2.2 *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. survey

Acacia ligulata Benth. and *Acacia tetragonophylla* F.Muell. have been identified as key perennial species in this study due to their presence in the various vegetation associations identified within the lease area (SBSJV, 1998) and availability of provenance seed. Three randomly placed 20 x 20 m quadrats were surveyed in each of the undisturbed areas adjacent to the pit sites. The heights of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. found in the quadrats were recorded and the growth status of all these plants was rated on a scale of 1-5, where 1 relates to vigorous growth, 2 growing and healthy, 3 stressed, 4 dying and 5 dead.

2.2.3 Most probable number of RNB

2.2.3.1 Soil sampling

In July 2007 and June 2008, six random profile soil samples were collected with a 50 mm diameter core borer to a depth of 0-400 mm in each of the sample areas described in section 2.2.1.1. The borer was dusted down with a cloth and surface sterilised with 70% (v/v) ethanol between each sample. The samples were pooled and homogenized

at each site; a pooled site subsample was collected in a sterile 120 mL screw cap jar and stored at 4°C for no longer than one week.

2.2.3.2 Seed and growth jar preparation

Sufficient seed of *A. ligulata* Benth. (narrow leaf form NS-9153) (Nindethana Seed Suppliers, Albany) and *A. tetragonophylla* F.Muell. (collected in November 2006 from within Shark Bay Salt lease area) were scarified by immersion immediately in water that had boiled (2 min), surface sterilised in 70% (v/v) ethanol (3 min), rinsed in four changes of sterile deionised water and left to imbibe in the final rinse water (30 min). Sterilised seeds were placed on 0.9% (w/v) agar, protected from light and kept at 28°C for 24 hs and then at room temperature until radicals emerged. Germinated seedlings were transferred aseptically, one per 120 mL screw cap container measuring 42 mm in diameter and 105 mm in height, containing 50 mL of sterile 2:3 yellow/river sand treated with 10 mL of sterile nutrient solution (final concentration composition: $MgSO_4.7H_2O$ 306.9 mg L^{-1}, KH_2PO_4 67.9 mg L^{-1}, K_2SO_4 437.6 mg L^{-1}, FeEDTA 62.4 mg L^{-1}, $CaSO_4.2H_2O$ 204.0 mg L^{-1}, H_3BO_3 92.6 µg L^{-1}, $Na_2MoO_4.2H_20$ 3.6 µg L^{-1}, $ZnSO_4.7H_20$ 107.6 µg L^{-1}, $MnSO_4.5H_20$ 8.4 µg L^{-1}, $CoSO_4.6H_20$ 28.1 µg L^{-1} and $CuSO_4.5H_20$ 25.0 µg L^{-1}) as modified from Howieson *et al.* (1988) for the generally reduced nutritional requirements of Australian native flora. Plants were maintained at 22°C in a glasshouse and after four weeks of growth, jar lids removed, sterile capped 5 mL pipette tips inserted into the soil as watering tubes and soil surface covered with sterile polyurethane beads. Plants were watered with sterile H_2O as required.

2.2.3.3 Infection test and RNB enumeration

The plant infection test to determine most probable number (MPN) was performed according to Brockwell (1982) using an initial dilution of 20 g of soil suspended in 40 mL sterile 1% (w/v) sucrose, a further six, three fold dilutions were prepared. Plants were inoculated 14 days post sowing after sufficient plants had emerged. Four containers sown with *A. ligulata* Benth. were prepared as positive controls and inoculated with 3 g of a bentonite clay inoculant containing 1.3 x 10^6 cells g^{-1} of GL2L, a

strain isolated from nodules in July 2006 from *A. ligulata* Benth. growing at Pit G (Section 3.2.1). Similarly, three grams of the clay inoculant with a mixed preparation containing authenticated *A. tetragonophylla* F.Muell. isolates 1a13, 2a11, 3a23, 4a13 5a16, 6a15, 7a23 and 8a11 that had been trapped from soils collected from the Shark Bay Salt lease area (Section 3.2.2), was used to inoculate the positive *A. tetragonophylla* F.Muell. controls at 3.9 10^7 cells g^{-1}.

The clay inoculant for *A .ligulata* was prepared with a pure culture of GL2L grown on CRS-M1 agar containing (g L^{-1}) Mannitol, 5; D-glucose, 5; Yeast extract, 1.25; $MgSO_4$, 0.8; NaCl, 0.1; $CaCl_2.2H_2O$, 0.2; K_2HPO_4, 0.0174; KH_2PO_4, 0.0136; $FeSO_4.7H_2O$, 0.005; Agar, 15 and trace elements (mg L^-) $Na_2B_4O_2$, 2.34; $MnSO_4.4H_2O$, 2.03; $ZnSO_4.7H_2O$, 0.22; $CuSO_4.5H_2O$, 0.08; $Na_2MnO_4.2H_2O$, 0.13 (Howieson *et al.*, 1988) from -20°C glycerol stock (section 3.2.1.2) for 4 days at 28°C. This was used to inoculate a 300 mL CRS-M1 broth at pH 7.0 (Howieson *et al.*, 1988), incubated for 3 days at 28°C and shaken at 200 rpm. The culture broth was added to a pre-inoculant preparation and then mixed with bentonite provided by Alosca®Technologies Pty Ltd, which had been sterilised twice at 121°C for 30 min. The resultant mixture was left at 28°C until completely dry and then was crushed to fine granules. The bacterial count of the inoculant was determined by the Miles and Misra method (Miles *et al.*, 1938). Inoculant was prepared for *A. tetragonophylla* F.Muell. was prepared in the same way with the exception that the original culture broth was a mixuture composed of eight 40 mL individual CRS-M1 broths inoculated with isolates 1a13, 2a11, 3a23, 4a13 5a16, 6a15, 7a23 and 8a11.

Four containers for both *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. were uninoculated as negative controls. Plants were examined for the presence or absence of nodules eight weeks after inoculation and the number of RNB with 95% confidence limits for each soil were estimated using the MPNES program (Woomer *et al.*, 1990).

2.3 Results

2.3.1 Chemical characteristics of soils

There were marked differences in the chemical characteristics between the pit and undisturbed soils at site Q1. The concentrations of nitrate, phosphorus and organic carbon in the pit soil were reduced compared to those in the undisturbed soil while the concentrations of potassium, sulfur and the conductivity of the pit soil were considerably greater than those of the undisturbed soils (Table 2.3). Concentrations of phosphorus and organic carbon were similarly reduced in the pit soils of P and G; however the re-vegetated area in pit G had similar concentrations of organic carbon to that of the undisturbed soil (Table 2.3). Iron concentrations at pit P were one third of those in the adjacent undisturbed soil while at pit G they were three times greater than the levels in the undisturbed soil (Table 2.3). At site R, the concentrations of nitrate, phosphorus, potassium, sulfur, organic carbon and iron were all reduced in the pit soil in comparison to the adjacent undisturbed soil. The soils at all sites were alkaline with pH (0.01 M $CaCl_2$) ranging from 7.9 at G revegetated area to 8.1 at pits Q1 and R and the undisturbed sites of G and P (Table 2.3).

2.3.2 Rainfall and Temperature

2.3.2.1 Rainfall

Rainfall in the SBSLA can vary significantly from year to year and historically the annual rainfall ranges from 140 mm (2002) to 370 mm (1984) with a long-term average of 225 mm (Figure 2.2). However, annual rainfall in 2006 and 2007 was 58.7 mm and 79.3 mm respectively, the two lowest rainfall years since meteorological recording in the area begun while the highest annual rainfall of 513.6 mm in 2008 (Figure 2.2). The majority of the rainfall was between the months of May and August, though events do occur during summer and can be attributed to cyclonic weather as seen for 2008, 2004 and 2000 (Figure 2.3).

Table 2.3: Physical and chemical properties of soils from excavated borrow pits, adjacent undisturbed sites and revegetated sites within the Shark Bay Salt lease area

Physical and Chemical characteristics	Soil site								
	G			P		Q1		R	
	Pit	Revegetated	Undisturbed	Pit	Undisturbed	Pit	Undisturbed	Pit	Undisturbed
Texture[a]	1.5	1.5	1	1.5	1.5	1.5	1.5	1.5	1.5
Colour[b]	BROR	BROR	LTBR	LTBR	LTBR	LTBR	LTBR	BRWH	LTBR
Nitrate (mg kg^{-1})	1	1	1	1	3	1	10	1	3
Ammonium (mg kg^{-1})	1	1	1	1	1	1	1	1	1
Phosphorous (mg kg^{-1})	2	2	16	3	6	4	13	4	10
Potassium (mg kg^{-1})	47	44	20	53	54	292	99	19	69
Sulfur (mg kg^{-1})	4.1	4	6.9	5.7	9	141	19.7	4.1	12.2
Organic Carbon (%)	0.0	0.16	0.12	0.24	0.63	0.17	0.61	0.1	0.56
Iron (mg kg^{-1})	167	141	54	32	96	96	100	122	151
Conductivity (dS m^{-1})	0.091	0.081	0.106	0.091	0.093	2.294	0.202	0.072	0.125
pH (0.01 M $CaCl_2$)	8	7.9	8.1	7.8	8.1	8.1	7.9	8.1	7.9
pH (H_2O)	9.4	9.3	9.3	9.2	9.2	9	8.6	9.5	9.1

[a] 1 course sand, 1.5 sandy loam
[b] LTBR light brown, BROR brown-orange, BRWH brown-white

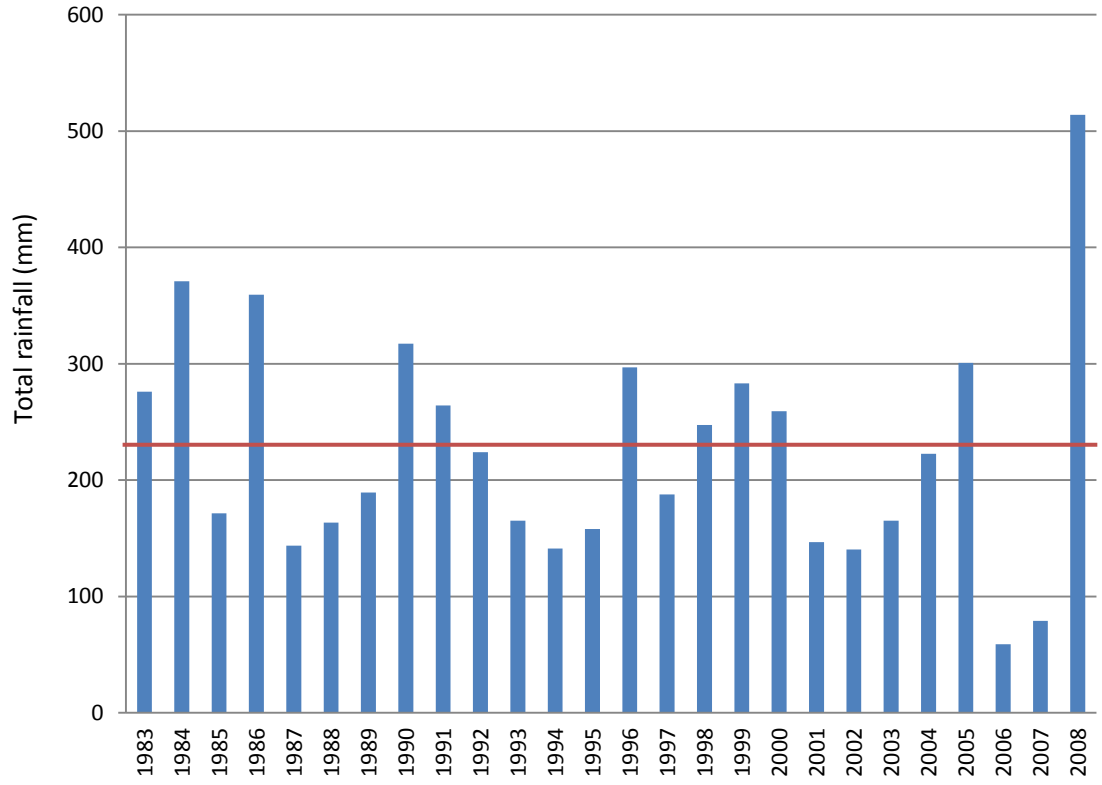


Figure 2.2: Rainfall of Shark Bay salt lease area from 1983 to 2008. The red line represents average annual rainfall of 225 mm. Field studies in this thesis were conducted during 2006, 2007 and 2008.

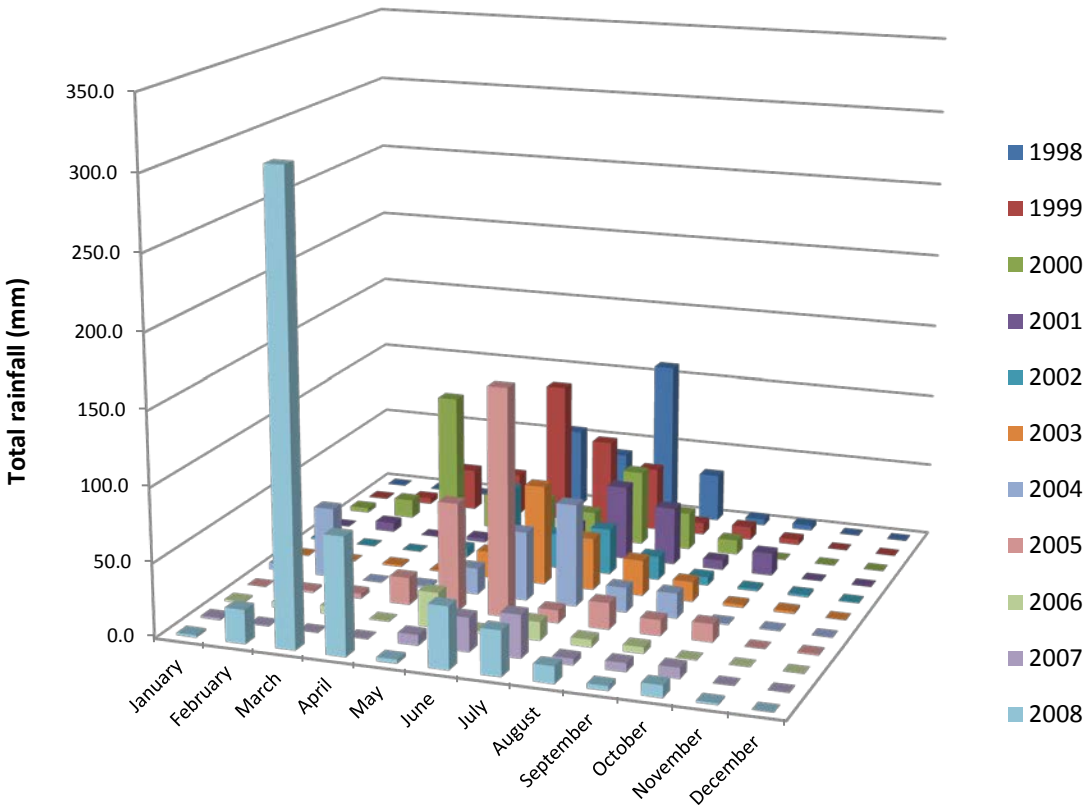


Figure 2.3: Monthly rainfall distribution at Shark Bay Salt lease area for the years 1998 to 2008

2.3.2.2 Temperature

At site P, there was little difference in temperature of the soil subsurface to a depth of 10 cm over the 12 month period from 17 July 2007 to 21 June 2008 (Figure 2.4). The undisturbed soil subsurface average temperature over this time was 24.5°C compared to 23.7°C in the pit P subsurface soil. The lowest recorded temperature at both sites was 11.3°C while the highest was 40.5°C and 38.7°C for undisturbed and pit subsurface soils respectively.

The average temperature for the seven months from 21 June 2008 to 3 February 2009 was 22.3°C for the soil surface at both pit P and the undisturbed area and there was little difference in the daily temperatures between the sites (Figure 2.5). The lowest recorded temperature for both soil surface sites was 4.1°C while the highest recorded was 57.1°C and 55.0°C for the undisturbed and pit P soil surface respectively.

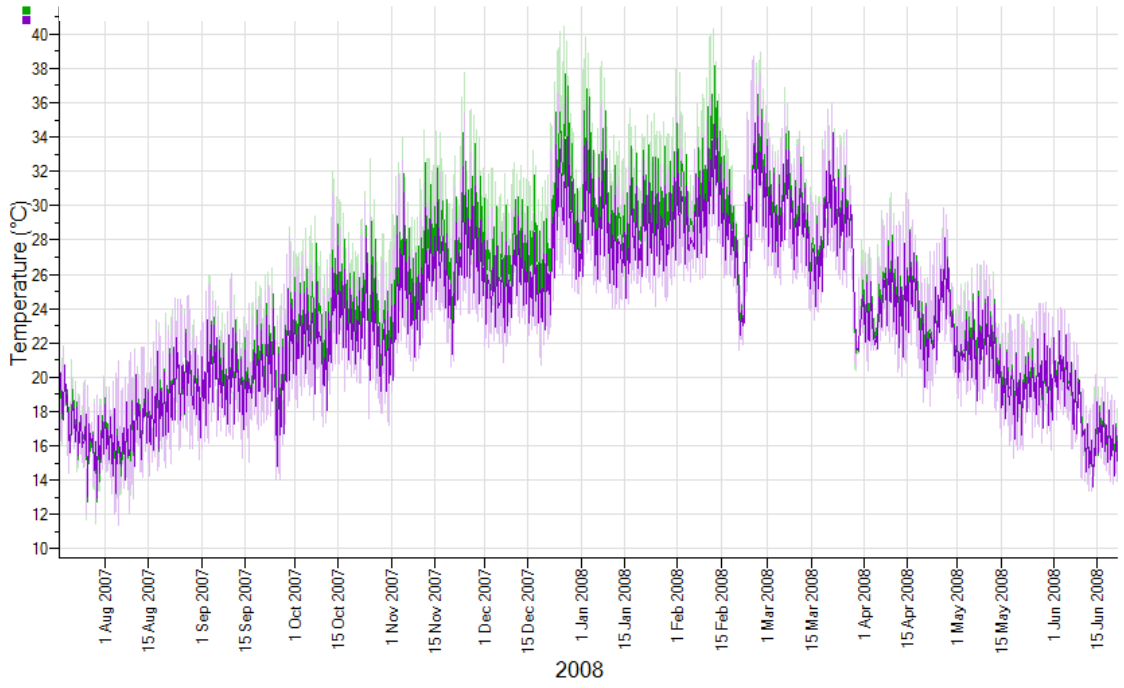


Figure 2.4: Temperature of the soil subsurface at 10 cm depth at Pit P (purple) and an adjacent undisturbed (green) area within the Shark Bay Salt Lease area. Temperature recorded from 17 July 2007 to 21 June 2008.

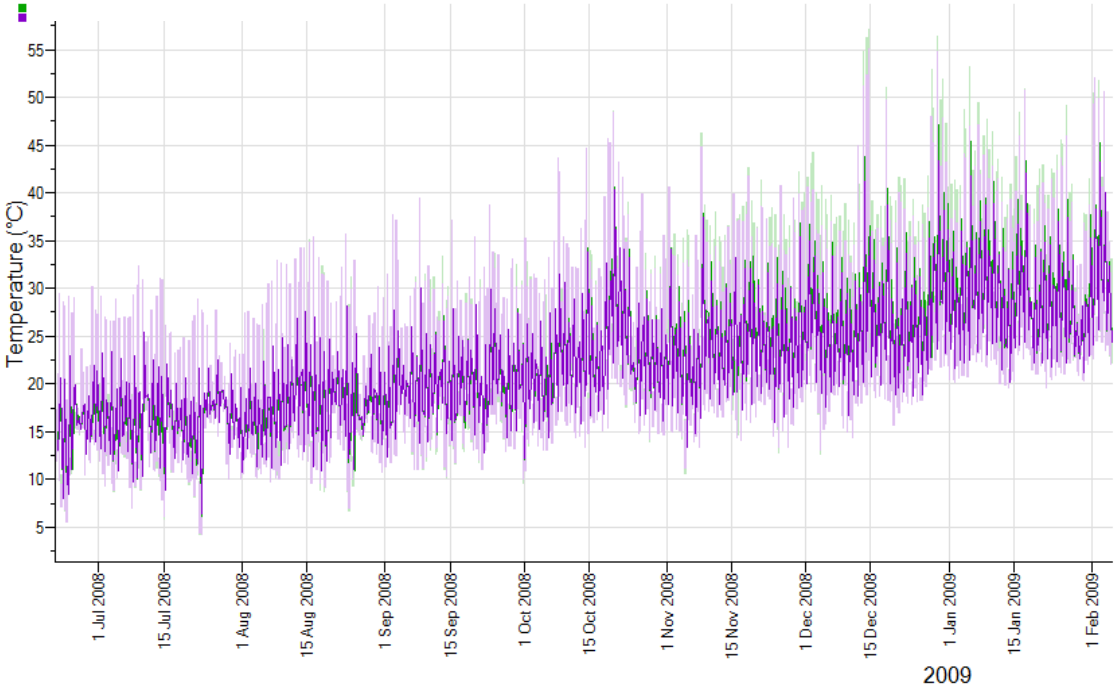


Figure 2.5: Temperature at the soil surface of pit P (purple) and an adjacent undisturbed (green) area within the Shark Bay Salt Lease area. Temperature recorded from 21 June 2008 to 3 February 2009.

2.3.3 Vegetation survey

2.3.3.1 Flora survey and vegetation profile

No vegetation was present in pit Q1, while P and G had 1.3% and 4.8% average total foliage cover in the quadrats surveyed. The vegetation, when present, was sparsely distributed and plants did not exceed 5 cm in height (Figures 2.6, 2.8 and 2.10). The undisturbed areas adjacent to pits P and G had similar foliage cover with 56.9% and 56.1%, while 63.5% was recorded at area Q1. The vegetation structure of the undisturbed sites was composed of different species in clusters with interspatial areas of no ground cover (Figures 2.7, 2.9 and 2.11). Undisturbed areas of P and Q1 had low growing plants that reached a maximum height of 60 cm and 80 cm, whereas larger plants were present at undisturbed area G, reaching up to 194 cm (Figures 2.7, 2.9 and 2.11).

The undisturbed areas at site G contained 33 floral species, 22 species were found in the area of P and in the area of Q1 there were 18 different floral species (Table 2.4). Ten species were identified in each of the pit sites P and G (Table 2.4). *Calotis multicaulis* (Turcz.) Druce, *Triodia plurinervata* N.T. Burb and the weed *Euphorbia peplus* L. were found at all sites (excluding pit Q1) (Table 2.4). Two other weed species were present, *Erodium cicutarium*(L.)L'Her. in the undisturbed G area and *Eragrostis barreleri* Daveau. in all the undisturbed areas (Table 2.4). Three species occurred frequently in pits P and G. *Triodia plurinervata* N.T. Burb., mentioned previously, was found in pits P and G quadrats at a frequency of 40% and 47% respectively (Table 2.4). *Angianthus acrohyalinus* Morrison. was found in 73% and 100% and *Euphorbia drummondii* Boiss. occurred in 67% and 80% of the quadrats in pits P and G respectively (Table 2.4). These two species were present in the adjacent undisturbed areas of P and G, but not Q1 (Table 2.4).

2.3.3.2 *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. survey

In 2008, when the site surveys were conducted, the larger *A. ligulata* Benth. plants appeared stressed or dying in all the quadrats in the undisturbed areas (Figure 2.12a, c, e). Site P had the largest number of *A. ligulata* Benth. present with a total of 15 plants (Figure 2.12c). Nine of the 15 *A. ligulata* Benth. were vigorous and of these, eight were recently germinated plants (Figure 2.12c). All the *A. tetragonophylla* F.Muell. found in the sites were vigorous or visibly growing, mature and well established (Figure 2.12b, d, f) and site G had the largest number of specimens with 18 plants (Figure 2.12b).

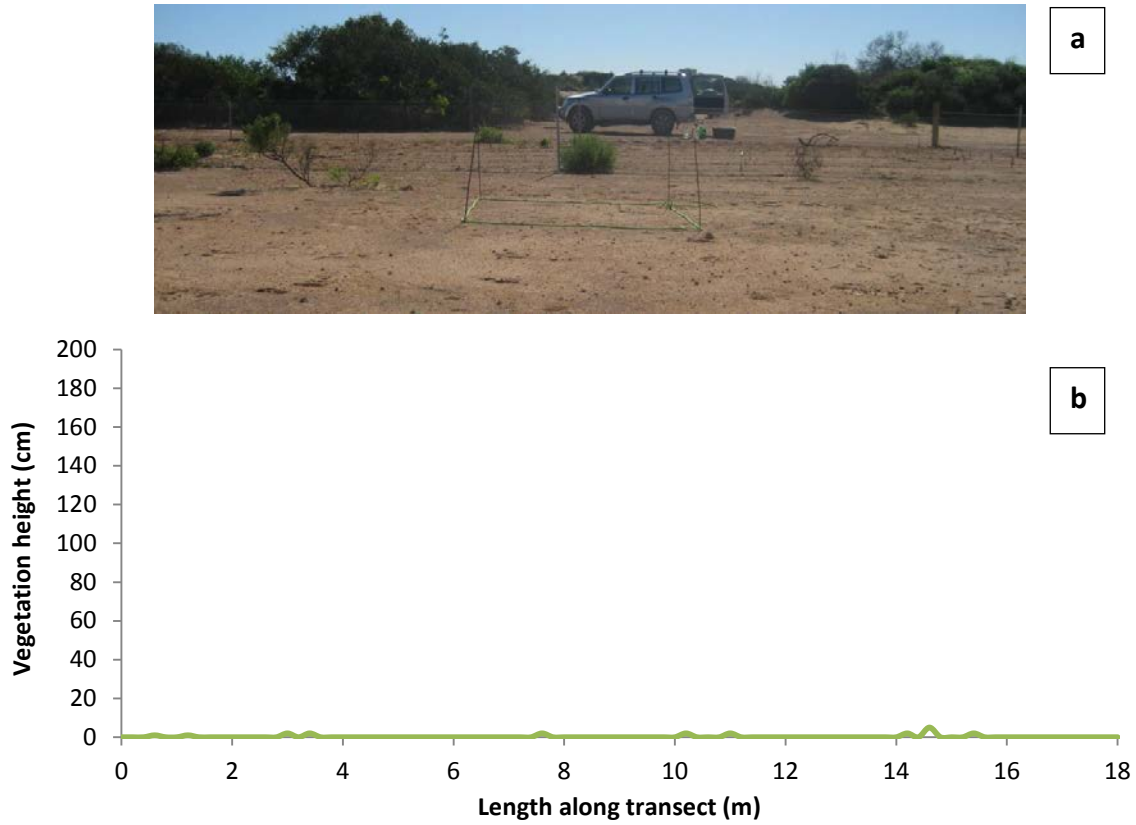


Figure 2.6: G pit transect 2 photo (a) and graph of height of flora along transect 2 (b)

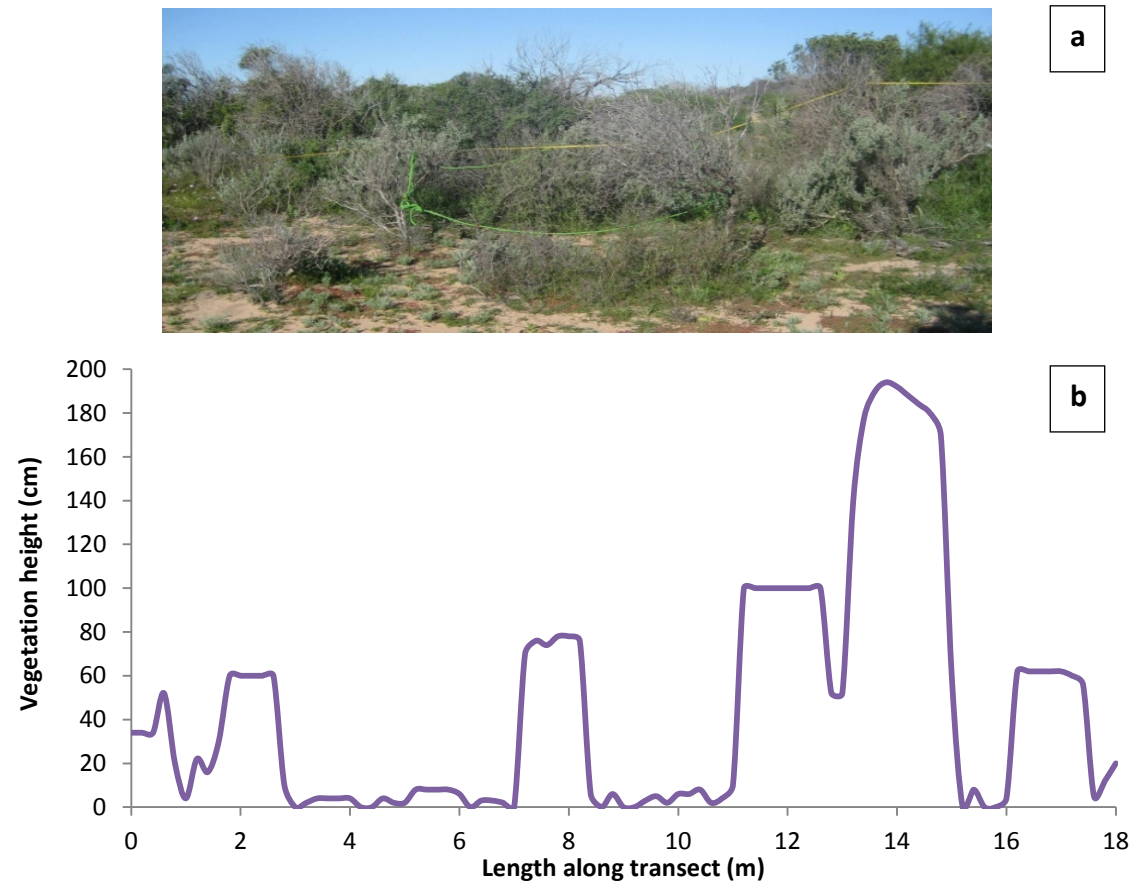


Figure 2.7: G undisturbed transect 2 photo (a) and graph of height of flora along transect (b)

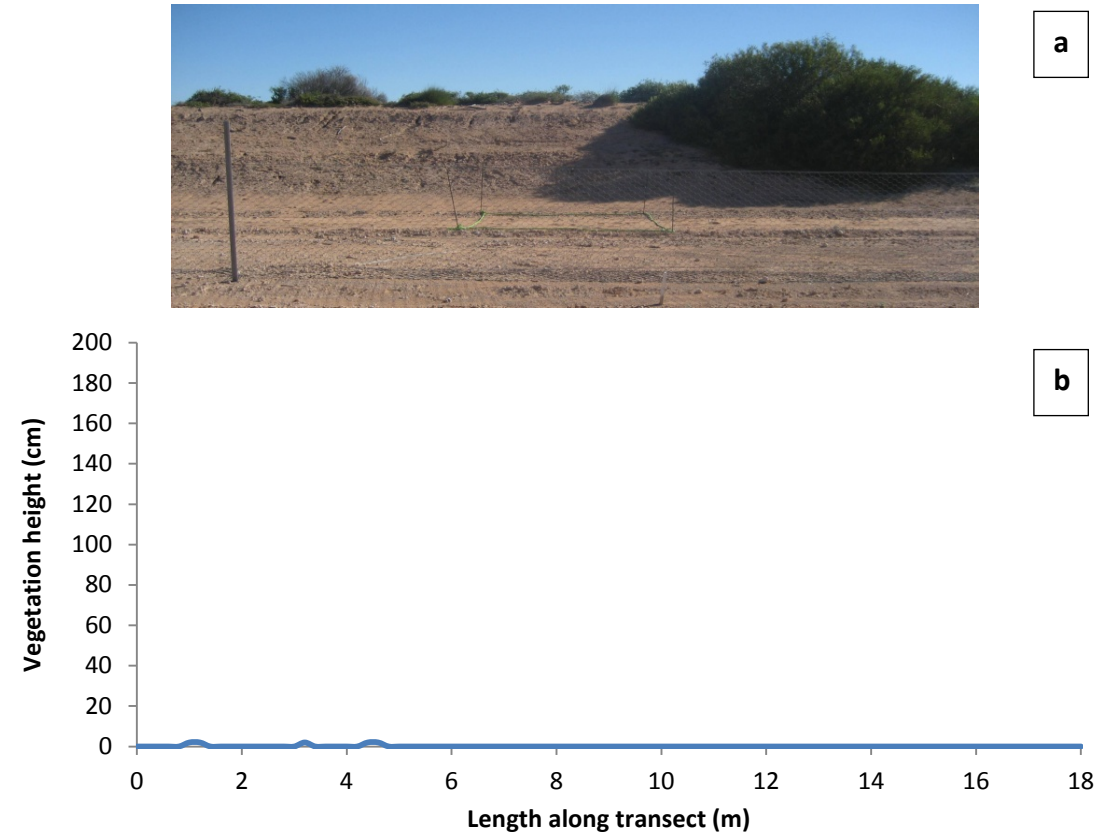


Figure2.8: P pit transect 2 photo (a) and graph of height of flora along transect 2 (b)

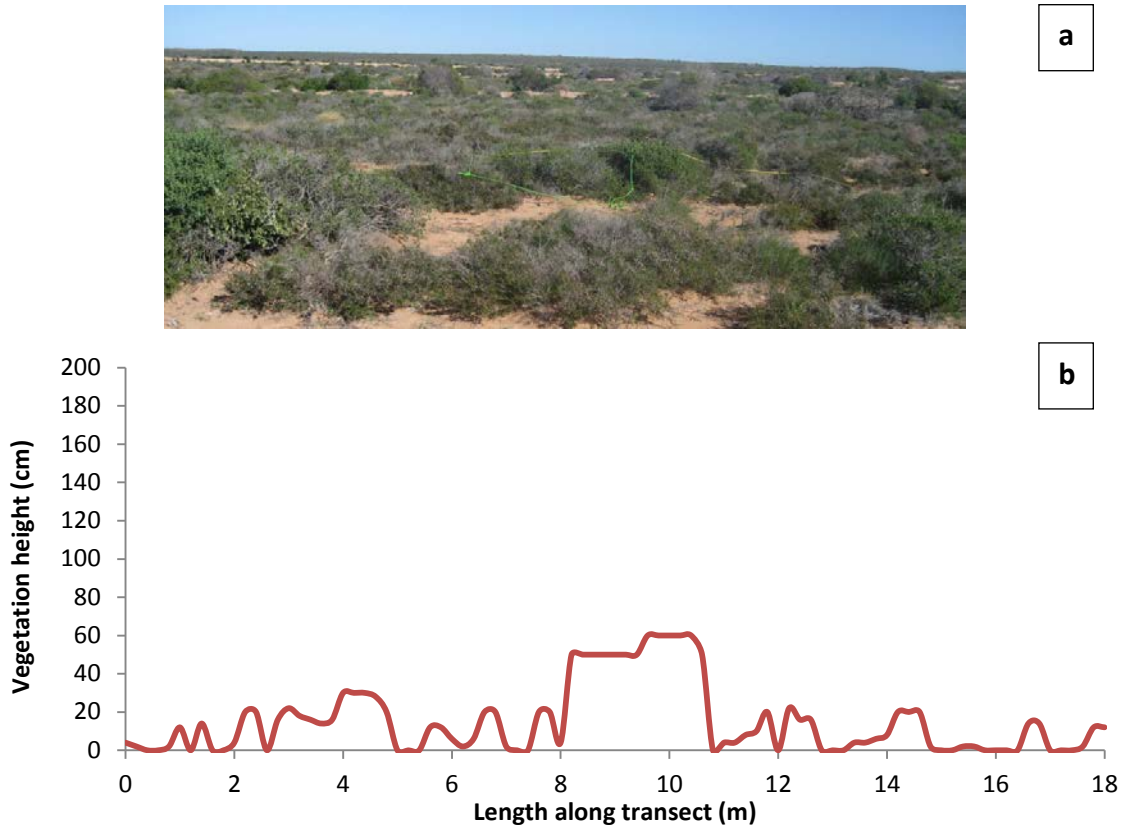


Figure2.9: P undisturbed transect 2 photo (a) and graph of height of flora present along transect 2 (b)

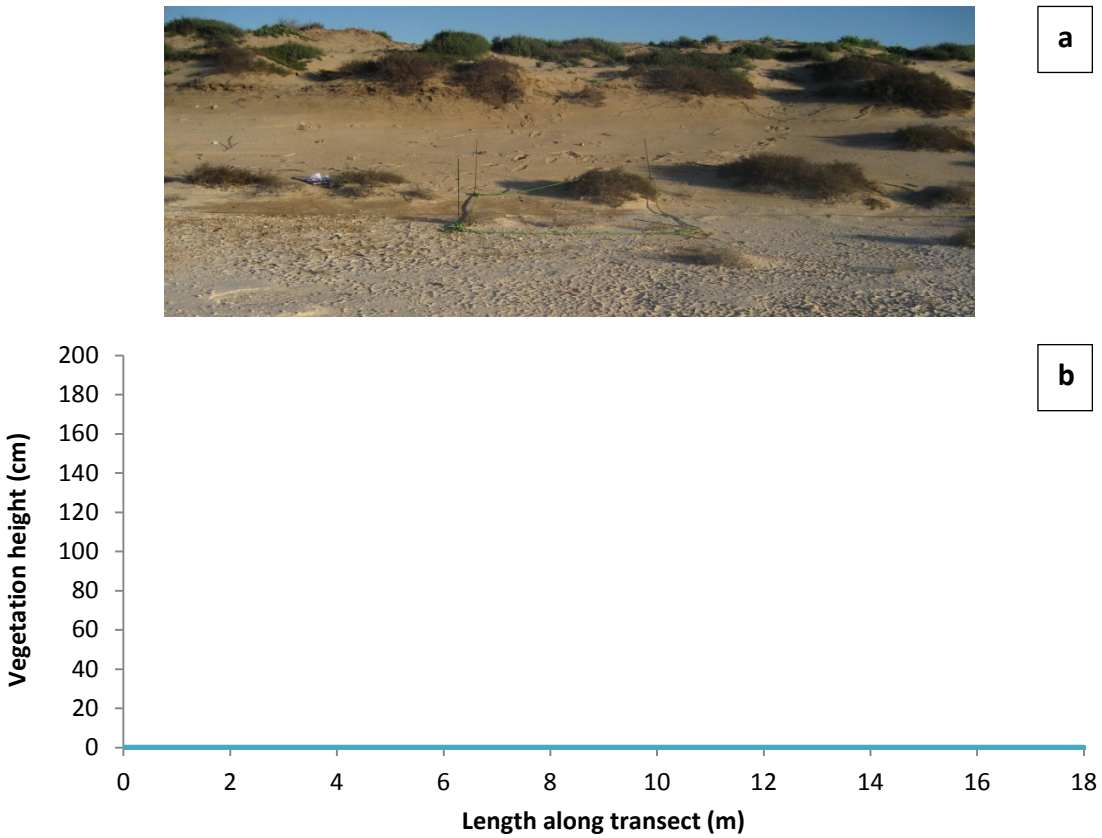


Figure 2.10: Q1 pit transect 2 photo (a) and graph of height of flora along transect 2 (b). No vegetation detected along transect.

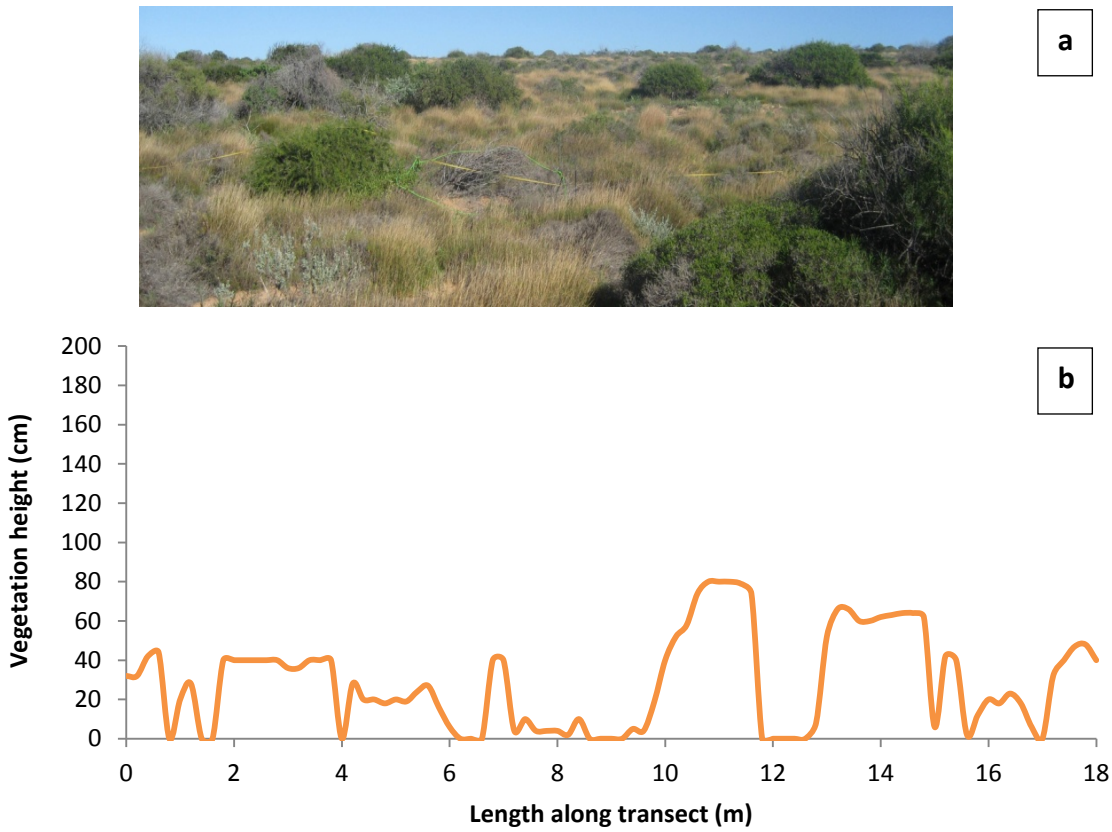
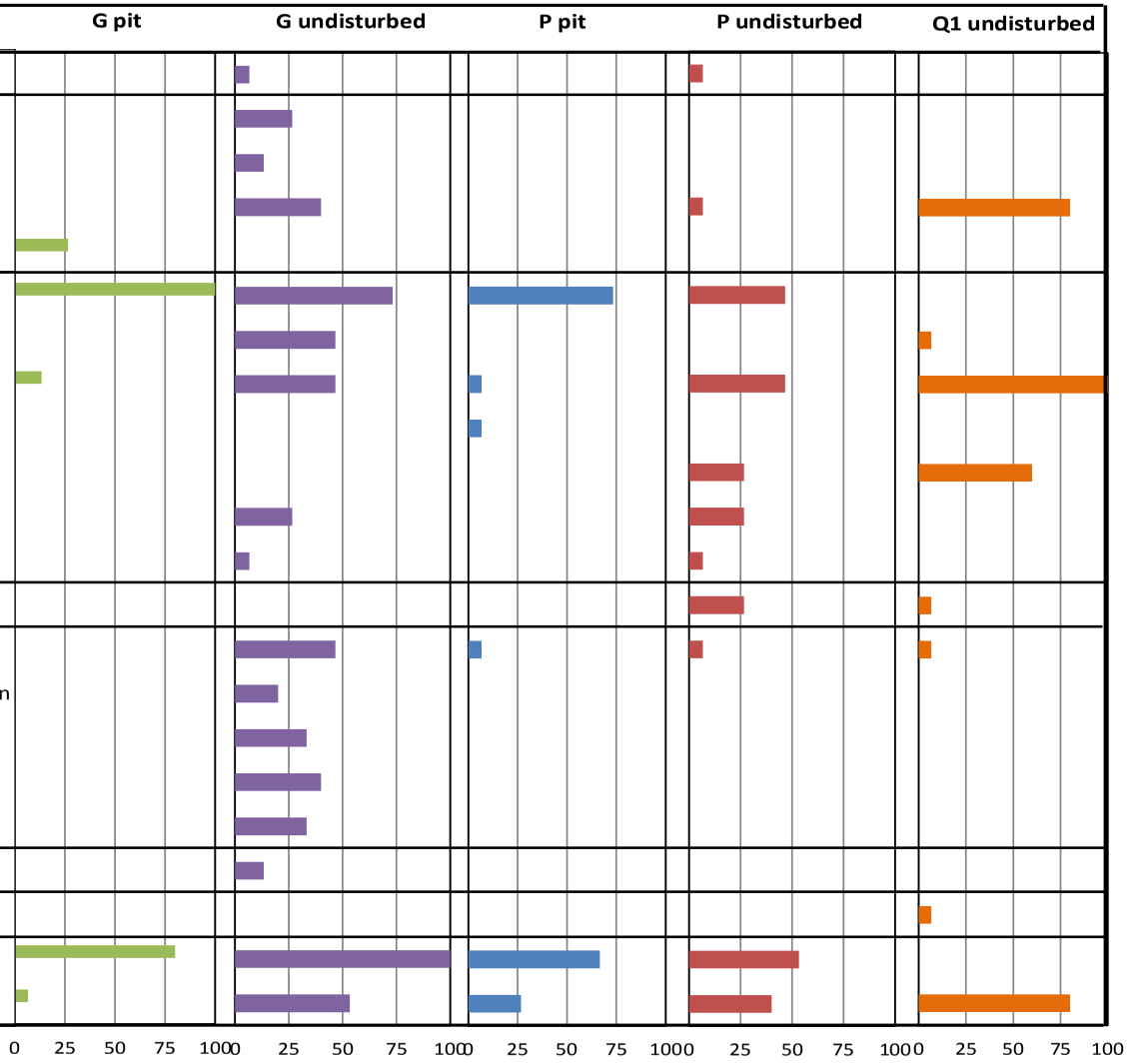


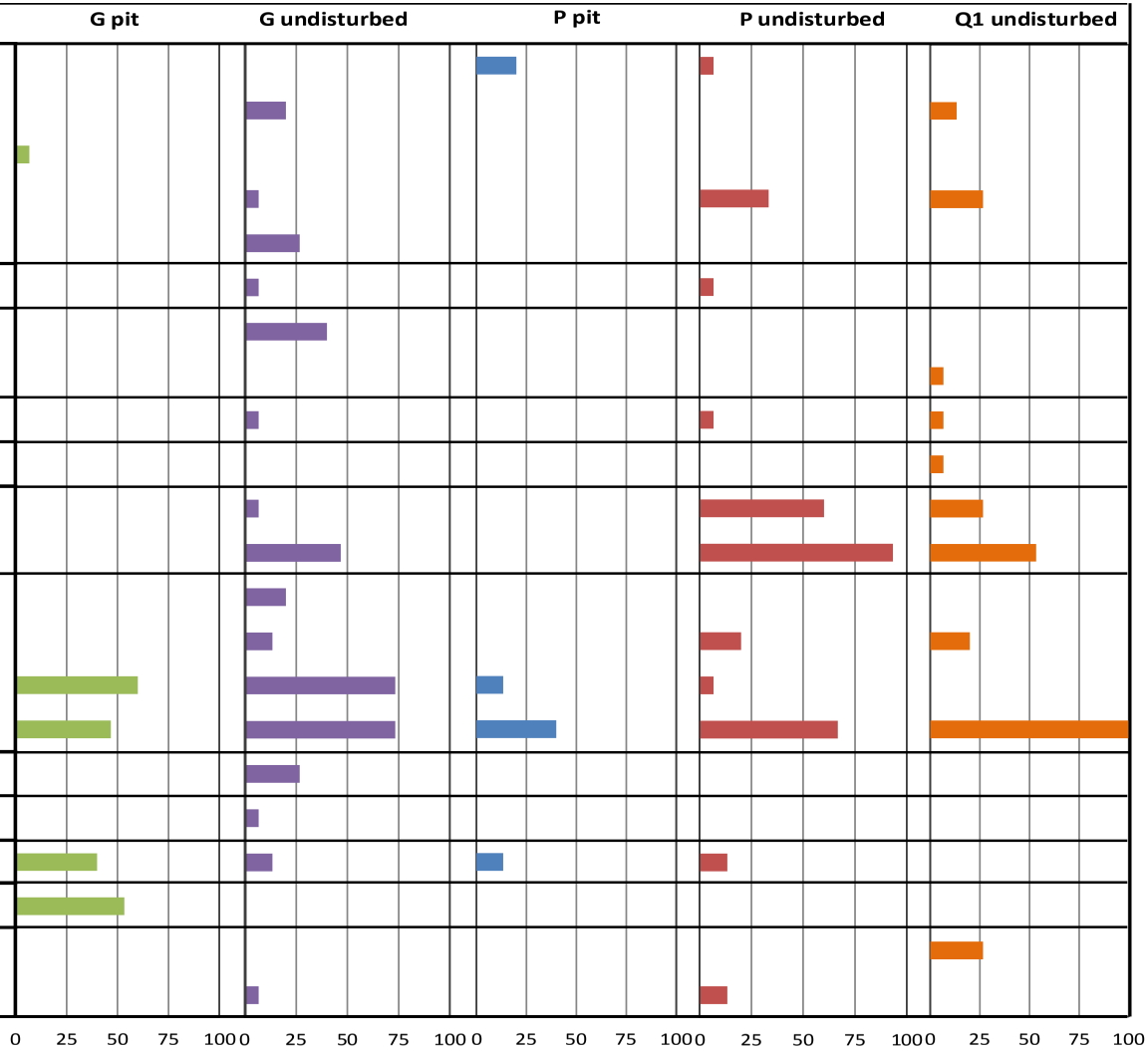
Figure 2.11: Q1 undisturbed transect 2 photo (a) and graph of height of flora along transect (b)

Table 2.4: Flora species occurrence at pit and undisturbed areas of Shark Bay lease area. Average total transect frequency of flora as a percentage.

Phyla/ Family	Species
Bryophyta	Bryophyta
Amaranthaceae	*Ptilotus divaricatus* (Gaudich.) F.Muell.
	Ptilotus obovatus (Gaudich.) F.Muell.
	Ptilotus nobilis (Lindl.) F.Muell.
	Ptilotus sp.
Asteraceae	*Angianthus acrohyalinus* Morrison
	Brachyscome ciliocarpa W.Fitzg.
	Calotis multicaulis (Turcz.) Druce
	Cephalipterum drummondii A.Gray
	Pembertonia latisquamea (F.Muell.) P.S.Short
	Rhodanthe sp.
	Waitzia podolepis (Gaudich.) Benth.
Boraginaceae	*Halgania littoralis* Gaudich. Halgania littoralis
Chenopodiaceae	*Atriplex bunburyana* F.Muell.
	Chenopodium gaudichaudianum (Moq.) Paul G.Wilson
	Maireana stipitata Paul G.Wilson
	Salsola tragus L.
	Threlkeldia diffusa R.Br.
Dasypogonaceae	*Acanthocarpus preissii* Lehm.
Dioscoreaceae	*Dioscorea hastifolia* Endl.
Euphorbiaceae	*Euphorbia drummondii* Boiss.
	Euphorbia peplus L.*



Phyla/Family	Species
Fabaceae	*Acacia ligulata* Benth.
	Acacia tetragonophylla F.Muell.
	Acacia sp.
	Mirbelia ramulosa (Benth.) C.A.Gardner
	Swainsona pterostylis (DC.) Bakh.f.
Frankeniaceae	*Frankenia pauciflora* DC.
Geraniaceae	**Erodium cicutarium* (L.)L'Her.
	Erodium cygnorum Nees
Goodeniaceae	*Scaevola tomentosa* Gaudich.
Malvaceae	*Alyogyne cuneiformis* (DC.) Lewton
Myrtaceae	*Melaleuca cardiophylla* F.Muell.
	Thryptomene baeckeacea F.Muell.
Poaceae	*Cenchrus ciliaris* L.
	Eragrostis barrelieri Daveau*
	Paractaenum novae-hollandiae P.Beauv. subsp.†
	Triodia plurinervata N.T.Burb.
Santalaceae	*Santalum spicatum* (R.Br.) A.DC.
Scrophulariaceae	*Eremophila oldfieldii* F.Muell
Solanaceae	*Solanum orbiculatum* Poir.
Surianaceae	*Stylobasium spathulatum* Desf.
Zygophyllaeae	*Zygophyllum fruticulosum* DC.
	Zygophyllum simile H.Eichler



† full name- *Paractaenum novae-hollandiae* P.Beauv. subsp. *novae-hollandiae*

* weed or introduced species

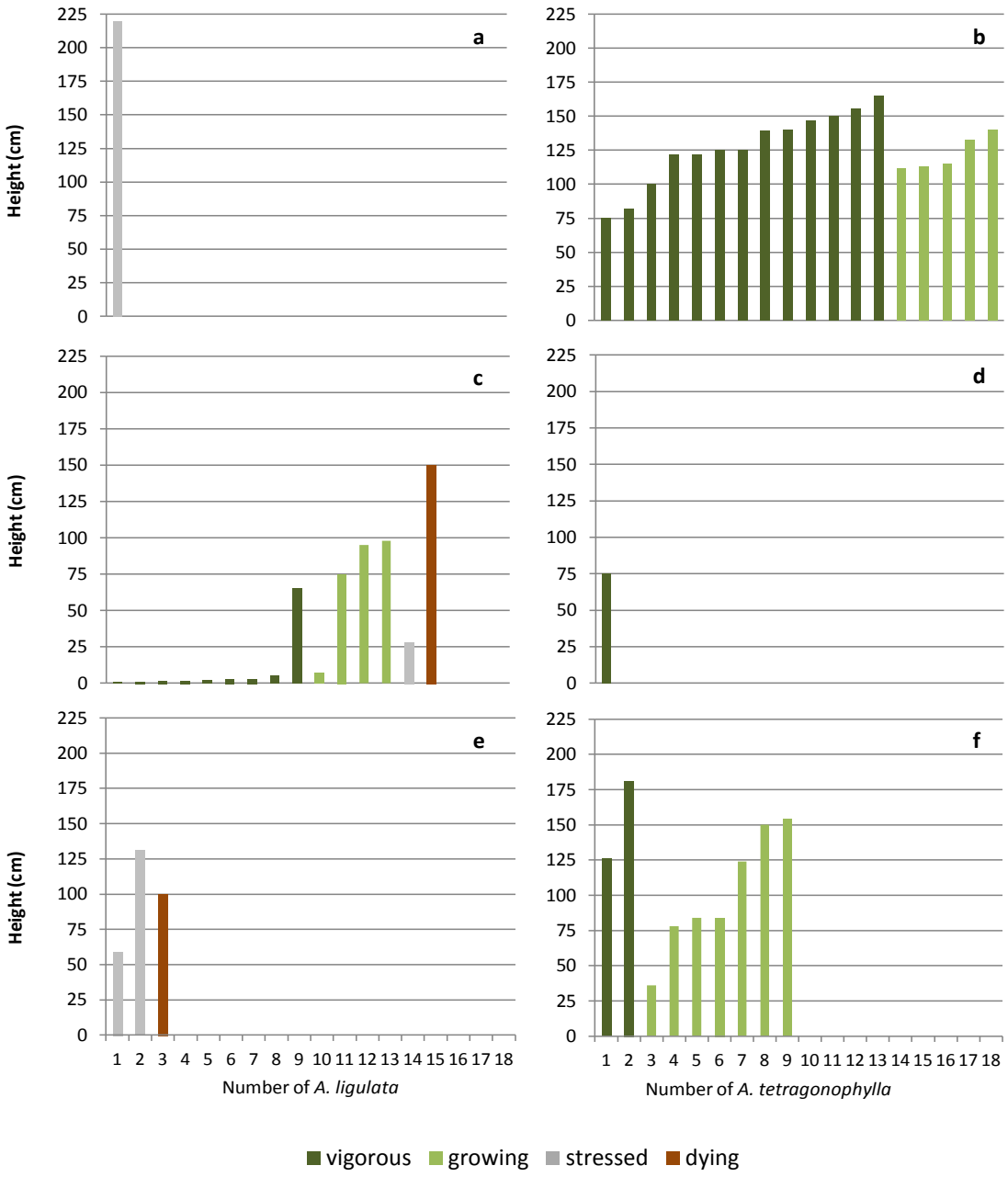


Figure 2.12 Height (cm) and health status of individual *A. ligulata* Benth. (a, c and e) and *A. tetragonophylla* F.Muell. (b, d and f) plants in the undisturbed areas adjacent to pits G (a and b), P (c and d) and Q1 (e and f). Health status was based on a visual assessment of the plants.

2.3.4 Most probable number of RNB in soil

In 2007 and 2008, populations of RNB were greater in undisturbed soils compared to pit soils at all three sites, with the exception of pit G in 2007 (Figure 2.13). RNB were not detected on *A. ligulata* Benth. or *A. tetragonophylla* F.Muell. from soils collected in 2007 from pits P and Q1 as well as Q1 2008 soil *(*Figure 2.13). Significantly greater

numbers of RNB that nodulate these two species were estimated in 2008 in comparison to numbers seen in 2007, with the exception of *A. ligulata* Benth. inoculated with site P soils (Figure 2.13). Other than in soil from the undisturbed sites P and Q1, at all other sites there were larger populations of RNB that nodulated *A. tetragonophylla* F.Muell. than nodulated *A. ligulata* Benth. and notably so in 2008 from undisturbed soil at site G (Figure 2.13). Soil collected in 2007 from the re-vegetated site in pit G had higher numbers than those in both pit and undisturbed soils.

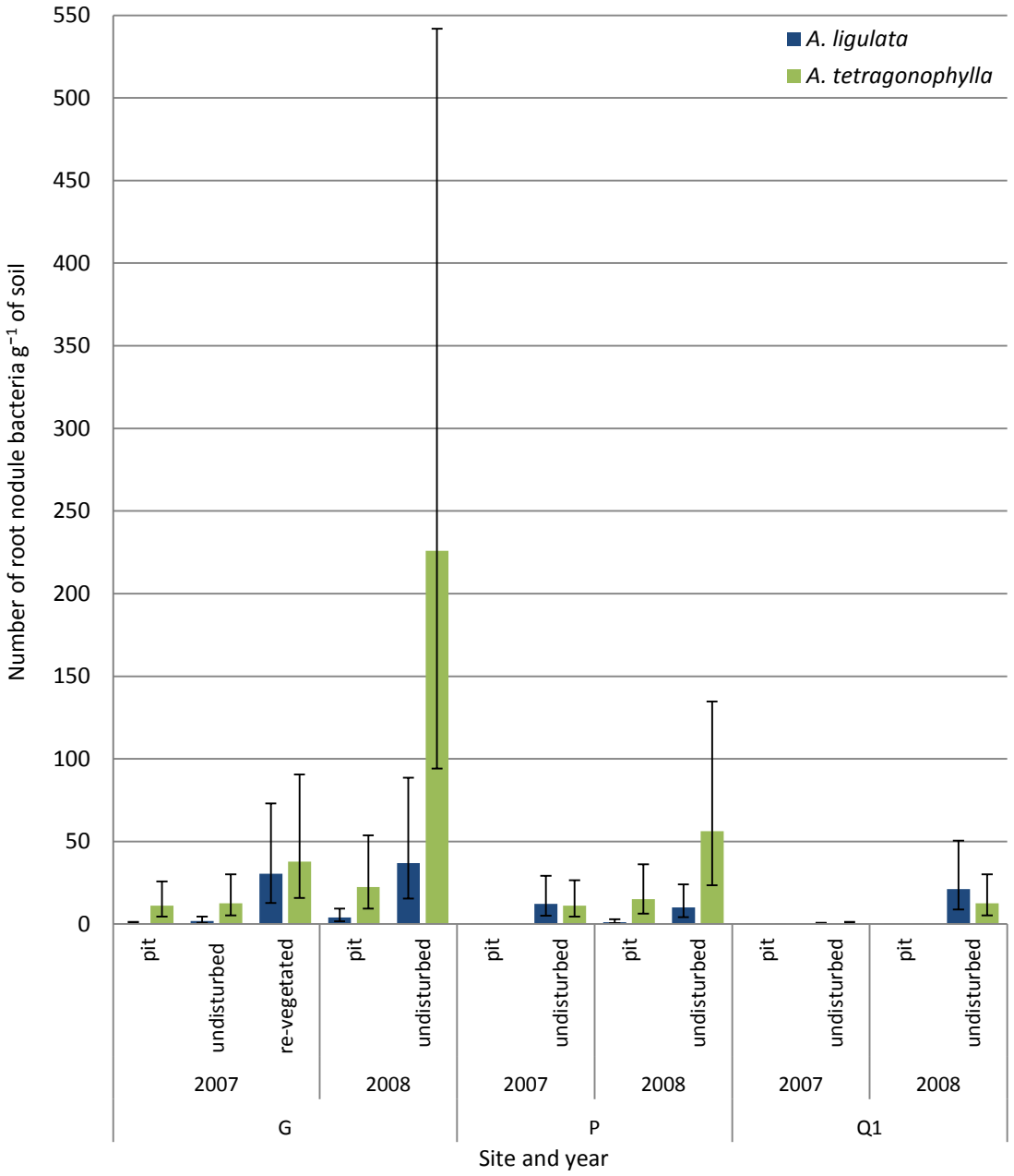


Figure 2.13: Most probable number of root nodule bacteria in soils from pit and adjacent undisturbed sites within Shark Bay lease area, detected on *Acacia ligulata* Benth. and *Acacia tetragonophylla* F.Muell. in soils collected in July 2007 and June 2008. Vertical bars represent range of confidence limit values (*P*=0.05).

2.4 Discussion

In this chapter, the removal of soil, subsoil and regolith to create borrow pits and the effects of this disturbance on the abiotic and biotic characteristics of Shark bay Salt lease area was assessed. At the time of this study, Shark Bay Salt reported pit area Q1 as disturbed, having been deactivated five years prior and declared pit areas G, P and R as no longer disturbed (SBSJV, 2005). However, with the exception of a fenced area at G, the selected pit sites were devoid of an established floral community. This is despite the various attempts at rehabilitation in G and P, which had been inactive for up to 15 years. The pit disturbance has also been deleterious to the soil biota, with reduced populations of RNB that nodulate *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. when compared to populations in the adjacent undisturbed area soils.

The distribution of vegetation in the undisturbed sites of G, P and Q1 was a mosaic of perennial species providing canopy with understory annual species and sparsely vegetated interspatial areas (Figures 2.7, 2.9 and 2.11). This vegetation patchiness is often associated with low rainfall and the movement of water across the landscape (Ludwig *et al.*, 1999; Tongway & Ludwig, 1997). Canopied areas are termed “islands of fertility” in arid ecosystems and are associated with greater nutrient availability and soil microbial activity (Bennett & Adams, 1999; Charley & West, 1975; Davidson & Morton, 1984; Zhao *et al.*, 2007) as well as increased water holding capacity (Muller & Muller, 1956).

Not only is the microbial biomass under canopies greater than interspatial areas but the microbial composition is diverse and different (Kaplan *et al.*, 2013). RNB, like many other microbes, are heterogeneous in an arid landscape, however the soil collected from the undisturbed areas at G, P and Q1 were composite samples of both canopied and interspatial areas at these sites. With the RNB undetectable in a number of pit sites, and *A. ligulata* Benth. RNB ranging from 0.52 to 36.92 cells g^{-1} and *A. tetragonophylla* F.Muell. between 0.55 to 225.95 cell g^{-1} in 2007 and 2008, these composite RNB estimates are comparable to other arid biomes that have been similarly assayed. In the soils of Zambia, Malawi and Zimbabwe, RNB of *Sesbania*

sesban were generally less than 50 cells g^{-1} (Bala *et al.*, 2002). In South Western Spain, RNB able to nodulate *Phaseolus vulgaris* L. cv. Arrocina ranged from between 3.6 to 42 cells g^{-1} (Rodriguez-Navarro *et al.*, 2000). RNB of *Psorothamnus spinosus* were undetectable by MPN methods in the soils of the Sonoran Desert of California by Jenkins *et al.* (1988b). Thrall *et al.* (2007) also reported undetectable numbers of RNB that nodulate *Acacia salicina* Lindley. and *Acacia stenophylla* A.Cunn in a number of soils from central New South Wales but up to 1.17×10^6 cells g^{-1} in others.

The presence of complex microbial communities of the soil surface in the form of biological soil crust (BSC) are also features of arid ecosystems and are major contributors to not only nutrient availability but, dependant on BSC composition, also the movement and retention of water across the landscape (Belnap, 2006; Eldridge & Greene, 1994; Pointing & Belnap, 2012). Booth (1941) found that the water holding capacity was much greater in soils that had a BSC as opposed to those soils without a crust. Bryophyta occurred in the undisturbed areas of both G and P (Table 2.4) and is a recognized component of BSC in arid Australia (Büdel, 2003). It is highly likely that other BSC organisms were present in the undisturbed areas when the floristic survey was conducted, but these can be difficult to detect when not hydrated and easily confused with raindrop impact crusts. There was no observed BSC or BSC organisms in the pit areas. Once disturbed, the recovery rates of BSC organisms in low soil moisture conditions is very slow and can take decades (Pointing & Belnap, 2012).

The influence of soil moisture is particularly evident in the difference seen in the RNB populations between the years 2007 and 2008 (Figure 2.13). Rainfall was the single major difference between these two years with 79.3 mm and 513.6 mm for 2007 and 2008 respectively (Figure 2.2). Increased soil moisture not only promotes legume growth but also stimulates the proliferation of RNB (Rupela *et al.*, 1987; Woomer *et al.*, 1988a) and their ability to fix nitrogen (Sprent, 1972). The response of RNB to increased soil moisture was most evident in the populations in the undisturbed area of G, where there was a 19 and 18-fold increase in the RNB estimates on *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. respectively, from 2007 to 2008 (Figure 2.13). The estimates obtained would no doubt vary depending on the time the soils were

collected and the frequency and degree of rainfall prior to this. RNB populations in the top 150 mm of soil demonstrate seasonal fluctuations and generally decrease in number at greater depths in agricultural systems (Chatel & Parker, 1973; Rupela *et al.*, 1987). However, Virginia *et al.* (1986) found greater abundance of *Prosopis glandulosa* RNB at 5 m in the soil profile of the Sonoran desert and the RNB of *Acacia albida* have been detected in sufficient numbers at depths of up to 34 m in the Sahelian area (Dupuy & Dreyfus, 1992). Aside from the proximity of the soils collected to *P. glandulosa* and *A. albida*, both Dupuy & Dreyfus (1992) and Virginia *et al.* (1986) claimed that the depth of the water table at these sites sustained the RNB populations. The excavation of the pits is unlikely to have affected water infiltration due to the sandy soils present in these sites (Table 2.3); however, the lack of vegetation and MPN estimates of RNB suggests the ability to retain moisture in the pit soils had been reduced.

High evaporation rates was one of the principle reasons for the location of the salt ponds in Shark Bay, resulting in surface soils that can be severely depleted of soil moisture depending on rainfall and temperature. While the alleviation of drought increases RNB abundance, moist soil conditions coupled with high temperatures compromises RNB survival (Graham, 1992; Wilkins, 1967; Zahran, 1999). It is doubtful the populations of RNB in the pit and undisturbed sites were adversely affected by temperature. Surface and subsoil temperatures were between 17 and 22°C at the time the soils were collected in 2007 and 2008 with temperatures during the summer months up to 57°C on the surface and 40.5°C in the subsoil (Figures 2.4 and 2.5). While the summer soil temperatures recorded are suboptimal for forming and maintaining legume-RNB symbioses, Wilkins (1967) reported that RNB native to dryland soils of New South Wales were able to survive prolonged periods of up to 100°C and then with the onset of ideal conditions, effectively nodulated with their symbiotic host (Wilkins, 1967).

The MPN estimates may have been affected by both a provenance effect of the seed and the legume species associations at the different sites at Shark Bay Salt lease area. For the assay, RNB were trapped on *A. tetragonophylla* F.Muell. grown from

provenance seed whereas the *A. ligulata* Benth. seed was from an unknown site, most likely in southern Western Australia. Phenotypic variation due to location has been observed with *A. ligulata* Benth. and it is one of 12 species in the informal '*Acacia bivenosa* group' with which it readily hybridizes (Chapman & Maslin, 1992; Maslin, 2001). Therefore, while the seed may have been collected from an *A. ligulata* Benth. specimen, the seed genotype may be mixed. The delineation of provenance based on species genotype can be quite constrained, with Krauss and He (2006) demonstrating restricted dispersal of both *A. rostellifera* Benth. and *A. cochlearis* (Labill.)H.L Wendl.. These differences in genotype of *A. ligulata* Benth. may have had implications in the number of RNB estimated on this species, given that the MPN assay is known to produce a trapping bias effect, dependant on host species selection (Woomer *et al.*, 1988b). The MPN estimates of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. appear contrary to the probable significance to the nitrogen economy of their respective plant communities reported by Beadle (1964), where *A. tetragonophylla* F.Muell. is noted as probably insignificant due to the rareness of observed nodules in the field. However, numerous nodules have been detected on *A. tetragonophylla* F.Muell. roots following a flush of growth after sufficient rainfall (Brockwell *et al.*, 2005).

The undisturbed areas of G are associated with stands of *A. tetragonophylla* F.Muell. and occasional *A. ligulata* Benth. and *A. rostillifera* Benth., whereas *A. ligulata* Benth. is associated with the vegetation of P and Q1 (SBSJV, 1998). The vegetation survey in 2008 also recorded a greater diversity of legumes occurring in the undisturbed area of G and in higher frequency than the undisturbed areas of P and Q1 (Figure 2.4). The abundance of *A. tetragonophylla* F.Muell. RNB in 2008 was four and 18-fold greater in the undisturbed soils of G in comparison to those of P and Q1 (Figure 2.13). The difference was not as great with *A. ligulata* Benth. RNB in 2008 where the population in undisturbed G soils was 3.7-fold greater than in P and 1.75-fold greater than in Q1. This delineation effect of RNB populations with respect to geographic locality and host specificity has been widely reported (Barrett *et al.*, 2012; Burdon *et al.*, 1999; Thrall *et al.*, 2007). Thrall (2007) found with two closely related *Acacia* spp., that there was a clear difference in the specificity of the RNB associations, with MPN estimates

generally greater with *A. salicina* Lindley. than with *A. stenophylla* A.Cunn ex Benth. across 58 soil sites. It may be that *A. tetragonophylla* F.Muell. exhibits the traits of a generalist in regards to its range of RNB symbiotic associations, whereas *A. ligulata* Benth. may have a specialist approach. The seeding of other legumes such as *Kennedia* sp. and *Acacia* sp. with the revegetation efforts at G may have also unwittingly led to the introduction of RNB genotypes on the seed that *A. tetragonophylla* F.Muell*.,* as a generalist is able to form symbioses. If introduced RNB have been able to withstand the environmental conditions at this site and persist, the presence of suitable legume species would influence the community structure and population of RNB in the rhizosphere (Bais *et al.*, 2006; Graham, 2008).

The chemical properties of the pit and undisturbed area soils were determined from composite samples and found to be comparable to those of other soils in arid Australia (Bell *et al.*, 2003; Davidson & Morton, 1984). Low soil fertility is not unexpected in the Shark Bay Salt lease area where the Pleistocene limestone and sandstone would have been leached of nitrogen and the phosphorous bound to calcium ions, a characteristic of many of Australia's ancient soils (CALM, 2005; Lambers *et al.*, 2008; Nix, 1981). Although nitrogen, organic carbon and phosphorous were reduced in the pits in comparison to the undisturbed areas, it has not impeded sustaining an established revegetated area as seen in pit G, but may contribute to lower populations of soil microbes, including RNB (Bastida *et al.*, 2006). These nutrients, as well as potassium, calcium, magnesium, iron and sulfur are required in the symbiotic and saprophytic states of RNB for metabolism (O'Hara, 2001). The continued lack of vegetation and BSC in the pits could be contributory to differences in some of these chemical properties between the pit and undisturbed sites (Table 2.3).

The salinity of pit Q1 is at toxic levels and this would be a major obstacle to attempt revegetation with perennial legume species. It certainly was shown to affect RNB populations, as none were detected in either 2007 and 2008 (Figure 2.13). There is a strong correlation between RNB in alkaline conditions and their ability to tolerate salinity (Ruiz-Díez *et al.*, 2009; Shamseldin & Werner, 2005; Zahran, 1999). However, where the salinity was at a level that would sustain both legume flora and RNB,

nodulation and nitrogen fixation is likely to be constrained (Bordeleau & Prévost, 1994).

The activities in the pits since their excavation may be an additional hindrance in the rehabilitation of these areas. The rehabilitation methods adopted at pits G and P included deep ripping, fertilizing and seeding which are effective protocols in temperate areas and accepted as 'standard practice' (Mulligan *et al.*, 2006). Ripping or moonscaping may give short term results for water and seed trapping but can lead to instability and erosion, impeding the establishment of a BSC (Belnap, 2006). Australian native plants have highly variable nutritional requirements and the application of fertiliser can encourage weed invasion rather than improving seedling survival (EPA, 2005). Australian native plants themselves have the potential to become weed species outside of their natural areas of distribution, with over 50% of Western Australian taxa recognised as environmental weeds (Keighery & Vanda, 2004). Therefore, the seeding of G with a seed mix of south Western Australian species, while successfully established within a fenced area, may have long-term implication for the site. Weed infestations of *Nicotina* sp., *Schinus* sp. and the Western Australian, *Eucalyptus platypus* had already been reported at various sites within the Shark Bay Lease area (SBSJV, 1998; SBSJV, 2005).

The undisturbed areas were selected as ecosystems of reference for standards of comparison and evaluation to the pit sites (Aronson *et al.*, 1993). The Western Australian Rangeland Monitoring System (WARMS) determines ecosystem health by the composition and density of key perennial spp. (Pringle *et al.*, 2006) and *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. would be useful species for this in the area of Shark Bay Salt. Given the importance of soil microflora in nutrient cycling and acquisition in arid areas, their comprehensive study should be an additional criterion in determining ecosystem health. Attempts were made to undertake an assessment of soil microbial abundance and to identify the microbial groups with an Adenosine Triphospahte (ATP) assay and with terminal restriction fragment length polymorphisms (T-RFLP). However, these methods were unsuccessful with the ATP assay compromised by the formation of calcium phosphate precipitate due to the soil chemistry and the

extraction method for T-RFLP requiring small subsamples of soil coupled with the presumably low microbial populations.

Shark Bay Salt lease area represents a unique area to study as it has numerous excavated areas of varying age and the rehabilitation experiences there have been used as reference sites in the proposal of the 'Coburn Mineral Sand Project' adjacent to Shark Bay World Heritage Area (EPA, 2005). The high level of endemism in the area lends it a uniqueness, and in review of the proposal, Professor R Gilkes expressed concerns that the mined areas could not support a self-sustaining floral community unless there was substantial intervention at considerable expense (EPA, 2005).

The use of legumes, particularly *Acacia* spp. along with beneficial microbes such as mycorrhizal fungi and RNB in the rehabilitation of degraded land in Australia is widespread and largely successful (Barnet *et al.*, 1985; Bell *et al.*, 2003; Bell, 2001; Brockwell *et al.*, 2005; Thrall *et al.*, 2005). To re-establish key perennial species such as *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. in the pits, the re-introduction of RNB in sufficient numbers to ensure an effective symbiotic interaction would be beneficial. In the next chapter, RNB isolates were collected from Shark Bay Salt soils and their symbiotic effectiveness evaluated for the purposes of use as a seed inoculant for provenance legume species.

Chapter 3

RNB from Shark Bay Salt lease area soils: isolation, authentication and effectiveness

Bacteria are the dark matter of the biological world with 4 million mostly unknown species in a ton of soil.

-Edward O. Wilson, TED prize winner 2007

3.1 Introduction

Early efforts to characterize the RNB associated with Western Australian indigenous Leguminosae focused on the ability of the RNB described at the time to cross-infect the plant host species associated with these RNB (Lange, 1961). Marsudi *et al.*(1999) described 133 RNB isolates associated with *Acacia saligna* (Labill.)H.L Wendl.*,* a species with historical use in Australia and overseas for fodder, mine site rehabilitation and agroforestry. Both these studies were concentrated on the South-West region of Western Australia (WA). Further investigations of the RNB symbionts of Western Australian native legumes has largely focused on pasture potential (Ryan *et al.*, 2008), as inoculants for introduced agricultural species (Yates *et al.*, 2004) or as competition for commercial inoculants (Howieson & O'Hara, 2008).

Rehabilitation of mine sites utilizing legumes, particularly *Acacia* spp. is well established and usually involves seeding into a site with replaced topsoil (ALCOA, 2003; Gardner & Bell, 2007) and/or the addition of fertilizer (Osborne & Brearley, 1999). Bell *et al.* (2003) assessed inoculation of *Acacia* spp. with mycorrhizal fungi in mine sites at Eneabba and Boddington in Western Australia. To date there has been no further publications on investigations into the RNB of Western Australian legumes and their potential as inoculants for the rehabilitation of legumes in disturbed sites, particularly those in the dryland regions of Western Australia. The native legume inoculant product, Wattle Grow™ (Bio-Care Technology Pty Ltd) was developed and was composed of four *Bradyrhizobium* strains from selected RNB isolated from *Acacia* spp. of eastern Australia. This product has been used for restoration applications (Thrall, 2011) but is not in production at present.

The most widespread method used to determine N_2 fixation effectiveness is to measure the growth of inoculated plants compared to uninoculated controls grown under N-free conditions by harvesting and drying the foliage (Vincent, 1970). This method has been used for both agricultural and native legume symbiotic systems (Bever *et al.*, 2013; Burdon *et al.*, 1999; Ryan *et al.*, 2008; Terpolilli *et al.*, 2008; Thrall *et al.*, 2000; Yates *et al.*, 2004). *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. were identified in Chapter 2 as key perennial legume species of Shark Bay Salt lease area.

With this technique, in the first study of Western Australian RNB isolates obtained from these two legumes, via site collected nodules and trapped from soil, the effectiveness of the RNB isolates in promoting growth in these host species was assessed in conjunction with Wattle Grow™.

3.2 Materials and Methods

3.2.1 Isolation and symbiotic effectiveness of *A. ligulata* Benth. RNB

3.2.1.1 Collection of root nodules from *A. ligulata* Benth.

In July 2006, eight nodulated seedlings of *A. ligulata* Benth. in the undisturbed areas of R, Q1 and G (section 2.2.1.1) were excavated and nodules observed at an approximate depth of 100-150 mm. There were three plants each in sites G and Q and two plants at site R. Unruptured nodules, viable in appearance were collected and placed on cotton wool over silica gel in 5 mL screw top vials for desiccation and storage. *A. tetragonophylla* F.Muell. seedlings were indentified, one each at sites P and R, however neither plant had any nodules present.

3.2.1.2 Isolation of RNB

Desiccated nodules were imbibed in sterile water for 4 h, surface sterilised in 70% (v/v) ethanol (20-30 s) and 4% (w/v) sodium hypochlorite (2-3 min), with time variability allowing for nodule size differences, and thoroughly rinsed in six changes of sterile deionised (DI) water. Nodules were macerated and the contents streaked onto CRS-M1 plates (section 2.2.3.3). Nodule isolates were incubated at 28°C and monitored daily over ten days for colony growth. Individual colonies were sub-cultured onto CRS-M1 plates until pure bacterial cultures were obtained. From each individual plant, up to three isolates were collected from the original nodule contents. Glycerol suspensions of isolates were prepared with 0.89% (w/v) saline to a final concentration of 18% (w/v) glycerol and stored at both -20°C and at -80°C.

3.2.1.3 Authentication of RNB isolated from *A. ligulata* Benth.

Seeds of *A. ligulata* Benth. collected from Shark Bay Salt lease area were scarified, sterilized and germinated as described in section 2.2.3.2. Germinated seeds were sown aseptically to a depth of 10 mm using sterile wooden applicator sticks into 3 kg pots filled with 1:1 mix of yellow and washed river sand, steam sterilised and leached of excess nitrogen as described by Howieson *et al* (1988). Pots received 25 mL of N-free modified nutrient solution (section 2.2.3.2) and were covered with plastic film to exclude airborne contamination.

Thirteen root nodule isolates (G11, G12, G13, G21, G22, Q13, Q31, Q32, R11, R12, R21, R22 and R24) were grown on CRS-M1 plates from the -20°C glycerol stock and incubated for five days at 28°C. A bacterial suspension prepared by washing growth off culture plates into 15 mL of 1% (w/v) sucrose solution was agitated until homogenised. Pots were inoculated with 4 mL of bacterial suspension at 10^6 to 10^8 colony-forming units (cfu) mL^{-1} directed at the sown seeds. Three replicate pots were prepared for the 13 isolate treatments and for uninoculated nitrogen-fed and nitrogen-starved control pots. After seedling emergence, a sterile capped watering tube (25 mm in diameter and 250 mm in length) was placed centrally to $^2/_3$ depth of the sand mix and the surface covered with sterile polyethylene beads. Randomised pots were maintained in a glasshouse at 24°C with 25 mL of modified nutrient solution (section 2.2.3.2) weekly, an additional 2 mL of 0.1 M KNO_3 was administered to the nitrogen-fed controls and all pots watered as required.

Plants were harvested at 56 days post inoculation (dpi). Plant condition, nodule presence, appearance and location was observed. From each pot, two nodules were retrieved from two plants (a total of four nodules) which were stored for no longer than 48 h in 25 mL microcentrifuge tubes at 4°C. The plant stem and foliage were dried for five days at 60°C in a Qualtex thermostat incubator after being separated from the rootstock at the hypocotyl.

3.2.1.4 RPO1-PCR fingerprinting of root nodule occupants

Bacteria were isolated from nodules in the authentication trial as described in section 3.2.1.2. Cell suspensions were prepared of these re-isolated bacteria as well as the original isolates from CRS-MR1 culture plates. Cells were suspended in 1 mL 0.89% (w/v) saline then pelleted at 21 000 x g for 2 min and the supernatant discarded. This process was repeated twice more. Cell pellets were suspended in a final volume of 200 µL of 0.89% (w/v) saline. The cell suspensions were standardised to optical density (OD) 6 at 600 nm and stored at -20°C.

Fingerprinting was conducted with the primer RPO1 (AATTTCAAGCGTCGTGCCA) designed by Richardson *et al.*(1995) to target the *nifHDK* promoter consensus region. RPO1-PCR reactions modified from Richardson *et al.*(1995) consisted of 1 µL of cell suspension, 4 µL of 5 x Fisher-Biotech polymerization buffer [composition of 1 x buffer: 67mM Tris-HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 0.45% (v/v) Triton X-100, 0.2 mg mL^{-1} gelatin and 0.2 mM dNTP], 2.5 µM of primer, 3 mM $MgCl_2$, 2.5 U *Taq DNA* polymerase (Invitrogen Life Technologies) and made up to a final volume of 20 µL with UltraPure grade water (Fisher Biotech).

PCR was conducted on a *I*cycler (BIORAD) with cell lysis at 95°C for 5 min; 5 cycles of 94°C for 30 s, 50°C for 10 s and 72°C for 90 s; 30 cycles of 94°C for 30 s, 55°C for 25 s and 72°C for 90 s with final extension for 5 min at 72°C. A one-sixth volume of 6x Promega gel-loading buffer [composition of 6x buffer: 15% (w/v) Ficoll® 400, 0.03% (w/v) bromophenol blue, 10 mM Tris-HCl (pH 7.5), 50 mM EDTA, 0.03% (w/v) xylene cyanol FF and 0.4% (w/v) orange G] was added to the PCR products, which were electrophoresed in 2% (w/v) agarose gel containing 83 µL L^{-1} SYBR®Safe DNA gel stain (Invitrogen Life Technologies) in TAE buffer (40 mM Tris-HCl, 4 mM Sodium acetate, 1 mM EDTA [pH 7.9]). Electrophoresis was conducted at 70 V for 2 h with 1 Kb DNA marker (Promega, G5711) in tanks containing buffered 1xTAE (40 mM Tris-Acetate, 1 mM EDTA, pH 8.0). Visualization of bands was in a UV transilluminator.

3.2.1.5 Statistical analysis

After analysis, dry plant shoot weight data was presented as a percentage of the nitrogen-fed control. The yield variance of *A. ligulata* Benth. with each root nodule isolate was analysed with 2-way analysis of variance (ANOVA) and where applicable the Fisher's least significant difference (LSD) test with α=0.05 was performed with IBM® SPSS® version 21.

3.2.2 Trapping RNB from Shark Bay Salt lease area soils

3.2.2.1 Selection of trap species

Four legume species were chosen as trap hosts to isolate RNB from Shark Bay Salt lease area soils. *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. as key perennial species (section 2.2.2.2) with widespread Eremean region distribution (Figure 3.1) and *Acacia rostillifera* and *Templetonia retusa,* which extend from the coastal South West region (Figure 3.1) to the Shark Bay Salt lease area (SBSJV, 1998).

3.2.2.2 Collection and preparation of soil

In July 2006, soil was collected from undisturbed sites at R, Q1 and G (section 2.2.1.1) and a fenced rehabilitated area at Pit G. In each of the four areas, soil samples were taken from the surface and subsurface (approximately 300 mm in depth) at six randomly selected sites. The six surface soil samples were pooled, homogenised, stored in calico bags and kept cool in an insulated tub for transport. This procedure was repeated for subsurface samples and the samples stored at 4°C for no more than 28 days.

3.2.2.3 Glasshouse trapping of RNB

Pots were prepared with three substrate layers. The first (bottom) layer was a 1:1 mix of yellow and washed river sand. The middle layer was 20 mm of sampled soil (3.2.2.2) and the third (top) was a further layer of the 1:1 mix of yellow and washed river sand.

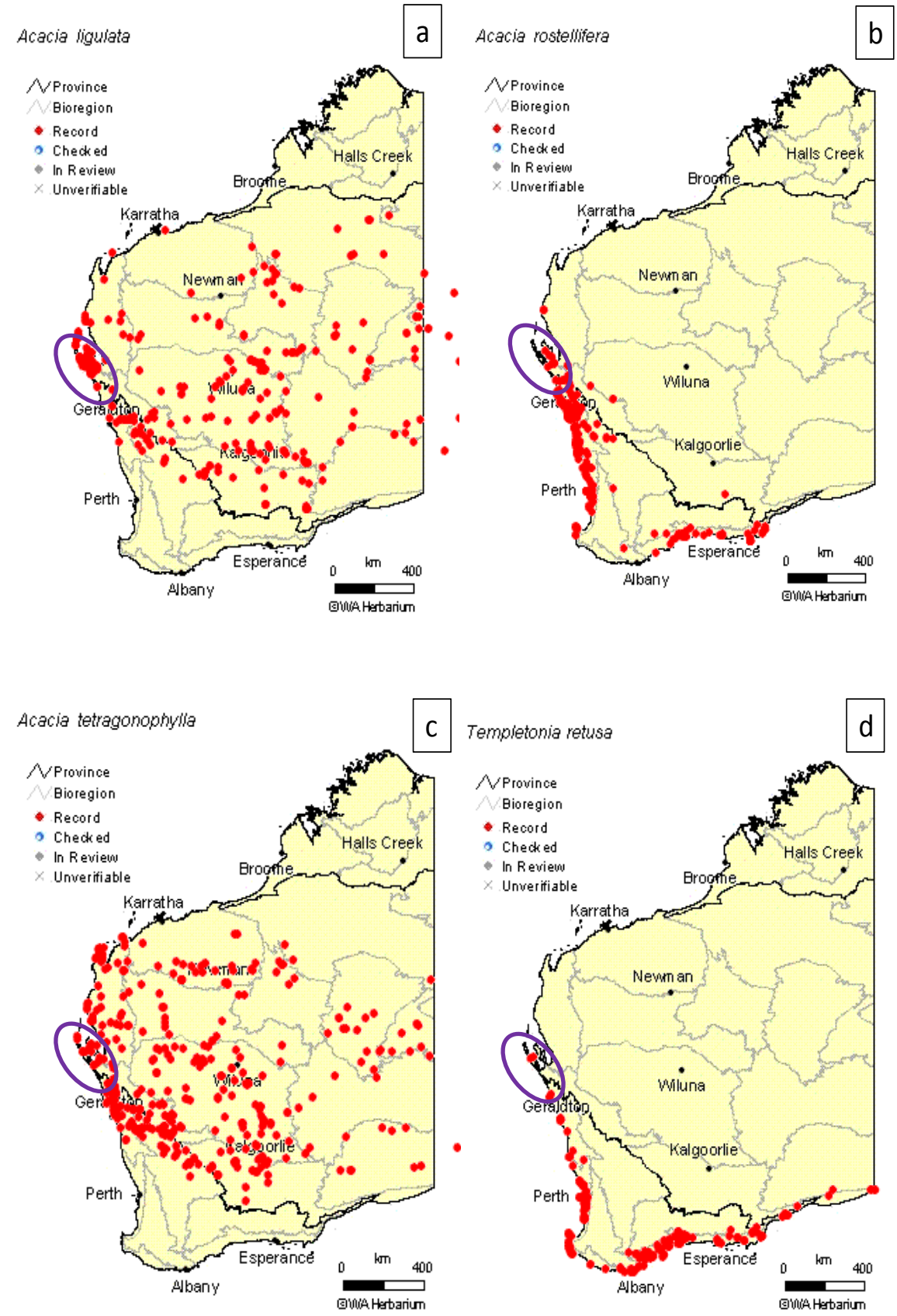


Figure 3.1: Western Australia distribution maps of a) *Acacia ligulata* Benth., b) *Acacia rostellifera* Benth., c) *Acacia tetragonophylla* F.Muell. and d) *Templetonia retusa* (Vent.)R.Br.. Location of Shark Bay illustrated by purple ovals. Images used with the permission of the Western Australian Herbarium (Gioia, 2005) (http://florabase.dec.wa.gov.au) Accessed on Thursday, 28 February 2013.

The first layer was added to 3 kg pots to 80 mm and steam sterilised along with additional sand mix for the third layer, the first layer was leached of excess nitrogen with two rinses of boiling water and allowed to cool prior to the middle layer of soil and top 10 mm layer of sand mix being added. Three replicate pots of each soil treatment (four sites each with surface and subsurface soils) were prepared in addition to control pots of nitrogen-fed and nitrogen-starved treatments containing only the 1:1 sand mix. All pots received sterile H_2O to water holding capacity.

Seeds of *A. ligulata* Benth.*, A. rostellifera* Benth.*, A. tetragonophylla* F.Muell. and *T. retusa* (Vent.)R.Br. (Table 3.1) were scarified, surface-sterilised, germinated and sown as described in section 2.2.3.2 into four separate split pot host treatments. Seeds were sown in the following combinations: 1) *A. tetragonophylla* F.Muell. (Shark Bay) and *A. tetragonophylla* F.Muell. (Newman), 2) *A. ligulata* Benth. (Shark Bay) and *A. ligulata* Benth. (northwest Victoria), 3) *A. ligulata* Benth. (unknown) and *A. rostellifera* Benth. and 4) *T. retusa* (Vent.)R.Br. (Esperance/Hopetoun). On seedlings emergence, treatment and control pots were randomised, watering tubes and beads added and maintained with the nutrient and watering regime as described in section 3.2.1.3. Plants were harvested 84 days post sowing with nodule appearance and plant vigour observed. Where possible two nodules per plant were collected and stored temporarily in a 2.5 mL capped tube, any remaining nodules were desiccated for long-term storage in 5 mL vials. Bacteria was isolated from nodules and stored as described in section 3.2.1.2.

Table 3.1: Host species used throughout thesis and their Australian seed provenance

Species*	**Seed provenance**	**Supplier/ collector**
Acacia ligulata Benth.	Shark Bay salt lease area	Colin Thomas (Shark Bay Salt)
Acacia ligulata Benth.(narrow leaf form)	Unknown	Nindethana seed service
Acacia ligulata Benth.(marpoo)	Northwest Victoria	Nindethana seed service
Acacia ligulata Benth.†	Laverton, WA	Kimseed International Pty Ltd
Acacia rostellifera Benth.§	Geraldton, WA	Nindethana seed service
Acacia rostellifera Benth.	Geraldton, WA	Kimseed International Pty Ltd
Acacia tetragonophylla F.Muell.	Shark Bay salt lease area	Colin Thomas (Shark Bay Salt)
Acacia tetragonophylla F.Muell.	Newman, WA	Nindethana seed service
Templetonia retusa (Vent.)R.Br.	Esperance/Hopetoun, WA	Nindethana seed service

* species ecotype in parentheses, † Seed not used in RNB trapping experiments, §Seed quality poor with low rates of germination.

3.2.3 Authentication and N_2 fixation effectiveness of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. RNB isolates and Wattle Grow™

3.2.3.1 Experimental design

Isolates obtained from the trap plants of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. were authenticated and screened for N_2 fixation effectiveness to identify multiple isolates with potential as inoculants on these two species. The ability of these isolates to cross infect different hosts and their N_2 fixation effectiveness was compared with Wattle Grow™.

3.2.3.2 *A. ligulata* Benth. RNB isolates

Pots and seeds of *A. ligulata* Benth. (Shark Bay lease area), *A. rostellifera* Benth. (Nindethana seed service), *A. tetragonophylla* F.Muell. (Shark Bay lease area) and *T. retusa* (Vent.)R.Br. (Table 3.1) were prepared as described in section 3.2.1.3. Pots were sown in split pot design with germinates of each host species occupying a quadrant of the pot. The 32 isolates obtained from *A. ligulata* Benth. (Table 3.2) were prepared for inoculation as in section 3.2.1.3. The commercial *Acacia* inoculant, Wattle Grow™ (Bio-Care Technology Pty Ltd), was prepared by suspending 20 g of the product in 40 mL of CRS-M1 broth media and agitating on a shaker at 200 revolutions per minute (rpm) for 3 h. Three replicate pots were prepared for each isolate treatment and nitrogen-fed and nitrogen-starved controls. Inoculated treatments received 10 mL of Wattle Grow™ or isolate suspension with bacterial cells ranging between 10^6-10^8 cfu mL^{-1}. Protection of pots from airborne contamination with beads, addition of watering tubes, randomisation, maintainance with nutrients and watering regime was as described in section 3.2.1.3.

Plants were harvested at 56 dpi following the procedure in section 3.2.1.3. Bacteria were isolated from nodules and glycerol suspensions prepared as described in section 3.2.1.2. Nodule occupancy determined by PCR-RPO1 fingerprinting and statistical analysis of dried plant shoots was as described in sections 3.2.1.4 and 3.2.1.5 respectively.

3.2.3.3 *A. tetragonophylla* F.Muell. RNB isolates

Isolates of *A. tetragonophylla* F.Muell. (Table 3.3) were assessed for N_2 fixation and cross-infection as described in section 3.2.3.2. Due to poor germination of *A. rostellifera* Benth. (Nindethana seed service), alternative seed was obtained from Kimseed International Pty Ltd. Pots were set up for inoculation with 27 isolates, along with Wattle Grow™ and control treatments. Enough viable seed of *A. rostellifera* Benth. was available to prepare 22 isolate inoculated treatments, Wattle Grow™ and control pots. The randomly selected isolates not inoculated on *A. rostellifera* Benth. were 1a26, 2a2, 4a25, 5a25 and 7a2.

3.2.3.4 Nitrogen content of inoculated *A. ligulata* Benth. and *A. tetragonophylla* F.Muell..

Following statistical analysis of dry plant shoot weights of *A. ligulata* Benth. (section 3.2.3.2) and *A. tetragonophylla* F.Muell. (section 3.2.3.3), plant material from the treatments inoculated with Wattle Grow™, isolates 1b36, 2b35, 2b36, 3b44, 4b36, 6b45, 2a11, 3a23, 5a25, 6a12 and 6a15 (on *A. ligulata* Benth.) and isolates 1a11, 1a26, 3a23, 4a13, 5a11, 5a25, 6a1, 6a12, 6a15, 1b36 and 3b33 (on *A. tetragonophylla* F.Muell.) and the nitrogen-starved un-inoculated controls were prepared for nitrogen content assay. These treatments were selected primarily due to the increased dryweights produced. Dried plant material of each replicate was pooled to provide sufficient material for analysis and two pooled replicates submitted for analysis on a Leco F528 Nitrogen Analyzer (CSBP soil and Plant Laboratory, Perth, Australia).

3.3 Results

3.3.1 Isolation, authentication and symbiotic effectiveness of *A. ligulata* Benth. RNB

The nodules collected from the roots of eight *A. ligulata* Benth. ranged in colour from pale to dark brown and were elongated from 1-5 mm in length or formed multi-lobed clusters (coralloid) 8 mm in diameter. If the nodules were coralloid, these were present

as a single multi-lobed cluster, otherwise the nodules were numerous (up to eight) and were distributed along the tap root. The nodules were present on the roots at a depth of between 100-150 mm from the soil surface. Thirteen gram negative isolates that grew to colonies of 1-2 mm in 2 to 4 days were obtained from the nodules, nine of these isolates resulted in nodulation when inoculated onto *A. ligulata* Benth. (Table 3.2).

Table 3.2: Isolates obtained from nodules collected from *A. ligulata* Benth. seedlings grown *in situ* within the Shark Bay Salt lease area.

Isolate*	Origin of host	Nodule form	Growth rate (days)†	Authenticated on host§
G11	G	Elongated	4	+
G12	G	Elongated	4	+
G13	G	Elongated	4	+
G21	G	Elongated	3	-
G22	G	Elongated	4	+
G31	G	Coralloid	4	-
G32	G	Coralloid	3	-
Q12	Q	Coralloid	4	-
Q13	Q	Coralloid	4	+
Q31	Q	Coralloid	4	+
R11	R	Elongated	3	+
R12	R	Elongated	3	+
R22	R	Elongated	4	+

*Isolates with the same number after the letter prefix indicates that isolates were obtained from nodules of the same host plant

† Growth rate determined by the appearance of colonies 1-2 mm in diameter

§ + indicates nodulation of *A. ligulata* Benth. by isolates, - indicates no nodulation

Of the nine authenticated isolates, seven of these increased foliage dry weight ($P \leq 0.05$) in comparison to the uninoculated control (Figure 3.2). The most effective symbiosis that produced a yield 88% of the N-fed control was with isolate G22 (Figure 3.2). Consistent nodulation was produced with isolates G11, G12, G13 and G22, with remaining isolates collected from *A. ligulata* Benth. nodules at sites Q and R failing to nodulate all re-inoculated plants in the treatments. PCR-RPO1 fingerprinting confirmed nodule occupancy of *A. ligulata* Benth. was by the inoculated isolates and six different fingerprints were identified. G11, G12 and G13 were isolated from the same plant and had identical banding patterns. Similarly, R11 and R12 were isolates from the same plant with identical fingerprints. The observed nodule morphology of the authenticated isolates was coralloid or elongated, and varied in colour from pale brown to dark pink/brown (Figure 3.3) which was similar to the field observations.

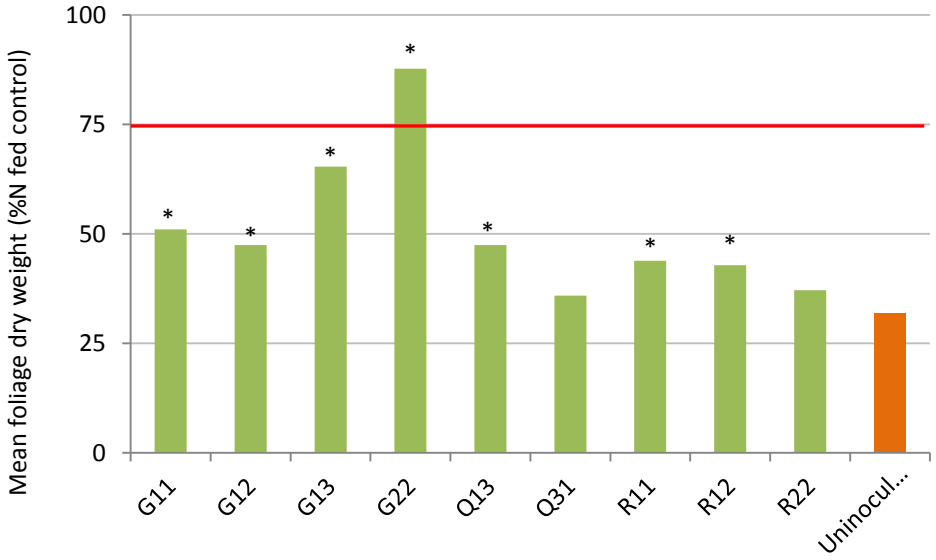


Figure 3.2: *A. ligulata* Benth. foliage dry weights as a percentage of the N fed control of plants nodulated by isolates obtained from Shark Bay Salt *A. ligulata* Benth. seedlings. The red line corresponds to 75% of the N fed control above which nodulation is considered effective (Terpolilli *et al.*, 2008). An * indicates significant difference to the uninoculated control ($P \leq 0.05$).

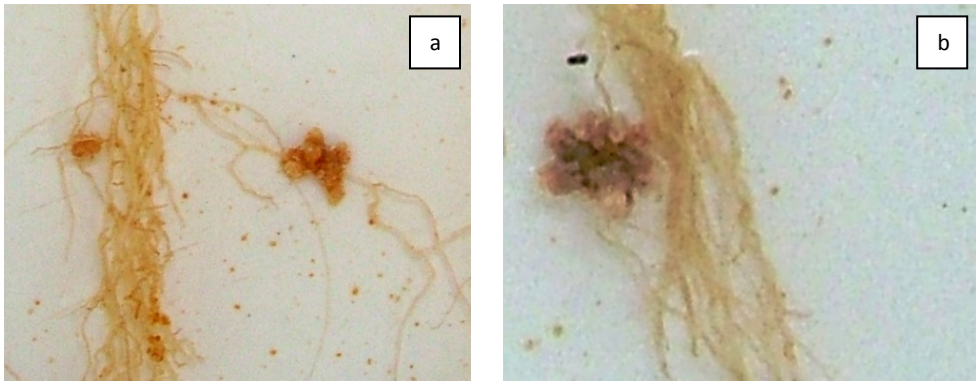


Figure 3.3: Nodules on *A. ligulata* Benth. showing pale brown elongated and coralloid (a) and dark pink/brown coralloid (b) morphology

3.3.2 RNB trapped from Shark Bay Salt lease area soils

All of the soil treatments resulted in nodulation and large vigorous trap hosts (Figure 3.4). Nodules on the *Acacia* spp. varied from elongated and coralloid (Figure 3.4 insert a and b) to squat pear shaped (Figure 3.4 insert c) and were of a dark cork-like appearance tending towards pale at the nodule tips. *T. retusa* (Vent.)R.Br. nodules were woody, pale brown to grey and elongated or spherical (Figure 3.4 insert d).

From nodules of *A. ligulata* Benth. grown from Shark Bay and Northwest Victoria sourced seed, there were 32 pure culture isolates of *A. ligulata* Benth. obtained across

the eight different soil treatments and of these isolates, 22 were from the Shark Bay seed *A. ligulata* Benth. (Table 3.3). Across the eight different soils there were 27 pure culture isolates obtained from *A. tetragonophylla* F.Muell. nodules, 19 of these isolates were trapped from *A. tetragonophylla* F.Muell. grown from Shark Bay seed (Table 3.4). There were 21 and 18 pure culture isolates obtained from *A. rostellifera* Benth. and *T. retusa* (Vent.)R.Br. respectively. All isolates grew colonies to 1-2 mm in diameter within 4 days.

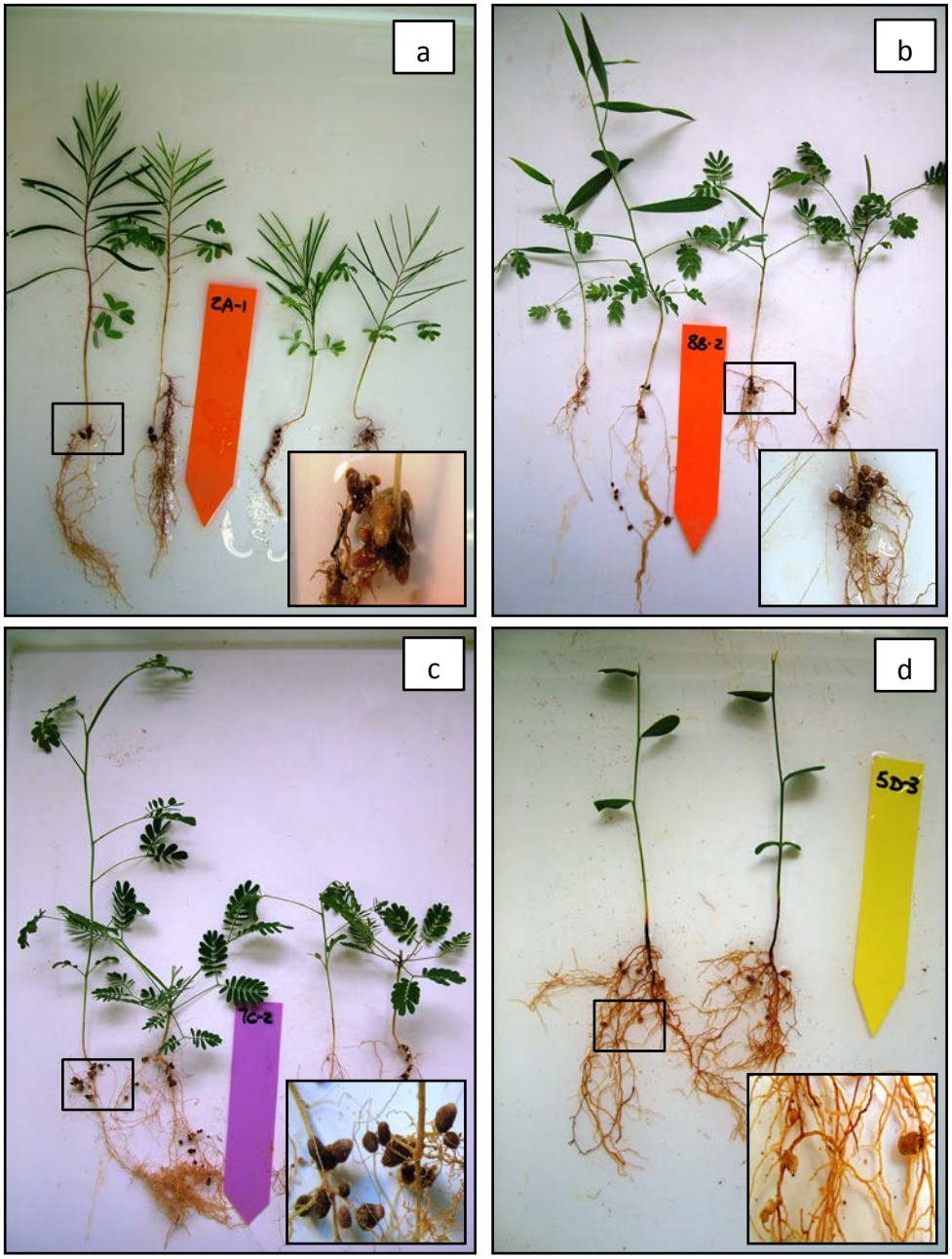


Figure 3.4: Nodules trapped from Shark Bay Salt soil with trap hosts *A.tetragonophylla* F.Muell. from Shark Bay and Newman (a), *A. ligulata* Benth. from Shark Bay and Northwest Victoria (b), narrow leaf form of *A. ligulata* Benth. and *A. rostellifera* Benth. (c) and *T. retusa* (Vent.)R.Br. (d) grown with Shark Bay Salt lease area soils. Plants arranged left and right of the treatment marker and location of nodule image inserts indicated by the black boxes on the root systems.

Table 3.3: The site and soil prolfile origin of the RNB isolates trapped on *Acacia ligulata* Benth. and assessed for N_2 fixation potential

Isolate	Soil origin*	Soil profile§	Seed provenance of trap plant
1b34	R	surface	Shark Bay
1b35	R	surface	Shark Bay
1b36	R	surface	Shark Bay
1b41	R	surface	Northwest Victoria
1b42	R	surface	Northwest Victoria
1b45	R	surface	Northwest Victoria
2b31	R	subsurface	Shark Bay
2b35	R	subsurface	Shark Bay
2b36	R	subsurface	Shark Bay
2b41	R	subsurface	Northwest Victoria
3b33	Q1	surface	Shark Bay
3b34	Q1	surface	Shark Bay
3b41	Q1	surface	Northwest Victoria
3b44	Q1	surface	Northwest Victoria
4b31	Q1	subsurface	Shark Bay
4b32	Q1	subsurface	Shark Bay
4b33	Q1	subsurface	Shark Bay
4b34	Q1	subsurface	Shark Bay
4b36	Q1	subsurface	Shark Bay
4b41	Q1	subsurface	Northwest Victoria
5b31	G	surface	Shark Bay
5b32	G	surface	Shark Bay
5b33	G	surface	Shark Bay
5b42	G	surface	Northwest Victoria
5b43	G	surface	Northwest Victoria
6b31	G	subsurface	Shark Bay
6b32	G	subsurface	Shark Bay
6b34	G	subsurface	Shark Bay
6b36	G	subsurface	Shark Bay
6b45	G	subsurface	Northwest Victoria
7b33	G †	surface	Shark Bay
8b3	G †	subsurface	Shark Bay

* Shark Bay lease area described in section 2.2.1.1

† Revegetated pit area

§ Surface soils collected from 0-50 mm, subsurface soils collected from 250-300 mm

Table 3.4: The site and soil prolfile origin of the RNB isolates trapped on *Acacia tetragonophylla* F.Muell. and assessed for N_2 fixation potential

Isolate	Soil origin*	Soil profile§	Seed provenance of trap plant
1a11	R	surface	Shark Bay
1a13	R	surface	Shark Bay
1a15	R	surface	Shark Bay
1a26	R	surface	Newman
2a11	R	subsurface	Shark Bay
2a15	R	subsurface	Shark Bay
2a2	R	subsurface	Newman
3a21	Q	surface	Newman
3a23	Q	surface	Newman
4a13	Q	subsurface	Shark Bay
4a15	Q	subsurface	Shark Bay
4a16	Q	subsurface	Shark Bay
4a25	Q	subsurface	Newman
5a11	G	surface	Shark Bay
5a12	G	surface	Shark Bay
5a15	G	surface	Shark Bay
5a16	G	surface	Shark Bay
5a25	G	surface	Newman
6a1	G	subsurface	Shark Bay
6a11	G	subsurface	Shark Bay
6a12	G	subsurface	Shark Bay
6a15	G	subsurface	Shark Bay
7a2	G†	surface	Newman
7a23	G†	surface	Newman
8a1	G†	subsurface	Shark Bay
8a11	G†	subsurface	Shark Bay
8a14	G†	subsurface	Shark Bay

* Shark Bay lease area described in section 2.2.1.1

† Revegetated pit area

§ Surface soils collected from 0-50 mm, subsurface soils collected from 250-300 mm

3.3.3 Authentication and N_2 fixation effectiveness of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. RNB isolates and Wattle Grow™

All of the 32 *A. ligulata* Benth. trapped isolates inoculated onto *A. liguata* Benth. were confirmed to be the nodules occupants by PCR-RPO1 fingerprinting. *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. were mostly consistently nodulated by these isolates. Both *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. produced deep verdant foliage in response to some of the *A. ligulata* Benth. isolates but plant size varied across all the treatments. All *A. ligulata* Benth. isolates, with the exception of eight, produced inconsistent nodulation on *T. retusa* (Vent.)R.Br. with as few as one plant among the replicates being nodulated (Figure 3.5). Insufficient seed of *A. rostellifera* Benth. germinated to produce statistically valid results.

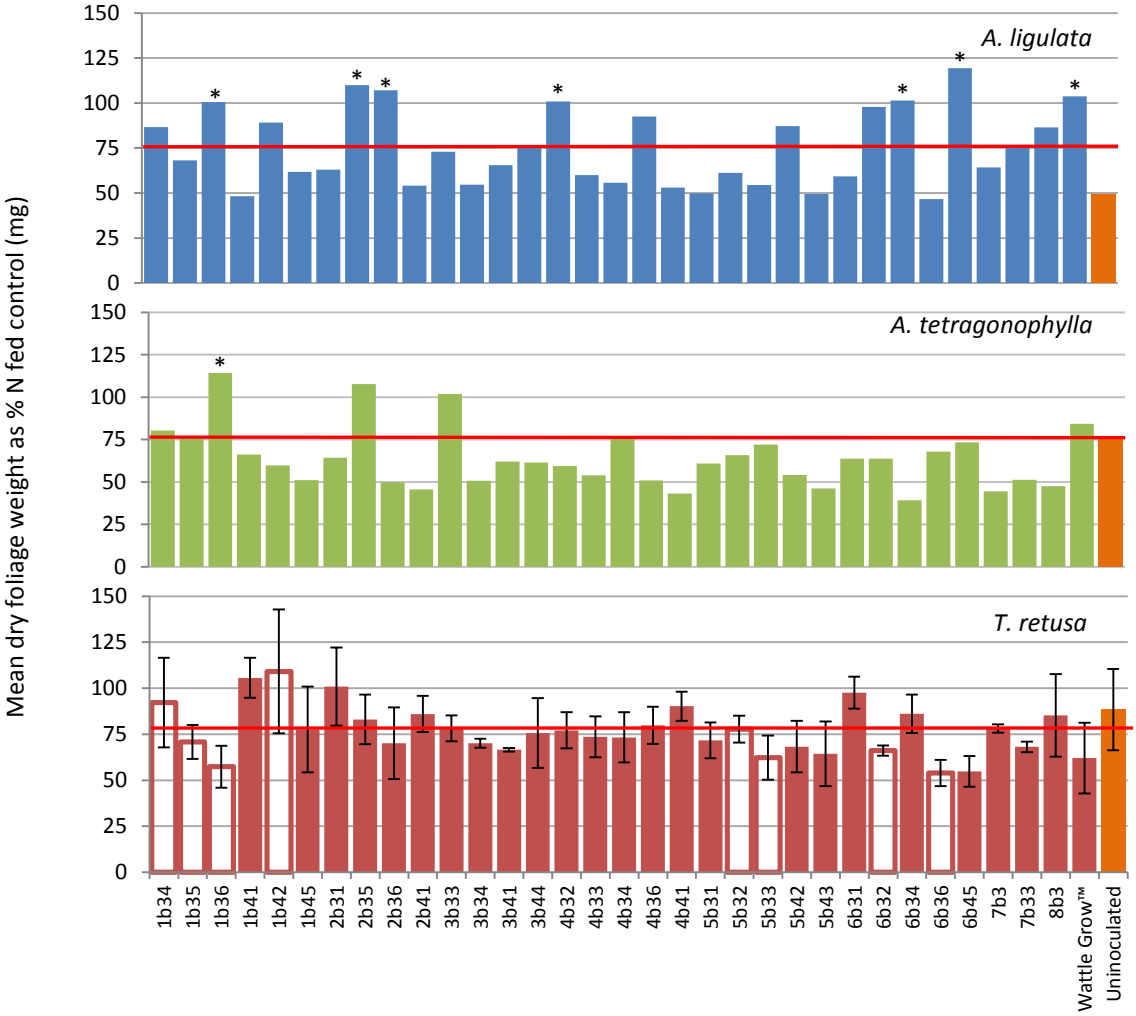


Figure 3.5: Foliage dry weights as % of N fed control for *A. ligulata* Benth., *A. tetragonophylla* F.Muell. and *T. retusa* (Vent.)R.Br. inoculated with 32 *A. ligulata* Benth. isolates and Wattle Grow™. The red line corresponds to 75% of the N fed control above which nodulation is considered effective (Terpolilli *et al.*, 2008). Treatments that *T. retusa* (Vent.)R.Br. did not nodulate with are indicated by unfilled bars. An * indicates significant difference to the uninoculated control ($P \leq 0.05$) and vertical bars correspond to standard error of means.

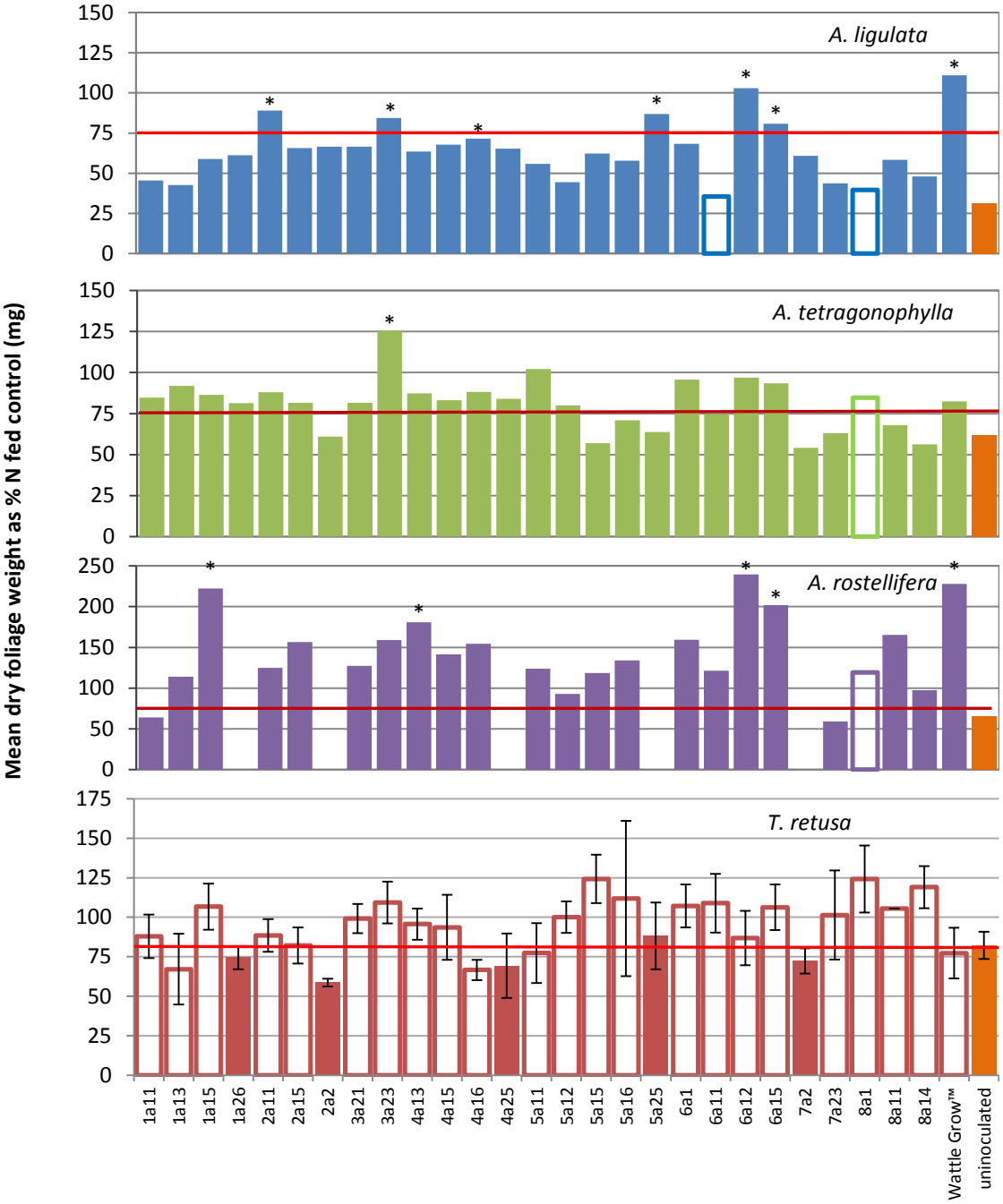


Figure 3.6: Foliage dry weights as % of N-fed control for *A. ligulata* Benth., *A. tetragonophylla* F.Muell. and *T. retusa* (Vent.)R.Br. inoculated with 27 *A. tetragonophylla* F.Muell. isolates, *A. rostellifera* inoculated with 22 isolates and Wattle Grow™. The red line corresponds to 75% of the N-fed control above which nodulation is considered effective (Terpolilli *et al.*, 2008). Treatments that did not nodulate with hosts are indicated by unfilled bars. An * indicates significant difference to the uninoculated control ($P \leq 0.05$) and vertical bars correspond to standard error of means.

Inoculation of *A. ligulata* Benth. with twelve of the *A. ligulata* Benth. isolates and the Wattle Grow™ resulted in plants with a mean dry foliage weight that was greater than 75% of the N-fed control. Growth of *A. liguata* Benth. was significantly increased in comparison to the uninoculated control ($P \leq 0.05$) by isolates 1b36, 2b35, 2b36, 4b32,

6b34, 6b45 and Wattle Grow™ (Figure 3.5). There were significant growth responses in comparison to the uninoculated control ($P \leq 0.05$) by *A. ligulata* Benth. to the *A. tetragonophylla* F.Muell. isolates 2a11, 3a23, 4a16, 5a25, 6a12, 6a15 and Wattle Grow™ and all, with the exception of 4a16 were greater than 75% of the N-fed control (Figure 3.6).

Of the twelve treatments selected of both *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. isolates inoculated onto *A. ligulata* Benth., the most effective were 3a23, 6a12, 6a15 and 6b45 with foliage containing over 2.5% nitrogen (Figure 3.7) and there was a 38% difference in foliage production with ranges between 81% to 119% of the N-fed control for these isolates. The *A. ligulata* Benth. dry matter weights of treatments 1b36, 4b36, 5a25 and Wattle Grow™ range between 87% and 111% of the N-fed control for these isolates while the nitrogen concentrations were less than 1.9% and not significantly different from the uninoculated plants (Figure 3.7). There was no significant correlation ($P > 0.05$) between the dried foliage matter of *A. ligulata* Benth. of selected treatments including Wattle Grow™ and uninoculated and the percentage nitrogen values of these plants (Figure 3.8).

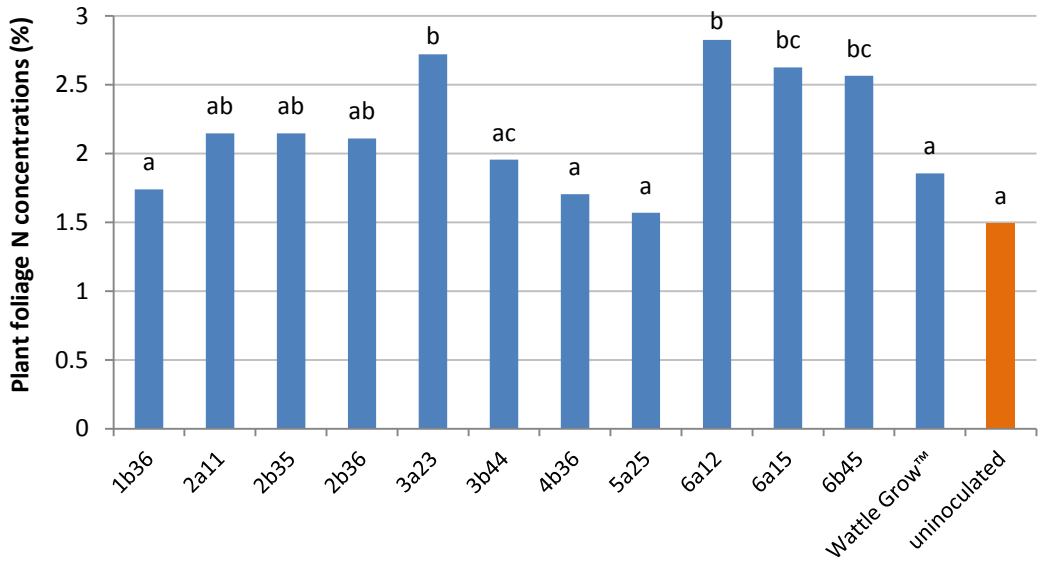


Figure 3.7: Plant foliage nitrogen concentration of *A. ligulata* Benth. inoculated with Shark Bay RNB isolates and Wattle Grow™. According to Fisher's LSD test ($P \leq 0.05$) treatments which share a letter are not significantly different.

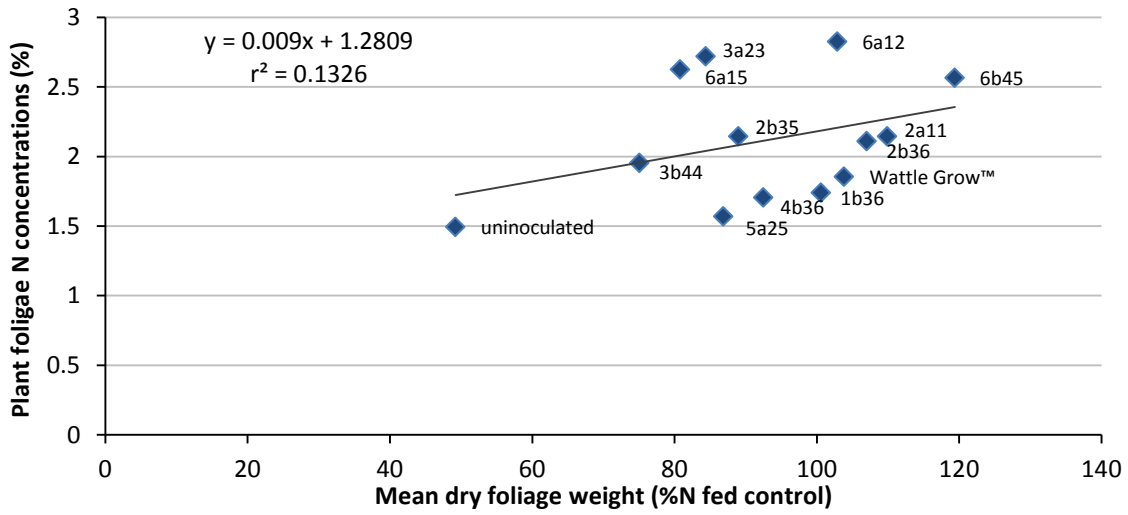


Figure 3.8: Regression graph of *A. ligulata* Benth. plant foliage dry weights (% N control) and the percentage nitrogen concentrations of these plants. Data boxes show the inoculant treatments of the Shark Bay RNB isolates and Wattle Grow™ as well as the uninoculated treatment. According to ANOVA there was no significant (P>0.05) correlation.

Of the 27 *A. tetragonophylla* F.Muell. trapped isolates, 26 were authenticated by PCR-RPO1 fingerprinting. The isolate 8a1, did not produce nodules when re-inoculated onto its original trap host or with any of the remaining three hosts (Figure 3.6). The hosts, *A. ligulata* Benth., *A. tetragonophylla* F.Muell. and *A. rostillifera* Benth. produced deep verdant foliage in response to some of the *A. ligulata* Benth. isolates but plant size was varied across all the treatments. Isolate 6a11 did not nodulate with either *A. ligulata* Benth. or *T. retusa* (Vent.)R.Br. and of the remaining isolates only 1a26, 2a2, 4a25, 5a25 and 7a2 were able to nodulate with *T. retusa* (Vent.)R.Br. (Figure 3.6) and as with the *A.ligulata* Benth. isolates, nodulation was inconsistent.

A. tetragonophylla F.Muell. foliage production after inoculation with 18 of the 27 *A. tetragonophylla* F.Muell. isolates and Wattle Grow™ was greater than 75% of the N-fed (Figure 3.6) however, only 3a23 showed a significant response when compared to the uninoculated control (P≤0.05). *A. tetragonophylla* F.Muell. inoculated with *A. ligulata* Benth. isolate 1b36 showed significant increase in dry weight in comparison to the uninoculated control (P≤0.05) (Figure 3.5). The other *A. ligulata* Benth. isolates inoculated on *A. tetragonophylla* F.Muell. that resulted in dry matter greater than 75% of the N-fed control were Wattle Grow™ and isolates 1b34, 1b35, 2b35, 3b33 and 4b34 (Figure 3.5).

Nitrogen concentrations of *A. tetragonophylla* F.Muell. plants ranged between 0.8 and 1.7% and were lower than those of *A. ligulata* Benth.. The *A. tetragonophylla* F.Muell. plants with greater than 1.5% N and a significant difference to the uninoculated control ($P \leq 0.05$) were those inoculated with 1b36, 4a13, 6a12 and 6a15 (Figure 3.9) whose dry foliage was between 87% and 114% of the N-fed control. There was no significant difference in the N concentrations of treatments 1a26, 3b33, 5a25, Wattle Grow™ and the uninoculated control (Figure 3.9), dry matter comparison to the N-fed control of these inoculated plants was between 64% and 102%. There was some correlation indicated by the dry foliage weights of *A. tetragonophylla* F.Muell. and the N concentrations of the foliage with approximately 30% of the data within the variance of the regression line (Figure 3.10). However, it is not possible to either statistically accept or reject that there is no relationship between the dry foliage weights of *A. tetragonophylla* F.Muell. and the N concentrations of those same plants.

The dry foliage weights of Wattle Grow™ and 20 of the 22 *A. tetragonophylla* F.Muell. isolates inoculated on *A. rostillifera* Benth., were greater than 75% of the N-fed control and one treatment, 6a12, was as great as 239% of the N-fed control (Figure 3.6). Five treatments showed a significant response in comparison to the uninoculated control ($P \leq 0.05$), 1a15, 4a13, 6a12, 6a15 and Wattle Grow™.

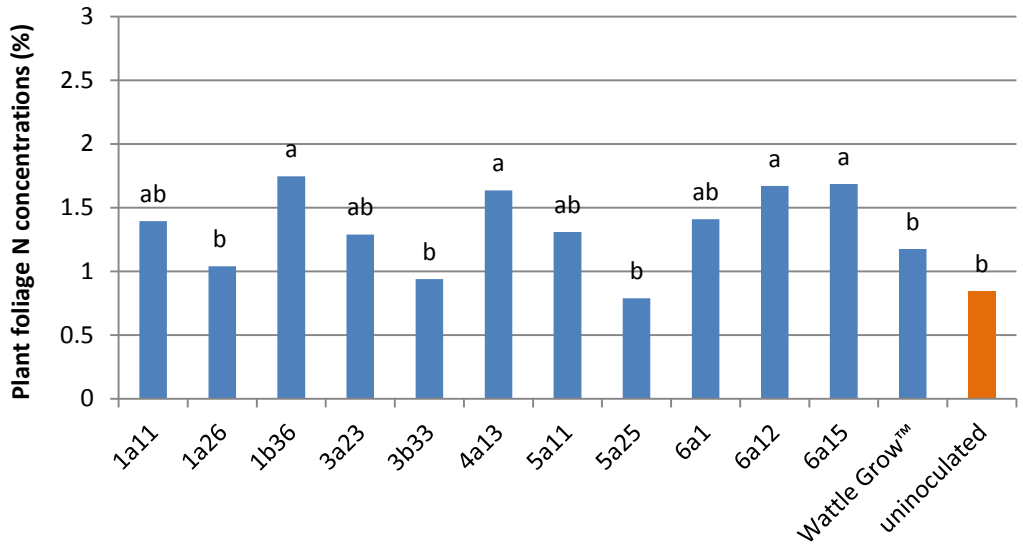


Figure 3.9: Plant foliage nitrogen concentration of *A. tetragonophylla* F.Muell. inoculated with Shark Bay RNB isolates and Wattle Grow™. According to Fisher's LSD test ($P \leq 0.05$) treatments which share a letter are not significantly different.

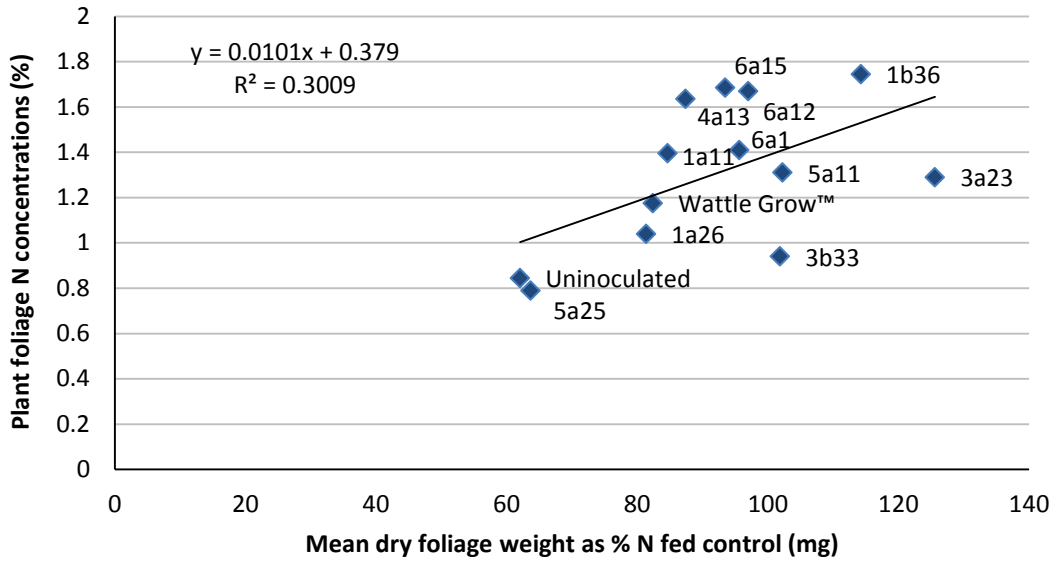


Figure 3.10: Regression graph of *A. tetragonophylla* F.Muell. plant foliage dry weights (% N control) and the percentage nitrogen concentrations of these plants. Data boxes show the inoculant treatments of the Shark Bay RNB isolates and Wattle Grow™ as well as the uninoculated treatment. According to ANOVA *P*=0.05, and therefore unable to reject the null hypothesis of no effect.

While *T. retusa* (Vent.)R.Br. did nodulate with Wattle Grow™ and with 24 of the *A. ligulata* Benth. and five of the *A. tetragonophylla* F.Muell. trapped isolates, this was inconsistent across the treatments and within the replicates. There was no significant difference in the mean dry foliage weights (P≤0.05) of *T. retusa* (Vent.)R.Br. when inoculated with *A. ligulata* Benth. isolates (Figure 3.5) and *A. tetragonophylla* F.Muell. isolates (Figure 3.6) and the plant growth of *T. retusa* (Vent.)R.Br. within the treatments was highly variable with many, including the uninoculated control, exceeding 75% of the N-fed control (Figure 3.5 and 3.6).

3.4 Discussion

In this chapter, isolates either collected from nodules on *A. ligulata* Benth. growing within the Shark Bay lease area or trapped from *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. grown in soils collected from the lease area were authenticated and screened for effectiveness together with Wattle Grow ™. The isolates readily cross-infected both *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. with all of *A. ligulata* Benth. isolates nodulating *A. tetragonophylla* F.Muell. and 25 of the *A. tetragonophylla* F.Muell. nodulating *A. ligulata* Benth.. Twenty-nine isolates

were able to nodulate, albeit inconsistently, with *T. retusa* (Vent.)R.Br.. The *A. tetragonophylla* F.Muell. isolates also readily nodulated *A. rostellifera* Benth. The effectiveness response of the isolates on the host plants was varied. There were differences in the response to isolates collected either from nodules of *in situ A. ligulata* Benth., and those trapped in glasshouse conditions. When inoculated onto *A. ligulata* Benth., 78% of the *A. ligulata* Benth. isolates collected from nodules collected at Shark Bay Salt were effective in comparison to the uninoculated control whereas 22% of the *A. ligulata* Benth. trapped isolates were effective in comparison to the uninoculated control.

The ability of the *A. tetragonophylla* F.Muell. isolates to cross-infect *A. ligulata* Benth. and *A. rostellifera* Benth., and the *A. ligulata* Benth. isolates to cross-infect *A. tetragonophylla* F.Muell. indicates host promiscuity with these isolates, which are provenant to where all three hosts overlap in their distribution. There is a degree of relatedness between *A. ligulata* Benth. and *A. rostellifera* which hybridize frequently and are sometimes difficult to distinguish between, being part of the informal *A. bivenosa* group (Chapman & Maslin, 1992). This may contribute to their similar patterns of promiscuity and ability to nodulate with the same *A. tetragonophylla* F.Muell. trapped isolates (with the exception of 6a11). *Acacia* spp. have been widely reported as exhibiting non-selectivity with RNB symbionts (Diouf *et al.*, 2010; Njiti & Galiana, 1996; Roughley, 1987) and this attribute contributes to numerous Australian *Acacia* spp. becoming invasive in Mediterranean areas, South Africa (Morris *et al.*, 2011; Richardson & Rejmánek, 2011) and within Australia (Adair, 2008).

T. retusa (Vent.)R.Br. did form indeterminate nodules with some of the isolates, though none were effective in N_2 fixation. *T. retusa* (Vent.)R.Br. is reported as nodulating ineffectively with *Ensifer fredii* USDA257 and NGR234 (Pueppke & Broughton, 1999) and not at all with *Bradyrhizobium* from *Lupinus* spp. (Lange, 1962). However, RNB associated with *T. retusa* (Vent.)R.Br. are reported as nodulating with exotic legume species such as *Lupinus* spp. and *Phaseolus* spp. (Lange, 1961) and effectively nodulating some *Cytisus* spp. (Perez-Fernandez & Lamont, 2003). Both *Lupinus* and *Cytisus* spp. are reported as being infected via crack-root entry giving rise to uniform infected tissue within the nodule, though some cells retain meristematic

activity (Sprent, 2007; Vega-Hernández *et al.*, 2001). *T. retusa* (Vent.)R.Br. may also display this unusual nodulation and nodule structure (Sprent, 2008).

Promiscuity by *Acacia* spp. does not necessarily confer the capacity to fix N_2 effectively. The inoculation response was different for *A. ligulata* Benth. and *A. tetragonophylla* F.Muell.. Of the isolates that resulted in nodulation, *A. ligulata* Benth. produced significantly increased foliage with 25% in contrast to the foliage increase of *A. tetragonophylla* F.Muell., which only occurred with 3% of isolates. This wide degree of growth response shown by the host plants to both the *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. isolates (Figures 3.6 and 3.9) and the statistical significance of the results was affected by, in some cases, extreme variability of individual plant dry weights. *Acacia mearnsii* D.Wild. nodulates with isolates obtained from a wide variety of legume hosts, but effectively fixed N_2 with comparatively few (Turk & Keyser, 1992). Similarly, Barnet and Catt (1991) reported that from a selection of isolates, only 36% produced significant foliage weights of *Acacia* spp. in comparison to uninoculated controls and that there was a large degree of variability between individual plants. Variation of inoculation growth response within a plant population has also been reported by Burdon *et al.* (1999) in 22 different *Acacia* spp. across south-eastern Australia. While *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. display non-selectivity with RNB isolates and a wide range of growth response to inoculation with these, inoculation with their own isolates resulted in 10% and 17% greater dry weights for *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. respectively. This effect has also been observed in *Acacia filicifolia* Cheel & M.B.Welch. and *Acacia silvestris* Tindale., species with restricted distribution in eastern Australia (Thrall *et al.*, 2000). This effect appears to be species dependant, *Acacia stenophylla* A.Cunn and *Acacia salicina* Lindley. have a similar widespread distribution through eastern Australia and when inoculated with whole soil *A. stenophylla* A.Cunn responded positively to its own soils whereas *A. salicina* Lindley. grew equally well in both (Barrett *et al.*, 2012).

Heterogeneity in the seeds may account for the statistical variance in plant growth seen in this study and those of others. This may account for the poor correlation between N concentrations in the plant foliage and the dry foliage weights of these same plants of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. (Figures 3.7 and

3.10). A positive correlation between leaf area and plant dry mass and the concentration of N was found in several *Betula* spp. (Niinemets *et al.*, 2002). The N concentration values in *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. were determined from pooled samples and the variability in plant size within the treatments may certainly have affected the concentrations of N obtained for the above ground plant material.

There were marked differences between the N concentrations of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. which were in the range of 1.57-2.82% and 0.8-1.7% respectively. These differences may be associated with their different adaptations, responses and characteristics to the dryland environment. *A. ligulata* Benth. plants have shallow and extensive lateral root systems, require heat shock to germinate and grow rapidly until maturity, and while they may live more than 15 years, they generally are short lived (Florabank, 2008) which are characteristics consistent with a seeder life history (Table 3.5). In contrast, *A. tetragonophylla* F.Muell. exhibits the resprouter characteristics (Table 3.5) of a prominent tap root and a slow growth rate, with plants living to approximately 100 years (WWW, 2010). There is also typically a difference between mature seeder and resprouter species in the investment of foliage production in comparison to their root systems (Table 3.5). In addition to the fast growth of *A. ligulata* Benth., it was observed that there was retention of the juvenile leaf form in *A. ligulata* Benth. before adopting the characteristic narrow elliptic phyllodes (Figure 3.4). These both have a larger surface area than the reduced phyllode form of the slow growing *A. tetragonophylla* F.Muell. that is present immediately after the first set of leaves (Figure 3.4). In comparison between slow growing and fast growing *Acacia* spp., the amount of plant N was shown to be correlated to leaf form and inherent growth rate of the species (Atkin *et al.*, 1998). Schortemeyer *et al* (2002) reported that *A. tetragonophylla* F.Muell.*,* in contrast to other species, increased nitrogen per nodule mass with increased plant size. For certain slow-growing sclerophyllous legumes there may be an adaptation to demand less N and while nodulation occurs, the effects to the plant may not be initially obvious while the plant is juvenile.

The variation in plant size within the species and differences in N concentrations illustrates the difficulties in simple approaches such as dry weights of plant material to

determine the effectiveness of the isolates in fixing N_2 with *Acacia* spp. and possibly other native legumes with variable life-history characteristics. More recently, studies have exploited the correlations between photosynthesis and nitrogen concentrations in leaves to determine plant nitrogen in a non-destructive way with the use of chlorophyll meters in rice (Peng *et al.*, 1995) and corn (Dwyer *et al.*, 1995). However, Warren *et al.* (2000) showed woody schlerophylic species have highly variable concentrations of N and that this was poorly correlated to photosynthesis. Nitrogen fixation has also been studied by the acetylene reduction method in root nodules of numerous legumes including *Acacia.* spp. however, this method is not without problems which have included within-species variability and damage to nodules by acetylene (Hansen *et al.*, 1987; Lawrie, 1981).

Table 3.4: Life-history of Australian native plants. Comparisons between Seeder and Resprouter characteristics.

Characteristic	Seeder	Resprouter	Reference
Root system	extensive, lateral	prominent tap root	Pate *et al.*(1990)
Shoot:root dry weight ratio	greater ratio	lower ratio	Pate *et al.*(1990)
Growth rate	fast	slow	Hunter (2003)
Life span	short(<15 y)/moderate	long	Hunter (2003)
Seed germination	heat shock	heat shock	Bell *et al.* (1993)
Fire sensitivity	variable, coppice or killed	resprouter	Bell *et al.* (1993)

The PCR-RPO1 fingerprinting of the isolates gives a preliminary indication of the genetic diversity of RNB in the soils of the Shark Bay lease area (Appendix 2), though not diagnostic of the RNB genera present. The RPO1 primer is derived from a conserved *Rhizobium nif* promoter consensus element (Richardson *et al.*, 1995) and shown to successfully amplify a wide range of RNB genera including *Rhizobium, Ensifer, Mesorhizobium, Bradyrhizobium* (Vachot-Griffin & Thies, 2005) and *Burkholderia* (Gerding González, 2011). There are some issues experienced with this technique, due largely to the variance in amplification profiles produced (Vachot-Griffin & Thies, 2005), however it was an adequate tool for verifying Koch's postulates of nodule occupancy for the re-authentication of the isolates, by comparing the banding patterns of the inoculant to the RNB re-isolated from the nodules. An element of bias may have

influenced the RNB genotypes found to be nodulating with *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. due to trapping conditions in the glasshouse compared to what may have been obtained from plants grown directly in the soils of the Shark Bay lease area. Attempts were made to grow *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. in the undisturbed soils, though none survived the drought conditions of 2006 (Section 2.3.2.1). Duodu *et al.* (2006) used PCR-fingerprinting to reveal the diversity of RNB of *Trifolium repens* L. and *Trifolium pratense* L. grown directly in the field and the result was different to the plants inoculated with field soil.

The experiments in this chapter demonstrated that there are numerous and diverse RNB that nodulate the key legume species of the Shark Bay lease area, *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. and to a lesser degree, *A. rostillifera* Benth. and *T. retusa* (Vent.)R.Br.. It was shown that there were discrepancies between plant foliage biomass production and the nitrogen concentration of the foliage in relation to N_2 effectiveness. However, if considering any of these isolates as potential inoculants for future rehabilitation projects, effectiveness of N_2 fixation is just one of many factors to consider.

A. ligulata Benth. and *A. tetragonophylla* F.Muell. are known to nodulate readily in their distribution areas (Beadle, 1964; Brockwell *et al.*, 2005). Bever *et al.* (2013), in a study conducted in South-Eastern Australia, reported *A. ligulata* Benth. interaction with phylotypes of RNB within the *Bradyrhizobium*, *Burkholderia*, *Rhizobium* and *Ensifer* genera. *A. tetragonophylla* F.Muell. has been reported as being nodulated by a mix of *Bradyrhizobium* spp. isolated from several *Acacia* spp. of northern Western Australia (Bowen *et al.*, 1997; Schortemeyer *et al.*, 2002). These two species have a widespread distribution across dryland Australia, which includes a wide range of environmental conditions and ecosystems. The characteristics of Shark Bay lease area are markedly different to those of Eastern Australia and investigating the physiological and phylogenetic characteristics of the RNB associated with *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. within Shark Bay would contribute to the knowledge regarding the extent of the symbiotic interactions with RNB for these species.

Chapter 4

The characteristics of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. RNB isolates.

It is not the strongest of the species that survives, nor the most intelligent that survives. It is the one that is the most adaptable to change.

-Charles Darwin

4.1 Introduction

Prior to the Shark Bay area being considered as a world heritage site, extensive surveys of the flora, fauna, geology and topography were undertaken (UNESCO, 2002). These contributed to Shark Bay being one of the few World Heritage properties meeting all four outstanding natural universal values: demonstrating earth's evolutionary history, ongoing ecological and biological processes, outstanding natural phenomena, and essential habitats for in situ conservation of biodiversity (UNESCO, 2002). However, no study of the soil microbial communities has been undertaken.

The dynamic relationships between RNB, mycorrhizas and other beneficial microbes to plant community structure, establishment and continued survival is well documented (de Souza Moreira *et al.*, 2010; EPA, 2006; Klironomos, 2002; Murray *et al.*, 2001; Requena *et al.*, 1997; Thrall *et al.*, 2007; Wardle, 2006). In view of the significance of the Shark Bay World Heritage Area, an understanding of the interactions between beneficial microbes and the flora would be valuable to future rehabilitation projects in the surrounding areas and within the Shark Bay Salt lease area.

The few studies of the relationships of native legumes and their symbiotic RNB in the South-West of Western Australia indicate a predominance of nodulation with *Bradyrhizobium* spp. (Lange, 1961; Marsudi *et al.*, 1999). In the North-West of Western Australia, Yates *et al.* (2004) demonstrated *Bradyrhizobium* spp. and *Ensifer* spp. nodulating indigenous legumes.

Acacia ligulata Benth. and *Acacia tetragonophylla* F.Muell. have widespread distribution across Western and central Australia and neither *A. ligulata* Benth. nor *A. tetragonophylla* F.Muell. occur in Tasmania, and the later does not occur in Victoria. These two species of *Acacia* are common in the majority of vegetation associations of the Shark Bay Salt lease area and represent the few large perennial species found in the area (Bennett, 1996). *A. ligulata* Benth. is reported as being nodulated by RNB phylotypes of *Bradyrhizobium*, *Burkholderia*, *Rhizobium* and *Ensifer* (Bever *et al.*, 2013) isolated from other native legume species of South-eastern Australia (Thrall *et al.*,

2011). *A. tetragonophylla* F.Muell. has been reported as being nodulated by a mix of *Bradyrhizobium* spp. isolated from several *Acacia* spp. of northern Australia (Bowen *et al.*, 1997; Schortemeyer *et al.*, 2002). These represent RNB that are able to cross-infect with *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. and field observations show that these *Acacia* spp. readily nodulate throughout their distribution, particularly in improved conditions such as after rainfall events (Beadle, 1964; Brockwell *et al.*, 2005). To date, no RNB isolated from *A. ligulata* Benth. or *A. tetragonophylla* F.Muell. have been described in Western Australia or other states of Australia in which these two species are distributed.

This chapter reports on the phenotypic characteristics of RNB growth at a range of temperature, pH and NaCl concentrations, as well as the phylogenetic relationship and genetic diversity of a sub-set of root-nodule bacteria isolated from *A. ligulata* Benth. and *A. tetragonophylla* F.Muell.*.*

4.2 Materials and Methods

4.2.1 Isolate selection

The phenotypic characteristics of colony morphology and growth tolerance at a range of temperatures, pH levels and NaCl concentrations were assessed for all 25 authenticated isolates listed in Table 4.1. The data reported in Chapter 3 indicated these isolates show a degree of effectiveness on *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. and were therefore chosen for further experimention as field inoculants and for competitiveness and persistence under field conditions. The diversity of the isolates was assesses using RPO1 molecular fingerprinting and 16S rDNA RFLP patterns with sequencing of the 16S rDNA for a subset of ten isolates (Table 4.1).

Table 4.1: Selected isolates of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. and characterization assessments conducted. The √ indicates where an assay was performed and na indicates where an assay was not performed on the isolates.

Original host	Isolate	Phenotype				Genotype		
		Colony morphology	Temperature	pH	NaCl	RPO1	RFLP	16S rDNA
A. ligulata Benth.	1b36	√	√	√	√	√	√	√
	2b35	√	√	√	√	√	√	na
	2b36	√	√	√	√	√	√	na
	3b33	√	√	√	√	√	√	√
	3b44	√	√	√	√	√	√	na
	4b36	√	√	√	√	√	√	√
	5b42	√	√	√	√	√	√	na
	6b45	√	√	√	√	√	√	na
	7b33	√	√	√	√	√	√	√
	8b3	√	√	√	√	√	√	na
	G22*	√	√	√	√	na	√	na
A. tetragonophylla	1a11	√	√	√	√	√	√	na
	1a13	√	√	√	√	√	√	na
	1a26	√	√	√	√	√	√	√
	2a11	√	√	√	√	√	√	na
	3a23	√	√	√	√	√	√	√
	4a13	√	√	√	√	√	√	√
	5a11	√	√	√	√	√	√	na
	5a16	√	√	√	√	√	√	√
	5a25	√	√	√	√	√	√	na
	6a1	√	√	√	√	√	√	na
	6a12	√	√	√	√	√	√	√
	6a15	√	√	√	√	√	√	na
	7a23	√	√	√	√	√	√	√
	8a11	√	√	√	√	√	√	na

*Isolate obtained from nodules of *A. ligulata* Benth. grown *in situ* at SBSLA

4.2.2 Phenotypic characterization

4.2.2.1 Bacterial culture conditions

The selected *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. isolates were grown from -20°C glycerol stocks (Sections 3.2.1 and 3.2.2) for the phenotypic characterization assays unless otherwise indicated.

4.2.2.2 Colony morphology

Isolates were transferred from the -20°C glycerol stocks (Section 4.2.2.1) onto yeast mannitol agar (YMA) modified from Vincent (1970) (YMA composition: Mannitol 10 g L^{-1}, Yeast extract 1 g L^{-1}, K_2HPO_4 0.5 g L^{-1}, $MgSO_4$ 0.2 g L^{-1}, NaCl 0.1 g L^{-1}, $CaCl_2$ 0.05 g L^{-1}, Agar 15 g L^{-1}) and CRS-M1 (Section 2.2.3.3). The plate cultures were incubated at 28°C and daily observations of growth and colony morphology made for 7 days. Acid or alkaline production by isolates was determined by growth on YMA at pH 7 prepared with 5 mL L^{-1} universal indicator (Foster & Gruntfest, 1937).

4.2.2.3 Temperature

Isolates were streaked onto CRS-M1 plates from the -20°C glycerol stocks (Section 4.2.2.1), incubated at the following temperatures: 4°C, 10°C, 15°C, 20°C, 28°C, 37°C, 40°C, 42°C and 45°C and checked daily for up to 10 days with three replicates for each temperature. This was repeated with replicate plates prepared with isolates freshly grown from the -20°C glycerol stocks (4.2.2.1). Constant temperature rooms were used to incubate isolates at 4°C, 28°C and 37°C, Fisher & Paykel refrigerators were controlled with Shimaden digital thermostats for isolates incubated at 10°C, 15°C and 20°C, and a Ratek orbital shaker/incubator for isolates incubated 40°C, 42°C and 45°C.

4.2.2.4 pH

Isolates were inoculated onto CRS-M1 (Section 2.2.3.3) at pH 7.0 from -20° glycerol stocks (Section 4.2.2.1) and grown for 4 days at 28°C. These cultures were then used to inoculate CRS-M1 plates, at eight different pH (4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0). For all pH levels, with the exception of pH 4.0 and 5.0, CRS-M1 was prepared with universal indicator (5 mL L^{-1}). Media at pH 6.0, 7.0 and 8.0 were buffered with 10 mM HEPES and media at pH 9.0, 10.0 and 11.0 were buffered with 10 mM CHES. The CRS-M1 for pH 4.0 and 5.0 was prepared without agar and was buffered with 10 mM Homopipes and a 6% (w/v) agarose solution added post autoclaving. To assess the influence of the buffers on growth, CRS-M1 was prepared at pH 7.0 containing either

10 mM CHES or 10 mM Homopipes. Isolates were spot inoculated with sterile applicator sticks from the initial pH 7.0 CRS-M1 plates three times onto each pH treatment. There were three replicate spot plates prepared for all the pH levels assessed. The pH plates were incubated at 28°C and the growth monitored daily for 10 days. This procedure was repeated with isolates freshly grown from ⁻20° glycerol stock (Section 4.2.2.1).

4.2.2.5 NaCl

The ability of isolates to tolerate saline conditions was assessed on CRS-M1 (Section 2.2.3.3) at pH 7.0 prepared with NaCl concentrations of 0 mM, 150 mM, 300 mM, 450 mM, 600 mM and 750 mM. Using the same method for the pH assay, isolates were spot inoculated from CRS-M1 pH 7.0 culture plates prepared in section 4.2.2.4. Growth at 28°C was monitored daily for 10 days. This procedure was repeated with isolates freshly grown from -20° glycerol stock (Section 4.2.2.1).

4.2.3 Genotypic characterisation

4.2.3.1 Bacterial cell preparation

Cell suspensions for molecular studies were prepared with whole cells obtained from isolates grown for three days on CRS-M1. The cells were suspended in 1 mL 0.89% (w/v) saline solution and pelleted at 21 000 x g for 2 min with the supernatant discarded. The cells were resuspended and pelleted twice more and the cell pellets suspended in a final volume of 200 µL of 0.89% (w/v) saline. The concentration of the cell suspensions was standardized with 0.89% (w/v) saline to optical density (OD) 6.0 at 600 nm. The standardized cell preparations were stored at -20°C.

4.2.3.2 Molecular fingerprinting

Fingerprinting was conducted on 24 of the isolates (Table 4.1) with RPO1-PCR as detailed in section 3.2.1.4. A binary matrix was constructed from the presence or

absence of a band at different molecular sizes for each of the isolate banding patterns produced and the genetic distance calculated with AFLP SURV version 1.0 (Vekemans, 2002). An unweighted pair group with arithmetic mean (UPGMA) cluster analysis was then performed (PHYLIP software package) and cladograms visualized in MEGA5.2 (Tamura *et al.*, 2007).

4.2.3.3 RFLP of the 16S rDNA gene

The 16S rDNA gene of isolates (Table 4.1) was amplified using primers FGPS 6 and FGPS 1509' designed by Ponsonnet and Nesme (1994) (Table 4.2). 16S rDNA-PCR reactions consisted of 2 µL of cell suspension, 5 µL of 5 x Fisher-Biotech polymerization buffer [composition of 1 x buffer: 67 mM Tris-HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 0.45% (v/v) Triton X-100, 0.2 mg mL^{-1} gelatin and 0.2 mM dNTP], 0.5 µM of primer FGPS 6, 0.5 µM of primer FGPS 1509', 1.5 mM $MgCl_2$, 1.25 U *Taq DNA* polymerase (Invitrogen Life Technologies) and made up to a final volume of 25 µL with UltraPure grade water (Fisher Biotech).

PCR was conducted on an *I*cycler (BIORAD) with cell lysis at 95°C for 5 min; 30 cycles of 94°C for 30 s, 50°C for 30 s and 30°C for 45 s and a final extension of 72°C for 7 min. The annealing temperature was adjusted between 54.9°C to 57.1°C for some isolates as required to amplify 16S rDNA. To ensure that there was no non-specific amplification and the PCR product was suitable for digestion, an 8 µL aliquot was electrophoresed as described in section 3.2.1.4 with the exceptions that the gel contained 1.5% (w/v) agarose and the electrophoresis was run with 90V.

Following Laguerre *et al.*(1996), 8 µL of PCR products were digested with 10 U of four endonucleases, *Alu*I, *Hinf*I, *Msp*I or *Sau*3*A*I (Promega) and the recommended buffer to a final volume of 10 µL then reactions incubated at 37°C for 4 h or overnight. The DNA fragments were visualized after horizontal gel electrophoresis as described in section 3.2.1.4 with the exception that the gels were prepared with 3% (w/v) agarose and run for 40 V for 3 h with a 100 bp DNA Ladder (Promega). Determination of the band sizes was performed by Photo-Capt software (Vilbert-Lourmat, France).

4.2.3.4 16S rDNA gene sequencing

Based on RFLP groupings and preliminary phenotypic results, ten isolates [1a26, 1b36, 3a23, 3b33, 4a13, 4b36, 5a16, 6a12, 7a23 and 7b33 (Table 4.1)] were selected for 16S rDNA gene sequencing. The primers FGPS 6 and FGPS 1509' (Table 4.2) were used to amplify the 16S rDNA gene and this PCR product was verified (section 4.2.3.3). PCR products were purified with Qia-quick purification kit (QIAGEN) using product guidelines to a final elution in 30 µL of buffer EB (10 mM Tris-Cl, pH 8.5).

Individual PCR reactions using the primers listed in Table 4.2 contained 4 µL of purified PCR product, 4 µL BIG-dye terminator (version 3.1), 5 µM of primer and 1 µL UltraPure grade water (Fisher Biotech). The *I*cycler (BIORAD) conditions were: 2 min at 96°C and 25 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 min. Following the recommended protocol, products were precipitated using ethanol/EDTA/Sodium acetate and sequenced on an ABI Prism® model 377A DNA sequencer (Applied Biosystems).

Table 4.2: Primers used for sequencing the 16S rDNA region of RNB isolates of the Shark Bay lease area

Name	Sequence (5'→3')	Source
FGPS 6	GGAGAGTTAGATCTTGGCTCAG	Ponsonnet & Nesme (1994)
420F	GATGAAGGCCTTAGGGTTGT	Yanagi & Yamasato (1993)
800F	GTAGTCCACGCCGTAAACGA	Yanagi & Yamasato (1993)
1100F	AAGTCCCGCAACGAGCGCAA	Yanagi & Yamasato (1993)
1190R	GACGTCATCCCCACCTTCCT	Yanagi & Yamasato (1993)
820R	CATCGTTTACGGCGTGGACT	Yanagi & Yamasato (1993)
520R	GCGGCTGCTGGCACGAAGTT	Yanagi & Yamasato (1993)
FGPS 1509'	AAGGAGGGGATCCAGCCGCA	Ponsonnet & Nesme (1994)

Consensus sequences were generated using GeneTool-Lite version 1.0 (BioTools, Inc.) and compared to 16S rDNA sequences available on the **GenBank®** database (National Center for Biotechnology Information [NCBI]) using the BLAST search tool. The sequences of species found to be closely related and species type strains (Appendix 3) were aligned using ClustalW and included in a phylogenetic tree constructed using MEGA4 (Tamura *et al.*, 2007) with the neighbor joining method and a bootstrap value of 1000 replicates (showing only bootstrap values greater than 50%).

4.3 Results

4.3.1 Phenotypic characterization

4.3.1.1 Growth rate, pH reaction and colony morphology

All isolates were fast growers, producing visible colonies of 1 mm diameter by 3 days. The 25 isolates produced acid with growth on YMA plates containing universal indicator and changed the colour of the media from yellow/green (pH 7.0) to orange/red (pH 4-5). There were three types of colony morphology among the isolates when grown on YMA and CRS-M1 (Table 4.3). Isolate colonies were circular, entire and convex with a smooth glistening surface. The colony morphology of isolates assigned to type I were distinguished by colonies that were white, translucent, mucoid and composed of brown granules densely and evenly dispersed throughout (Figure 4.1a and d). Isolates with type II colony morphology were distinguished by their white, opaque, very mucoid colonies containing brown granules concentrated centrally which radiated out (like an iris). In the older, joined colonies, the granules formed snaky bands, and donut shapes in isolated colonies (Figure 4.1b and e). Isolates with type III colony morphology were distinguished by colonies that were white, extremely mucoid and mostly translucent with evenly dispersed brown granules (Figure 4.1c and f).

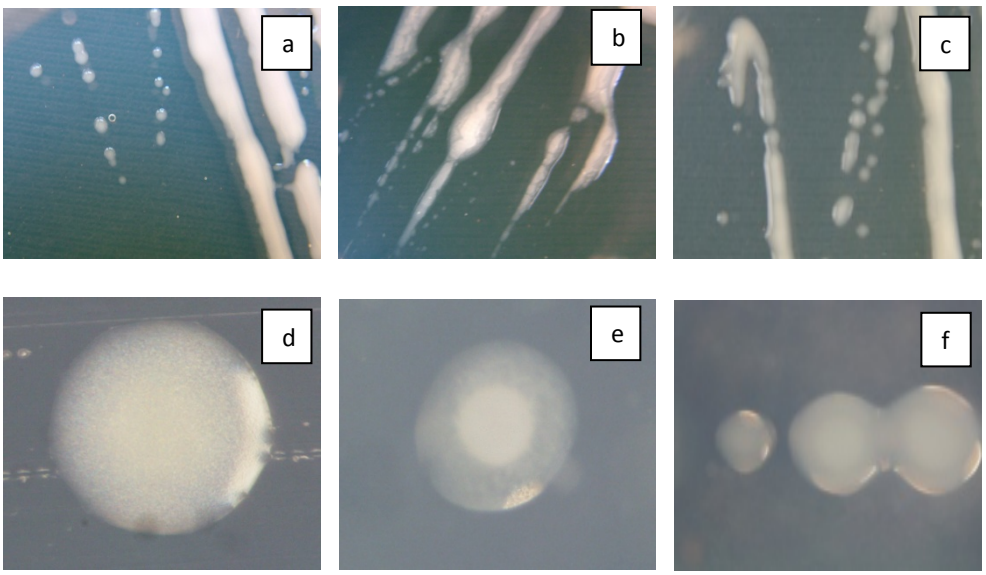


Figure 4.1: Colony morphology types of isolates grown on CRS-M1. Colony morphology type I represented by a and d, II by b and e and III represented by c and f.

4.3.1.2 Temperature

All isolates grew at 20°C and 28°C; none grew at 4°C, 10°C and 45°C (Table 4.3). Twelve of the 25 isolates (1a11, 5a16, 8a11, 7b33, 4a13, 3b33, 4b36, 3b44, G2L2, 6a12, 1a26 and 3a23) were able to grow at 15°C. All grew at 37°C, with the exception of 4a13 and 4b36. The 6 isolates, 7a23, 3b44, 5b42, 8b3, G2L2 and 3a23 grew at 40°C and these were also able to grow at 42°C, with the exception of 7a23 (Table 4.3).

4.3.1.3 pH

All 25 isolates grew in the range of pH 7.0 to pH 11.0 and only 6a15 and 2b35 failed to grow at pH 6.0 (Table 4.3). The isolates 1a26, 6a1, 6a12, 6b45, 7b33 and 8a11 produced colonies at pH 5.0 and had type I colony morphology (Table 4.3). No isolate grew at pH 4.0. The buffers added to the media did not impede growth; however less mucoid colonies were produced on media containing 10 mM CHES (pH 7.0, 9.0, 10.0 and 11.0).

4.3.1.4 NaCl

All isolates grew on media with 0 mM, 150 mM, 300 mM and 450 mM of NaCl, with the exception of 6a15, which did not grow on concentrations greater than 300 mM (Table 4.3). Isolate 8a11 did not grow on the media containing 600 mM and 750 mM NaCl and a further 3 isolates (5a11, 7b33 and 6a12) failed to grow 750 mM NaCl (Table 4.3).

Table 4.3: Physiological characterization of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. RNB isolates trapped from Shark Bay Salt lease area soils (Growth indicated by +, no apparent growth indicated by -)

Isolate		1a11	1a26	5a11	5a16	5a25	6a1	6a12	6a15	6b45	7b33	8a11	1a13	2a11	2b35	2b36	3b33	4a13	4b36	1b36	3b44	5b42	7a23	8b3	3a23
Morphology		I	I	I	I	I	I	I	I	I	I	I	II	II	II	II	II	II	II	III	III	III	III	III	III
Temperature (°C)	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	15	+	+	-	+	-	-	+	-	-	+	+	-	-	-	-	+	+	+	-	+	-	-	-	+
	20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	28	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	37	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+
	40	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
	42	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+
	45	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
pH	4.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	5.0	-	+	-	-	-	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
	6.0	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
	7.0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	8.0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	9.0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	10.0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	11.0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
NaCl (mM)	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	150	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	300	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	450	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	600	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
	750	+	+	-	+	+	+	-	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+

4.3.2 Genotypic characterization

4.3.2.1 Molecular fingerprinting

Using the RPO1 primer a high molecular diversity was shown among the *A. ligulata* Benth. *and A. tetragonophylla* F.Muell. isolates with 22 unique fingerprinting patterns produced by the 24 isolates. The relationships among these bacteria broadly fell into five groups (Figure 4.2). Isolates 6a12 and 6b45 had identical fingerprint patterns, as did the isolates 1a13 and 1b36 (Figure 4.2). Isolates 6a12 and 6b45 were trapped from pit site G subsurface soil and respectively isolated from *A. tetragonophylla* F.Muell. and *A. ligulata* Benth.. Isolates 1a13 and 1b36 were trapped from pit site R surface soil using *A. tetragonophylla* F.Muell. and *A. ligulata* Benth. respectively (Tables 3.2 and 3.3).

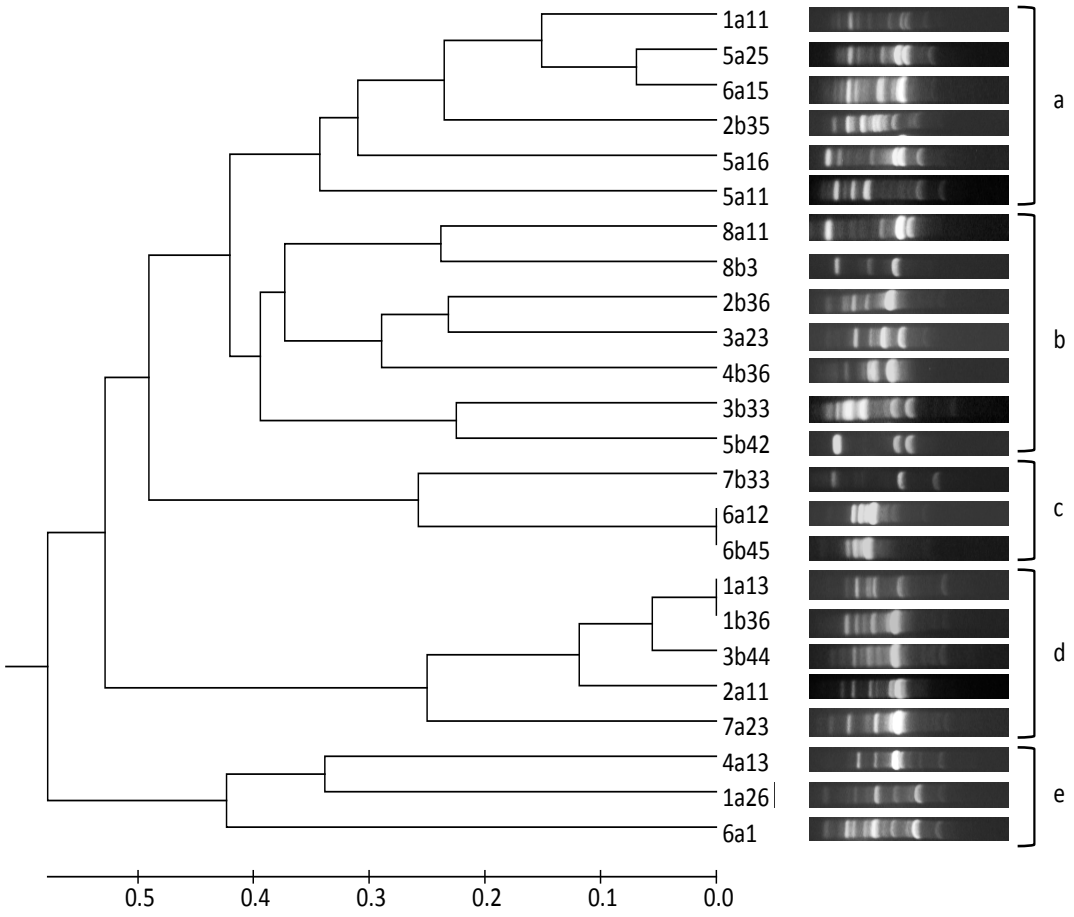


Figure 4.2: Cladogram of relationships between authenticated root nodule bacteria isolates of *Acacia ligulata* Benth. and *Acacia tetragonophylla* F.Muell. based on RPO1-PCR fingerprints. Cladogram created in MEGA5.2 (Tamura *et al.*, 2007) from genetic distances (Vekemans, 2002) and cluster analysis (PHYLIP software package).

Digestion of the 16S rDNA gene of the 25 isolates using the restriction enzymes *Alu*I and *Hinf*I each produced three distinct RFLP banding patterns in identical groupings, whereas four and five distinct RFLP banding patterns were seen for enzymes *Msp*I and *Sau*3AI, respectively (Appendix 4). Digestion with *Msp*I separated *Alu*I type A isolates into a further 3 groups. *Alu*I type B isolates were divided by *Msp*I and *Sau*3AI digestions into 2 separate groups. Isolate 3a23 was grouped separately from the other isolates because of the unique patterns produced with *Alu*I and *Sau*3AI (Table 4.4).

Table 4.4: The RFLP type groups of the isolates based on the 16S rDNA restriction patterns produced by *Alu*I, *Msp*I and *Sau*3*A*I digestions. The isolates were isolated on *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. from undisturbed area soils adjacent to excavated pit G, R and Q1 sites. Gr represents a revegetated site within the greater disturbed pit area

Isolate	Original trap host	Soil origin[ŧ]	Restriction patterns			RFLP type	
			***Alu*I,**	***Msp*1**	***Sau*3*A*I**		
1a11	*A. tetragonophylla*	R	A	A	A	AAA	
5a11	*A. tetragonophylla*	G	A	A	A	AAA	
5a16	*A. tetragonophylla*	G	A	A	A	AAA	1
5a25	*A. tetragonophylla*	G	A	A	A	AAA	
6a15	*A. tetragonophylla*	G	A	A	A	AAA	
7b33	*A. ligulata*	Gr	A	B	A	ABA	2
8a11	*A. tetragonophylla*	Gr	A	B	A	ABA	
1a13	*A. tetragonophylla*	R	A	C	B	ACB	
1b36	*A. ligulata*	R	A	C	B	ACB	
2a11	*A. tetragonophylla*	R	A	C	B	ACB	
2b35	*A. ligulata*	R	A	C	B	ACB	
2b36	*A. ligulata*	R	A	C	B	ACB	
3b33	*A. ligulata*	Q	A	C	B	ACB	
3b44	*A. ligulata*	Q	A	C	B	ACB	3
4a13	*A. tetragonophylla*	Q	A	C	B	ACB	
4b36	*A. ligulata*	Q	A	C	B	ACB	
5b42	*A. ligulata*	G	A	C	B	ACB	
7a23	*A. tetragonophylla*	Gr	A	C	B	ACB	
8b3	*A. ligulata*	Gr	A	C	B	ACB	
G22	*A. ligulata*	G	A	C	B	ACB	
6a12	*A. tetragonophylla*	G	B	C	C	BCC	4
6b45	*A. ligulata*	G	B	C	C	BCC	
1a26	*A. tetragonophylla*	R	B	D	D	BDD	5
6a1	*A. tetragonophylla*	G	B	D	D	BDD	
3a23	*A. tetragonophylla*	Q	C	B	E	CBE	6

ŧ soil collected from undisturbed areas adjacent to excavated pit G, R and Q sites. Gr represents a revegetated site within the greater disturbed pit area.

Digestion with *Hinf*I and *Sau*3AI did not separate the isolates into further groups. The six rRNA-RFLP pattern types produced from the assembled results of digestions with the endonucleases *Alu*I and *Msp*I (Appendix 4) were arbitrarily named 1, 2, 3, 4, 5 and 6 (Table 4.4). No correlation between soil sites, original plant host and 16S rDNA-RFLP group was evident. However, there was a correlation between colony morphology and 16S rDNA-RFLP group. All the isolates of with RFLP type 3 along with type 6 (3a23) had a type II or III colony morphology while the remaining RFLP types 1, 2, 4 and 5 were grouped among those isolates with a type I colony morphology (Table 4.3).

4.3.2.2 16S rDNA phylogeny

A fragment no less than 1400 bp was successfully amplified for the ten selected isolates 1a26, 1b36, 3a23, 3b33, 4a13, 4b36, 5a16, 6a12, 7a23 and 7b33. Sequence comparisons of isolates with GenBank® sequences showed similarity to strains of root-nodule bacteria belonging to the genera of *Ensifer, Rhizobium* and *Neorhizobium.* Eight of the isolates clustered with *Ensifer,* one with *Rhizobium* and one with *Neorhizobium* (Figure 4.3).

Six isolates (4b36, 3b33, 7a23, 1b36, 3a23 and 4a13) formed a separate cluster within an *Ensifer* clade and were most similar to *E. meliloti* SEMIA 6162 (FJ025127), with 99.6%, 99.6%, 99.5%, 99.5%, 99.5% and 99.4% sequence similarity respectively (Figure 4.3). All of these isolates with the exception of 3a23 were RFLP type 3. Isolate 7b33 (RFLP type 2) shared 99.9% homology with *Ensifer meliloti* LILM2009 (FJ792814) and 5a16 (RFLP type 1) was 99.9% similar to *E. fredii* USDA 257 (CP003563) (Figure 4.3). The *Rhizobium* sp. ORS 1457 (AY500263) shared 99.8% sequence similarity with RFLP type 4 isolate 6a12, while 1a26 (RFLP type 5) shared 99.7% similarity with *N. huautlense* $SO2^T$ (AF025852) (Figure 4.3).

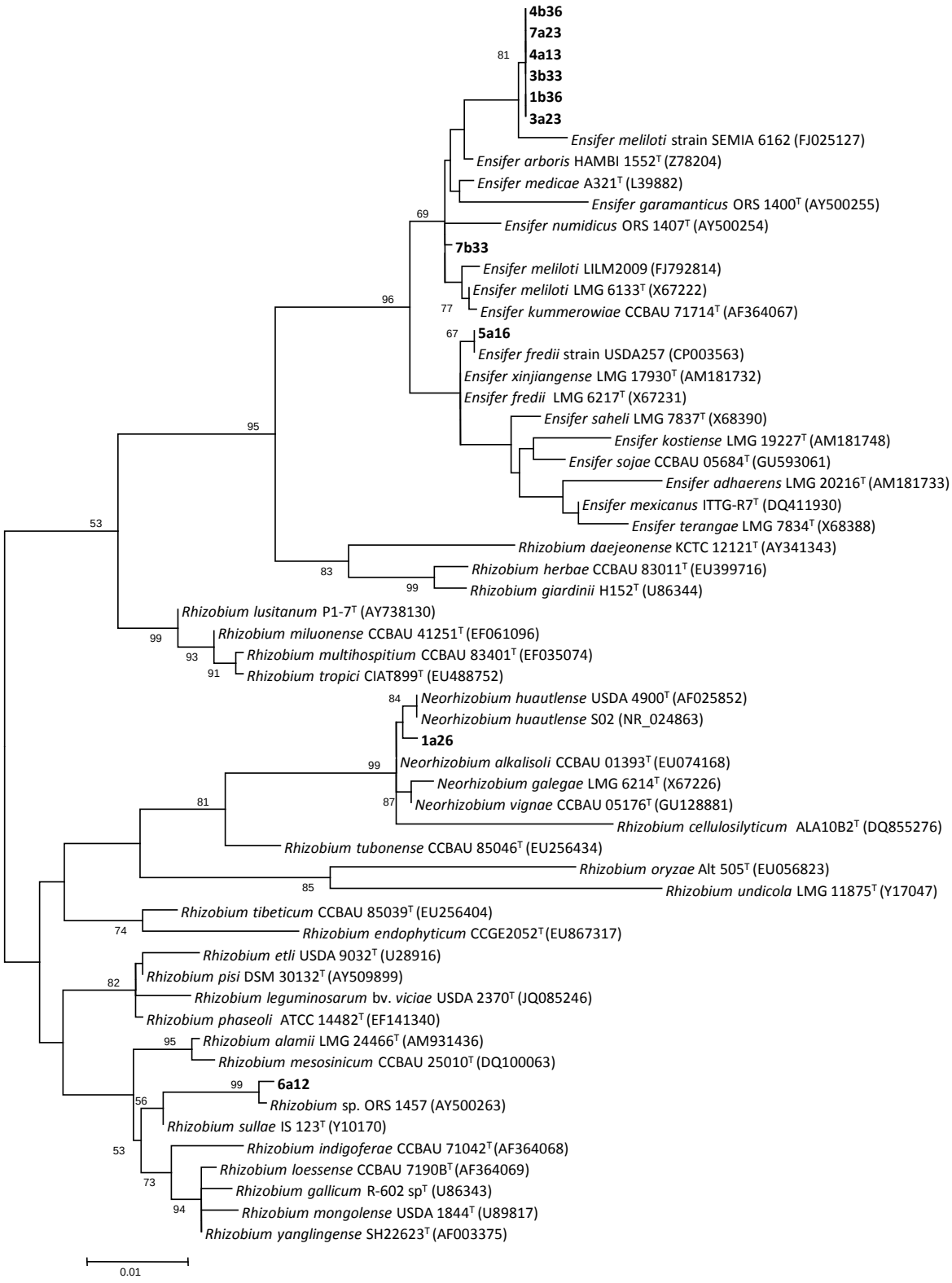


Figure 4.3: Neighbour joining phylogenetic tree using the Maximum Likelihood method of 16S rDNA sequences of isolates from this study (bold type) and other RNB obtained from GenBank® (accession numbers in parenthesis, type strains indicated by ^{T} adjacent to strain identification). Analyses were conducted in MEGA5 and there were 1489 positions in the final dataset. Branch values less than 50% are not shown.

4.4 Discussion

This chapter assessed the species and strain diversity and phenotypic characteristics of RNB that interacted with *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. which are provenant to SBSLA. SBSLA is an arid area of low rainfall and high temperatures with alkaline soils so it is not suprising that the isolates were tolerant to many of the environmental stresses characteristic of the site. The isolates were well adapted to the alkaline conditions, with all being able to grow at pH 11.0. The soils of the site are not saline with the exception of low-lying areas; however, the majority of the isolates tolerated up to 750 mM NaCl. Five of the isolates were able to grow at 42°C and all, with the exception of two isolates, grew at 37°C. The variability of the RPO1-PCR fingerprints indicate that the diversity of these isolates was considerable. Based on the 16S rDNA sequences, ten of the isolates were grouped within the *Ensifer, Rhizobium* and *Neorhizobium* genera and six formed a distinct cluster within *Ensifer.* No *Bradyrhizobium* were identified in this study, as all isolates were fast-growing and produced an acid reaction (Jordan, 1982). Although historically, a diverse range of Australian native legumes have been believed to be nodulated predominately by *Bradyrhizobium,* this implied bias may be largely due to the pH of the areas sampled at the time. The adaptability of the isolates to the arid environmental conditions of SBSLA is imperative to ensure that positive interactions occur with not only the perennial legumes such as *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. but with the many other annual and perennial legumes species found on site.

Legume-RNB interactions do occur in arid or dryland environments and in Australia, *Acacia* are the dominant plant species in many vegetation associations (Beadle, 1964; Ladiges *et al.*, 2006). For many RNB, the optimum temperature for growth in culture is between 28 and 31°C (Graham, 1992) though some RNB are able to persist at much greater temperatures (Kulkarni *et al.*, 2000; Shamseldin & Werner, 2005; Wilkins, 1967; Zahran, 1999). Wilkins (1967) isolated RNB from *Psoralea eriantha* Benth., *Lotus coccineus* Schlecht., and *Acacia rubida* A. Cunn. from dryland areas in New South Wales, which were still able to nodulate after exposure to 80°C for 5 h in dry soil conditions. *Rhizobium* spp. isolates of *Sesbania aculeate* L. survived for up to 4 and 2 h

at 50°C and 65°C temperatures, respectively (Kulkarni *et al.*, 2000). Shamseldin & Werner (2005) obtained isolates from *Phaseolus vulgaris* L. that grew up to a maximum of 42°C in culture. The data collected with the Tinytag™ Plus 2 Internal Temp/RH data loggers on site showed that the temperatures in the SBSLA soils up to a depth of 100 mm could exceed 40°C at times (Section 2.3.2.2) and that these high soil temperatures were not sustained for greater than 6 h (data not shown). Studies in the cellular response indicate that many RNB respond better to heat shock rather than exposure to sustained heat stress (Alexandre & Oliveira, 2011; Laranjo & Oliveira, 2011; Münchbach *et al.*, 1999).

The isolates with colony type III morphology generally had a higher tolerance to elevated temperature with five of the seven isolates in this group being able to grow at 42°C and one at 40°C (Table 4.3). All the isolates with colony morphology types I and II, with the exception of 4a13 and 4b36, grew at 37°C. Temperature affects the processes of nodulation via infection, nodule differentiation, nodule structure and function and the ability to infect a suitable host may be compromised (Graham, 1992; Hartel & Alexander, 1984). Desiccation is certainly detrimental to positive legume-RNB interactions in arid or dryland environments but given the ability of these RNB to persist at high temperatures in the soil, improved conditions such as rainfall can stimulate nodulation. In dryland areas, Beadle (1964) and Brockwell *et al.* (2005) observed the presence of newly formed nodules on *Acacia* spp. after rainfall. Soil moisture at 15% was found to be the optimum for the plant growth of *Acacia* spp. at 35°C and to be effectively nodulated by their respective RNB, with growth and nodulation being significantly reduced at 7.5 and 22.5% soil moisture content (Habish, 1970).

Numerous studies have reported that there are positive correlations between the tolerance of RNB to temperature and with the tolerance to alkaline pH and saline conditions (Kulkarni *et al.*, 2000; Laranjo & Oliveira, 2011; Shamseldin & Werner, 2005; Zahran *et al.*, 1994). Although the alkaline soils of Shark Bay Salt lease area were not saline with the exception of the low-lying pit Q1 (Section 2.3.1), 20 of the 25 isolates grew on 750 mM NaCl which includes all the isolates of types II and III colony

morphology (Table 4.3). Surange *et al.* (1997) have reported *Acacia farnesiana* L. the ability of isolates from alkaline soils to grow at pH 12.0 and tolerate salt concentrations of 856 mM. *Acacia* isolates from the Sudan could similarly grow up to pH 12.0 and tolerate saline conditions of 1.7 M NaCl (Zahran *et al.*, 1994). The Shark Bay Salt isolates showed considerable tolerance to pH, with 6 of 11 isolates having type I colony morphology group grow at pH 5.0 to 11.0. All the remaining isolates grew between the ranges of pH 6.0 to 11.0 with the exception of 6a15, which was the most sensitive to all three parameters of temperature, pH and salt (Table 4.3). In a geographically similar area to Shark Bay (within 100-300 km), Yates *et al.* (2004) obtained isolates from alkaline soils that also tolerated acid conditions of pH 5.0. Recently, core gene clusters involved in alkaline-saline adaptations have been identified in *Ensifer* spp. such as *pha2* (Na^+ resistance and alkaline pH) and *bet* (osmoprotectant) that are not present in *Bradyrhizobium* (Tian *et al.*, 2012). While it is possible to isolate *Bradyrhizobium* from alkaline soils (Raza *et al.*, 2001; Yates *et al.*, 2004), they were reported as displaying sensitivity to high alkaline pH (Graham, 1992; Jordan, 1982). *Lupinus* spp. inoculated with *Bradyrhizobium* form less nodules in alkaline soils compared to acid soils (Tang *et al.*, 1995) and at over 100 sites throughout France, the population was shown to reduce from more than 100 *Bradyrhizobium* g^{-1} soil in soils less than pH 6 to less than 1 *Bradyrhizobium* g^{-1} soil in soils at greater than pH 7 (Amarger *et al.*, 1984).

The sites where *Bradyrhizobium* have been isolated generally have acid to neutral soils and annual rainfall greater than 400 mm, which include the temperate areas of Western Australia (Lange, 1961; Marsudi *et al.*, 1999), South-eastern Australia including southern Queensland (Barnet *et al.*, 1985; Barnet & Catt, 1991; Lafay & Burdon, 1998; Lafay & Burdon, 2001; Lawrie, 1983) and tropical northern Australia (Bowen, 1956; Lafay & Burdon, 2007). Watkin (pers. comm) and Yates *et al.,* (2004) have isolated *Bradyrhizobium* strains from dryland alkaline soils in Western Australia. Though in comparison to other RNB genera, Bradyrhizobia were proportionally less. *Bradyrhizobium* bacteria have been found at considerable depth in the Sahelian area of West Africa with populations up to 1.3×10^3 g^{-1} of soil (Dupuy & Dreyfus, 1992). The soils from which the isolates were obtained in this study were collected to a depth of 300 mm and in a year with only 58.7 mm annual rainfall. The combination of alkaline

pH and desiccated soil conditions could have contributed to the lack of *Bradyrhizobium* strains in the Shark Bay soils.

The 16S rDNA phylogenetic data suggest that the isolates associated with *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. belong to the *Rhizobium, Neorhizobium* and *Ensifer* genera with greater than 99% homology to species or strains of these genera (Figure 4.3). Eight of the 10 isolates sequenced grouped with *Ensifer*, while 1b36, 3a23, 3b33, 4a13, 4b36 and 7a23 formed a cluster with *E. meliloti* SEMIA 6162 with isolate 7b33 being most similar to *E. meliloti* LILM2009. All of the clustered isolates produced type II or III colony morphology. Based on the sequences, it was difficult to assign a species to these isolates as all have greater than 99% similarity to each other and to the four type strains of *E. arboris, E. medicae, E. garamanticus* and *E. numidicus.* The remaining isolates, including 7b33, were all assigned to type I colony morphology and the relationship indicated by the phylogentic tree suggests they are disparate. The isolate 5a16 was most similar to *E. fredii* USDA 257 and was also 99% similar to the type strain of *E. fredii.* Isolate 1a26 shared greater than 99% homology to the type strain of *Neorhizobium huautlense,* formerly *R. huautlense* (Mousavi *et al.*, 2014). Isolate 6a12 was most similar to *Rhizobium* sp. ORS 1457 but was 99% homologous to the type strain of *R. sullae.* These strains that shared 16S rDNA homology with the isolates are geographically diverse and are symbionts of similarly diverse legume host species.

With the exception of *N. huautlense,* all the strains that shared similarity with the isolates were either salt-tolerant strains or isolated from salt tolerant plants in dryland areas. *E. meliloti* SEMIA 6162 and *E. fredii* USDA 257 originate respectively from *Prosopis juliflora* in Brazil and wild soybean from China (Binde *et al.*, 2009; Qi *et al.*, 2014), both of which are salt-tolerant plant species. *E. meliloti* LILM2009 is a salt tolerant strain isolated from *Phaseolus vulgaris* in Tunisia (Mnasri and Mhamdi unpublished). *Lotus cretus* is a salt-tolerant species growing in Tunisia from which *Rhizobium* sp. ORS 1457 was isolated (Zakhia *et al.*, 2004) and *R. sullae* is a symbiont of *Hedysarum coronarium* L. a species tolerant to drought, salinity and alkaline soils (Squartini *et al.*, 2002). *N. huautlense* is symbiont of *Sesbania herbacea* (Wang *et al.*,

1998), this legume and the other original hosts of *N. galegae* and *N. vignae* are legumes associated with damp sites and water bodies (Lindstrom, 1989; Ren *et al.*, 2011). The exception is the strain *N. alkalisoli* which was isolated from *Caragana intermedia* Kuang & H.C. Fu. plants growing in alkaline-saline soils in the drylands of the Tibetan plateau (Li Lu *et al.*, 2009). While the 16S rDNA of the isolates and strains show no correlation to either legume host or geography, there is an apparent relationship to saline tolerance and therefore to alkaline conditions.

Bever *et al.* (2013) cross-inoculated *A. ligulata* Benth. with strains of *Bradyrhizobium*, *Burkholderia*, *Rhizobium* and *Ensifer* collected from south-eastern Australia. As with this study, it was found that a number of the phylotypes corresponded to *E. meliloti* and *E. fredii* but also to *E. morelense, E. adhaerans* and *E. arboris* (2013). The *Rhizobium* phylotypes identified that nodulated *A. ligulata* Benth. were *R. gallicum, R. leguminosarum* and *R. mongolense* (Bever *et al.*, 2013) and these are not closely related to the phylotypes of *Rhizobium* sp. ORS 1457 and *N. huautlense* that nodulated *A. ligulata* Benth. in this study. To date, only *Bradyrhizobium* spp. isolated from several *Acacia* spp. of northern Australia have been reported as nodulating *A. tetragonophylla* F.Muell. (Bowen *et al.*, 1997; Schortemeyer *et al.*, 2002). *A. tetragonophylla* F.Muell. was similarly nodulated by the same isolates as *A. ligulata* Benth. and this is the first report of this species symbiotically associating with *Enifer, Rhizobium* and *Neorhizobium* phylotypes.

When describing and classifying prokaryotes, the use of 16S rDNA is still the most widespread, largely owing to the large datasets available (Tindall *et al.*, 2010). However, due to the highly conserved nature of 16S rDNA it can be difficult to distinguish between similar species (Eardly *et al.*, 2005; Gaunt *et al.*, 2001; Young *et al.*, 2004). The use of multiple loci, particularly housekeeping genes enables the discrimination between closely related strains (Haukka *et al.*, 1998; Mousavi *et al.*, 2014; Young *et al.*, 2004). All the isolates in this study showed more than 99% similarity of 16S rDNA to their respective genera type strains and yet the RPO1-PCR fingerprints show much greater genetic diversity in the isolates. To resolve these

relationships, particularly with the six clustered isolates, further study would be necessary to discern boundaries between the RNB species and isolates.

From the 25 isolates assessed, there were 22 unique fingerprints produced by RPO1-PCR. The RPO1 primer was selected in this study because of the numerous bands generated per isolate in comparison to the fingerprints produced with ERIC primers (data not shown) and this offered more discrimination in the analysis of the fingerprints. The use of RPO1-PCR has previously been reported as both more or less discriminatory than other Random Amplified Polymorphic DNA (RAPD) PCR reactions on different strains (Garau *et al.*, 2005; Sessitsch *et al.*, 1997). There was some correlation between the relationships implicated by the RPO1-PCR cladogram and those indicated by the 16S rDNA RFLP groupings. Isolates in the RPO1-PCR cladogram cluster group a, were all RFLP type 1 with the exception of 2b35. All isolates in group c were also all RFLP type 4 with the exception of 7b33 and group e were also RFLP type 5 with the exception of 4a13. Cluster groups b and d were composed of isolates that were RFLP type 3 with the exception of 8a11 and 3a23 in group b.

The major discordance between the RPO1 fingerprint groupings, the 16S rDNA sequences and the 16S rDNA RFLP is with the six isolates which form a cluster within *Ensifer* (Figure 4.3). All of those isolates with the exception of 3a23 were RFLP type 3, however this isolate was also in RPO1-PCR fingerprint group b along with other RFLP type 3 isolates in this cluster. The other anomaly in the 16s rRNA cluster is isolate 4a13, also RFLP type 3 but which was in RPO1-PCR fingerprint group e, along with 1a26 which shared 16S rDNA homology with *N. huautlense* (Figure 4.3). This variance between the sequence data and the fingerprint of the isolates is perhaps not surprising given that the RPO1-PCR fingerprints are produced from digested genomic DNA rather than a discreet section of 16S rDNA. Neither the RPO1-PCR cladogram nor the 16S rDNA tree showed any association of original host or soil site. The broad groupings of the isolates based on the relationships indicated by the cladogram also show no correlation to the physiological characteristics of these isolates.

Both the RPO1-PCR fingerprints and the 16S rDNA sequences of the isolates give an indication of the promiscuous relationship of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. with a diverse range of RNB symbionts. Geographic isolation in Australia has led to *Bradyrhizobium* populations quite distinct to those of other parts of the world and also to distinct clades within Australia with relation to temperate or tropical areas (Stępkowski *et al.*, 2012). This may also apply to other RNB genera and there may be further distinct communities in dryland ecosystems. However, there is a dearth of knowledge regarding the relationships of legumes in dryland Australia to their RNB symbionts and the distribution, diversity and taxonomy of these RNB. Understanding these relationships is important to maintain the integrity of this site given its proximity to the Shark Bay World Heritage Area.

The isolates are provenant to Shark Bay lease area and therefore suitable for re-introduction into the disturbed areas where the populations have been diminished. In order to determine the possible benefits of RNB inoculation of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell*.,* the next chapter studied the survival, vigour and nodulation of these species sown in several disturbed sites and treated with selected isolates delivered in various inocula carriers.

Chapter 5

Field establishment of *Acacia* spp. and RNB with various inoculation media.

Perhaps bacteria may tentatively be regarded as biochemical experiments; owing to their relatively small size and rapid growth, variations must arise much more frequently than in more differentiated forms of life, and they can in addition afford to occupy more precarious positions in natural economy than larger organisms with more exacting requirements.
— Marjory Stephenson *Bacterial Metabolism* (1930).

5.1 Introduction

The agricultural benefits of legumes and N_2 fixation to crop yield has been utilized since antiquity. It was only in the late 1800's that the first commercialized pure agar culture inoculants were used in an effort to improve crop yields (Date, 2001). Agar was used widely in the 1930's alongside peat, which has since become the most widespread form in which inoculants are applied either as a seed coating or directly to the soil (Albareda *et al.*, 2008; Date, 2001; Herridge, 2008). Peat can be a limited or unavailable resource and many countries are seeking quality inocula alternatives (Albareda *et al.*, 2008). Other products including waste materials, show potential to be utilized for application in inoculant cultures and solid preparations (Bashan, 1998; Lindström *et al.*, 2010).

While the biological limitations of chosen isolates are crucial to the successful production of a legume inoculant, so too are the physical and chemical characteristics of the carrier medium (Date, 2001; Stephens & Rask, 2000). Whatever the medium selected, the delivery of adequate cell numbers must be ensured (Stephens & Rask, 2000) as rhizobial survival can be markedly reduced depending on environmental conditions at the time of application (Herridge, 2008). In the rehabilitation of a dryland area such as the borrow pits in the SBLA, where topsoil reclamation is not possible and the topography and soil profile have been considerably altered, the re-introduction of soil microbes could prove to be beneficial to the slow growing perennial vegetation. With sporadic rainfall and high temperatures, it is imperative to select inoculant that increase the "shelf-life" of selected isolates in the soil until conditions are met that promote both bacterial growth and seed germination. In this chapter, peat, alginate and bentonite clay preparations are investigated to determine whether any have the necessary qualities to meet the rigorous conditions at SBLA.

Chapter 2 described the lack of recruitment of flora in the pit sites and showed that there are reduced RNB populations in these degraded sites compared to adjacent undisturbed vegetated areas within the SBLA exclusion zone. In Chapter 3, numerous isolates were identified that had a positive growth effect on *A. ligulata* Benth. and *A.*

tetragonophylla F.Muell.. The aim of this chapter was to re-introduce provenant RNB into the pit sites. The selected isolates were delivered in various inocula preparations and the effects on nodulation and field establishment of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. in selected disturbed pit sites was investigated.

5.2 Materials and Methods

5.2.1 Inocula preparation

Isolate 6a12 nodulates both *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. (section 3.3.4). A pure culture of this isolate was grown on CRS-M1 agar (section 2.2.3.3) from the -20°C glycerol stock (section 3.2.1.2) for 4 days at 28°C and used to inoculate a 300 mL CRS-M1 broth at pH 7.0 (section 2.2.3.3), incubated for 3 days at 28°C and shaken at 200 rpm. The bacterial count of the broth culture of 6a12 as determined by Miles and Misra method (Miles *et al.*, 1938) was 10^8 cfu mL^{-1}. This broth was used as the stock from which all subsequent inocula were prepared.

5.2.1.1 Clay

Bentonite clay was inoculated with a medium containing the stock broth and then processed as described in section 2.2.3.3 to contain 10^8 cfu g^{-1} in the final preparation. Control clay inocula were prepared in the same way using uninoculated medium.

5.2.1.2 Alginate bead

Using a method modified from Bashan (1986) and Bashan *et al.*(2002), alginate beads were prepared with an equal volume of 2% (w/v) sodium alginate (A0682 Sigma-Aldrich, Australia) mixed slowly for 1 h with 75 mL of the stock broth. The beads were formed by adding the inoculated sodium alginate solution dropwise to slowly stirred 0.1 M $CaCl_2$ and left to set for 1 h prior to rinsing in 0.89% (w/v) NaCl. Beads were separated from the suspension using Whatman No 4 filter paper. Control beads were

prepared identically with uninoculated CRS-M1 broth added to the sodium alginate suspension prior to forming the beads. Freshly prepared beads were temporarily stored in petri dishes at 4°C for no more than 24 h or dried at 28°C until desiccated (3-4 days) for long term storage. The alginate beads contained 10^9 cfu g^{-1} and was determined by Miles and Misra from a suspension of 0.1 g of fresh or dried alginate beads dissolved in 0.9 mL of 0.2 M KH_2PO_4 (adjusted to pH 7.0). Alginate beads to be used in a dissolved form for inoculation were prepared to the required volume by a 1 in 10 dilution of dried alginate beads in 0.2 M KH_2PO_4 (adjusted to pH 7.0).

5.2.1.3 Peat

A 150 g bag of sterile peat (Bio-Care Technology Pty Ltd) was injected with 75 mL of stock broth. The bag was resealed and incubated for 10 days at 28°C and contained 10^8 cfu g^{-1}. Uninoculated control peat was prepared by treating 150 g peat autoclaved twice at 121°C for 30 min with 75 mL of uninoculated CRS-M1 broth.

5.2.2 Inocula effect on nodulation of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell.

5.2.2.1 Experimental design

The nodulation potential of different carrier media containing 6a12 was assessed on *A. ligulata* Benth. and *A. tetragonophylla* F.Muell.. The experiment was a split–pot design with each half of the pot containing *A. ligulata* Benth. and *A. tetragonophylla* F.Muell., with seed and 1.5 L pots prepared as described in section 3.2.1.3. There were seven treatments: inoculated and uninoculated peat, inoculated and uninoculated clay, inoculated alginate beads and dissolved alginate beads and uninoculated alginate beads. There were three replications of each treatment sown with three germinated seeds of both *A. ligulata* Benth. and *A. tetragonophylla* F.Muell.. Inocula was applied at sowing at a rate of 1 g per germinate for peat and clay, 0.5 g per germinate for alginate beads and 1 mL per germinate of dissolved alginate beads. This ensured that each germinate in the inoculated treatments was inoculated with 10^8 cfu. Additional pots

containing inoculated peat were prepared for harvesting at 7, 11, 12, 14, 18 and 28 days post inoculation (dpi) to assess nodule initiation. Plants were maintained with nutrients and water until harvest at 42 dpi or as required for nodule initiation assessment as per section 3.2.1.3. At harvest, the plant foliage and root systems were separated at the hypocotyl and reserved.

5.2.2.2 Assessment of plant growth and nodulation

The plant foliage was dried and weighed as per section 3.2.1.3. The root systems were assessed for the presence or absence of nodules. Nodules present were scored for number, size and position in relation to the hypocotyl using a system modified from Howieson and Ewing (1989) as shown in Table 5.1. Statistical analysis of foliage dry weights and nodule scores were performed as described in section 3.2.1.5.

Table 5.1: Score of individual nodules determined by size and position on the root system of *Acacia ligulata* Benth. and *Acacia tetragonophylla* F.Muell. [modified from Howieson and Ewing (1989)].

Nodule size and position		Score
Nodule size (mm)	<2	**1**
	2-8	**3**
	>8	**5**
Nodule position	Tap root (0-5 cm)	**5**
	Tap root (>5 cm)	**2**
	Lateral (1 cm from tap)	**3**
	Elsewhere	**1**

5.2.3 2007 field establishment of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. with inocula carrying selected Shark Bay isolates

5.2.3.1 Field establishment experimental design

In July 2007, borrow pit sites G, P and Q1 (section 2.2.1.1) were prepared for sowing of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. with the aim of assessing the effect

of inoculation and inocula delivery on plant establishment. The borrow pits were ripped using a bulldozer equipped with a winged tine and rabbit-proof fencing erected around a 10x40 m area to exclude herbivores. The borrow pit sites were sown with *A. ligulata* Benth. seed inoculated with dried alginate beads and uninoculated seed and *A. tetragonophylla* F.Muell. seed inoculated with peat, clay, dried alginate and dissolved alginate bead treatments as well as uninoculated seed. There were ten plots per treatment and three replicates of each treatment sown in a randomized arrangement of plots spaced every 0.5 m along rows that were 1 m apart.

5.2.3.2 Seed and inoculant preparation

Inocula prepared for *A. ligulata* Benth. contained isolates 1b36, 2b35, 3b33, 4b36, 5b42, 6b45, 7b33 and 8b3, representatives of each soil collection site screened for effectiveness in section 3.2.4.2. Culture plates for each of the isolates were prepared on CRS-M1 agar from the -20°C glycerol stock (section 2.2.3.3) and incubated at 28°C for 3 days. From the plates, individual 70 mL CRS-M1 broths were inoculated, incubated and shaken at 28°C for 4 days at 200 rpm. The bacterial concentration of each CRS-M1 broth culture was determined to be between 10^6 and 10^8 cfu mL^{-1} by the Miles and Misra method (Miles *et al.*, 1938). The individual broths were combined to create a stock broth from which *A. ligulata* Benth. inocula were prepared. Dried alginate beads were prepared as per section 5.2.1.2. There was insufficient provenance seed of *A. ligulata* Benth. to prepare other inocula for sowing at each of the three sites and the two treatments were seed inoculated with dried alginate beads and uninoculated seed.

A stock broth containing isolates 1a13, 2a11, 3a23, 4a13, 5a16, 6a15, 7a23 and 8a11, selected from section 3.2.4.3, was prepared for *A. tetragonophylla* F.Muell. as described above and used in the inocula carriers, peat (section 5.2.1.3), clay (section 2.2.3.3) and both dried and dissolved alginate beads (section 5.2.1.2). Dissolved beads were prepared at site prior to sowing as described in section 5.2.1.2.

Seeds of *A. ligulata* Benth. (Shark Bay) and *A. tetragonophylla* F.Muell. (Shark Bay) were pre-treated for sowing by immersion for 2 min in water that had just reached boiling point. For each plot, four or five pre-treated seeds of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. respectively and an amount of the required inoculant to ensure between 10^7 to 10^8 cfu per seed was placed in a labeled 10x5 cm envelope. This was repeated for each treatment and both host species for the three borrow pit sites. Uninoculated and dissolved bead treatment envelopes were prepared with seed only. At sowing, a small depression was dug at the plot site into which the required envelope contents were placed and then covered with approximately 15 mm of soil. Dissolved alginate prepared at 10^8 cfu per seed was inoculated directly onto sown seeds before covering with soil. Plots were watered thoroughly at sowing and subsequently watered for two more days.

5.2.3.3 Plant evaluations and statistical analysis

The number of germinated plants was recorded at 4 months in November 2007 with growth status rated as described in section 2.2.2.2. Between five to seven representative plants of each treatment were carefully harvested to include as much of the root system as possible. The foliage above the hypocotyl was reserved for dry weight analysis as per section 3.2.1.3 and the presence or absence of root nodules noted. Statistical analysis was performed with 2-way analysis of variance (ANOVA) and where applicable the Fisher's least significant difference (LSD) test with α=0.05 was conducted with IBM® SPSS® version 21. Further growth status assessment of the juvenile *Acacia* spp. was conducted at 11 and 18 months in June 2008 and February 2009, respectively.

5.2.4 2008 field establishment of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. with inocula carrying selected Shark Bay isolates

5.2.4.1 Inocula preparation and sowing

Using methods described in section 5.2.3.2, a stock broth was prepared for *A. ligulata* Benth. containing isolates 1b36, 2b35, 2b36, 4b36, 6b45, 3b44, 2a11, 6a12 and 5a25 and for *A. tetragonophylla* F.Muell. the stock broth contained isolates 1a26, 1b36, 3a23, 3b33, 4a13, 5a11, 5a25, 6a12 and 6a15. Clay, alginate bead and peat inocula was prepared as described in section 5.2.3.2 for both *Acacia* species. Envelopes were prepared for the borrow pits as described in section 5.2.3.2 and contained five pretreated seed with the required amount of inocula at a rate of 10^8 cfu per seed. Seed envelopes were prepared for unioculated and dissolved bead treatments and the dissolved alginate inoculant prepared at site to apply at 10^8 cfu per seed (section 5.2.3.2). Seed was sown and inoculated in June 2008 as described in section 5.2.3.2, adjacent to the previous year's plantings in the fenced borrow pits.

5.2.4.2 Plant evaluations and statistical analysis

Plant number and growth evaluations were as described in section 5.2.3.3 and carried out at 4 months in October 2008 and at 8 months in February 2009. Five plants per replicate of each treatment were harvested at 4 months for dry weight statistical analysis as described in sections 3.2.1.3 and 5.2.3.3. Presence or absence of nodules noted and the nodules present were collected and stored as described in section 3.2.1.1.

5.2.5 Competitive ability of 2008 inocula to nodulate *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. and establishment of RNB isolates in pit sites

The re-introduction of isolates into the degraded pit sites was evaluated by comparing nodule occupancy of harvested plants from borrow pits G and P by RPO1-PCR fingerprinting and by conducting a MPN assay to enumerate the RNB population in site G soils.

5.2.5.1 Isolate occupancy of nodules

Bacteria were isolated as described in section 3.2.1.2 from stored nodules of the dissolved alginate bead and uninoculated treatments collected in section 5.2.4.2. These isolates as well as isolates used in the 2007 (section 5.2.3.2) and 2008 (section 5.2.3.4) inocula preparations were RPO1-PCR fingerprinted as per method in section 3.2.1.4. RPO1-PCR fingerprints were manually compared between nodule isolates, inocula isolates and other isolates previously RPO1-PCR fingerprinted in Chapter 3.

5.2.5.2 Most probable number of RNB

In October 2008, four months post sowing and inoculation, soil was collected from uninoculated, inoculated and undisturbed areas at site G. As described in section 2.2.3.1, subsamples of soil from the three areas were collected and homogenized. Enumeration of root nodule bacteria in the three areas on *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. was performed as described in section 2.2.3.3.

5.3 Results

5.3.1 Inocula effect on nodulation of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell.

Mean nodule scores of *Acacia* plants harvested at 42 dpi showed no significant effect ($P \leq 0.05$) of carrier media for either host (Figure 5.1). Although the mean nodule scores for *A. ligulata* Benth. were greater than those for *A. tetragonophylla* F.Muell. in the peat, clay and dissolved alginate treatments, this difference is largely attributed to larger *A. ligulata* Benth. nodules rather than nodule number or position. The wide variation in nodule size and number of *A. tetragonophylla* F.Muell. in replicates of the dissolved alginate treatment contributed to the large standard error of mean nodule score (Figure 5.1).

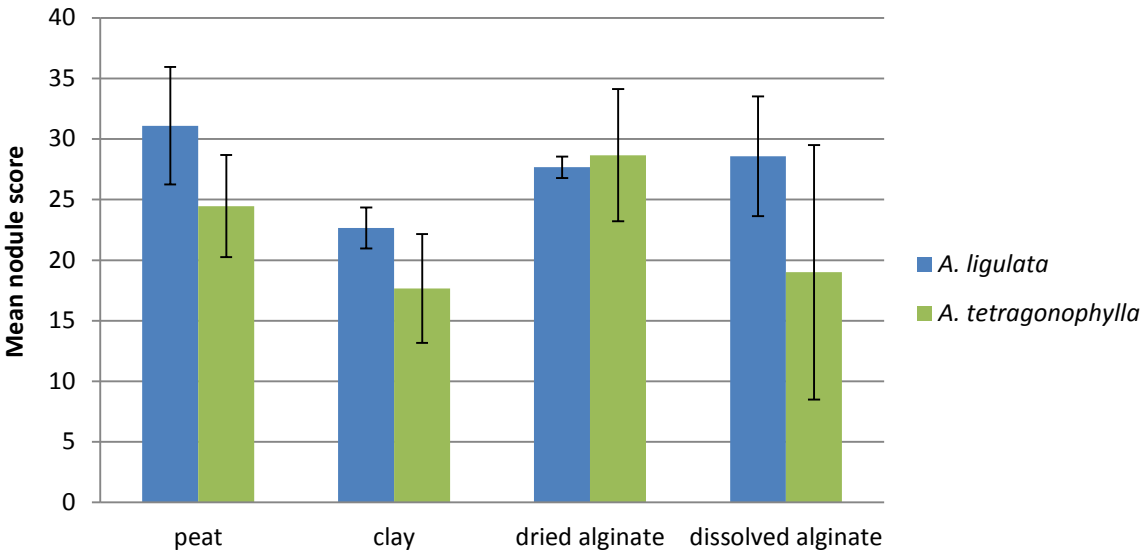


Figure 5.1: Nodulation score of *A. ligulata* Benth. (a) and *A. tetragonophylla* F.Muell. (b) inoculated with 6a12 in various inocula, harvested at 42 dpi. ANOVA ($P \leq 0.05$) detected no significant difference between treatments or host species. Values are shown with standard error of means (vertical lines).

There was a significant effect ($P \leq 0.05$) of carrier on the mean dry foliage weights of *A. ligulata* Benth., with the foliage dry weights of plants treated with inoculated and uninoculated clay being reduced by 16-20% and 26% respectively in comparison to plants treated with the inoculated and uninoculated peat, dried alginate and dissolved

alginate treatments (Figure 5.2). The foliage mass of inoculated *A. ligulata* Benth. was greater than the plants grown with the uninoculated carrier at 42 dpi, although this difference was not statistically significant.

Neither inoculation nor carrier had a significant effect ($P \leq 0.05$) on the foliage mass of *A. tetragonophylla* F.Muell.. However, alginate in both dried and dissolved forms resulted in greater mean foliage mass in comparison to the other respective inoculated and uninoculated treatments (Figure 5.2). Addition of inoculated and uninoculated clay resulted in reduced mean foliage dry weights of *A. tetragonophylla* F.Muell. in comparison to the respective peat, dried alginate and dissolved alginate treatments (Figure 5.2).

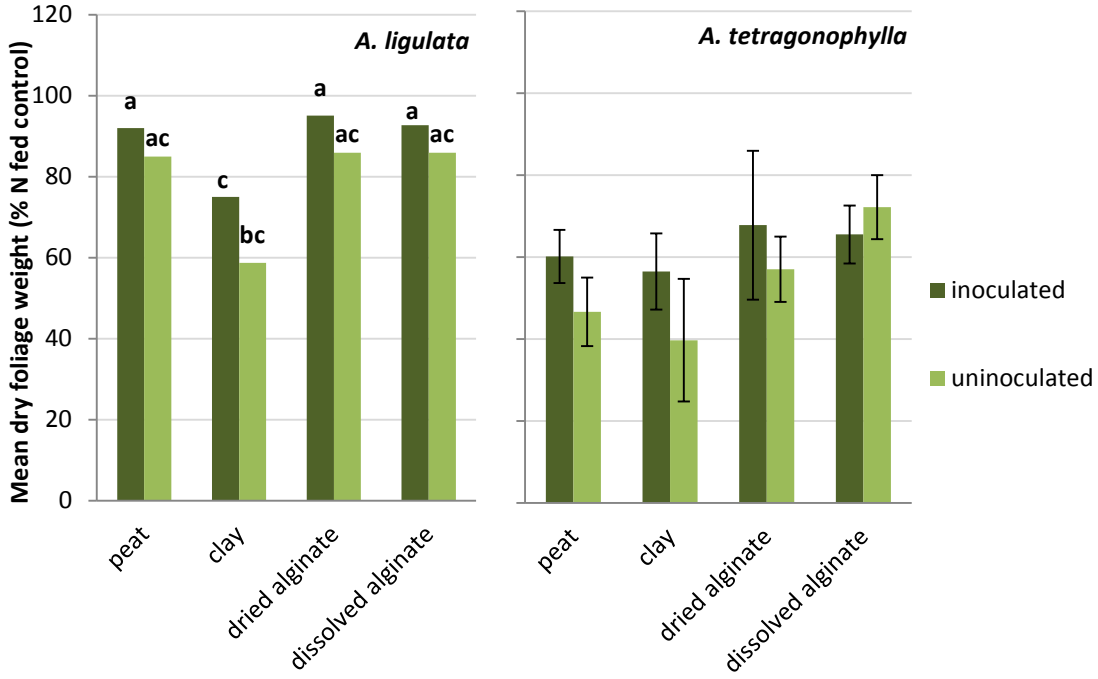


Figure 5.2: Mean dry foliage weight of *A. ligulata* Benth. (a) and *A. tetragonophylla* F.Muell. (b) inoculated with 6a12 in various inocula treatments and uninoculated treatments, harvested at 42 dpi. Treatments of *A. ligulata* Benth. that share a letter are not significantly different according to Fisher's LSD test ($P \leq 0.05$). ANOVA ($P \leq 0.05$) detected no significant difference between treatments for *A. tetragonophylla* F.Muell. and values are shown with standard error of means (vertical lines).

5.3.2 Plant field establishment with inocula (July 2007-Feburary 2009)

5.3.2.1 Plant establishment and condition status

The effect of inoculation at borrow pits G, P and Q1 on the survival rates of germinated *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. and their condition status was assessed at 4, 11 and 19 months post sowing and inoculation.

5.3.2.1.1 *A. ligulata* Benth.

There was a significant effect ($P \leq 0.05$) of both inoculation and borrow pit on the percentage of *A. ligulata* Benth. plants established at four months from the potential number of seed sown. The greatest number of seedlings observed was at G, with 62% and 59% of the seed sown germinated for dried alginate and uninoculated treatments, respectively and the lowest percentage was the uninoculated plants at Q1 with 33% of seed sown germinated (Figure 5.3). Inocula or borrow pit had no significant effect on the percentage of plants surviving to 11 and 19 months at G and P (Figure 5.3). No *A. ligulata* Benth. or *A. tetragonophylla* F.Muell. plants survived at Q1 to 11 and 19 months due to the pit being flooded with hypersaline water from an adjacent pond in March 2008 during a cyclone.

At four months, approximately half of all the surviving germinated *A. ligulata* Benth. were growing or vigorous with the exception of Q1 uninoculated plants where only 1% of the plants that successfully germinated were actively growing and all the remaining seedlings were stressed, dying or dead (Figure 5.3). The mean percentage of *A. ligulata* Benth. inoculated with dried alginate surviving to 11 months correlates to plant condition at four months at both G and P. Over half of these plants were stressed and the *A. ligulata* Benth. that were growing and vigorous at 11 months were in a similar condition at 19 months (Figure 5.3). All the uninoculated *A. ligulata* Benth. at P that survived to 11 months were vigorous or growing and the numbers of plants increased at 19 months by 2.6% and 4.4% for the dried alginate and uninoculated treatments respectively (Figure 5.3).

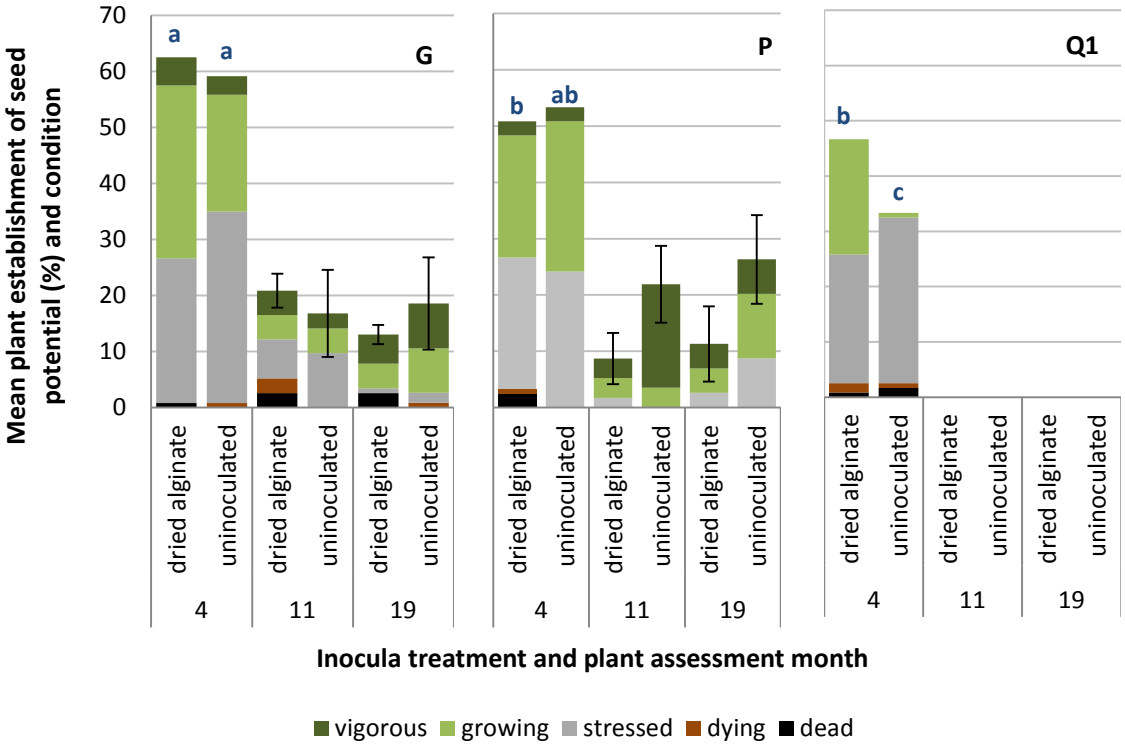


Figure 5.3: Mean percentage establishment and condition status assessment of *A. ligulata* Benth. plants sown in 2007 at pits G, P and Q1 at 4, 11 and 19 months post sowing and inoculation. Pit treatments at 4months that share a letter are not significantly different according to Fisher's LSD test ($P \leq 0.05$). The mean percentage values for 11 and 19 months account for plants harvested at 4 months and ANOVA detected no difference in the means between sites (excluding Q1) at 11 and 19 months respectively. Values are shown with standard error of means (horizontal lines).

5.3.2.1.2 *A. tetragonophylla* F.Muell.

There was no significant effect ($P \leq 0.05$) of inoculation or pit site on the mean percentage of *A. tetragonophylla* F.Muell. seedlings established at four months, due largely to the high degree of variation between treatment replicates. However at Q1, the percentage means of *A. tetragonophylla* F.Muell. seedlings at four months indicated an inoculation effect, particularly of dried and dissolved alginate treatments, respectively with 65% and 62% of germinated plants compared to established uninoculated seedlings at 21% (Figure 5.4). Less than a third of the germinated *A. tetragonophylla* F.Muell. that survived to 4 months were growing or vigorous at G, P and Q1 (Figure 5.4). Across inoculation treatments at G, the percentage of *A. tetragonophylla* F.Muell. surviving and growing at 11 months was not dissimilar to that at 19 months. Whereas at P, only inoculated plants survived to 19 months and in a reduced condition with less than 3% of the seed potential actively growing (Figure 5.4).

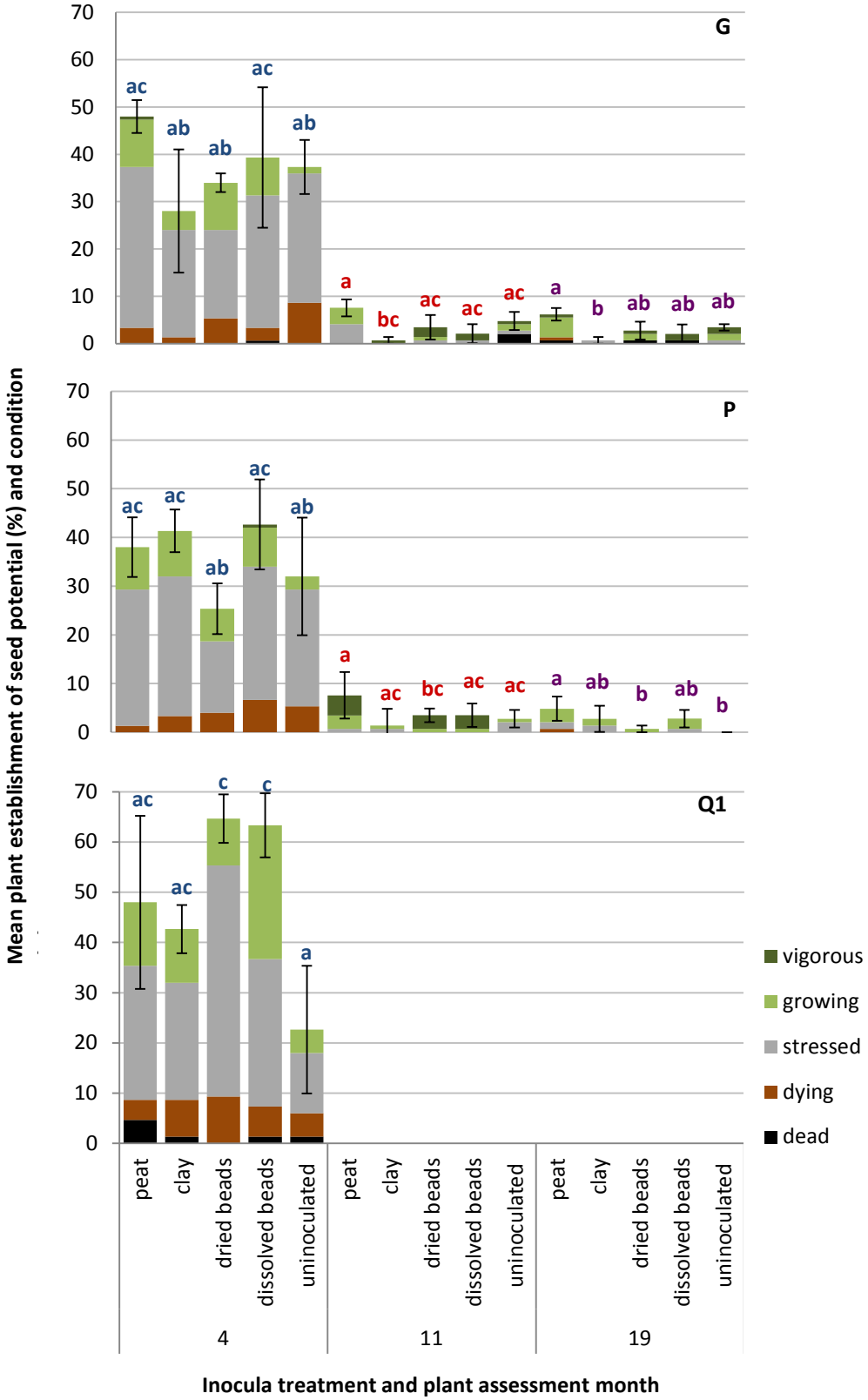


Figure 5.4: Mean percentage plant establishment and condition status assessment of *A. tetragonophylla* F.Muell. sown in 2007 at pits G, P and Q1 at 4, 11 and 19 months post sowing and inoculation. The mean percentage values for 11 and 19 months account for plants removed for assessment at 4 months. ANOVA detected no difference in the means between sites at each assessment time (excluding Q1 at 11 and 19 months) and values are shown with standard error of means (horizontal lines). Treatments at each assessment period that are not significantly different according to Fisher's LSD test ($P \leq 0.05$) share a letter at 4 (blue), 11 (red) and 19 (purple) months.

5.3.2.2 Plant growth

5.3.2.2.1 *A. ligulata* Benth.

The foliage weight at four months of harvested *A.ligulata* Benth. inoculated with dried alginate at G was significantly different ($P \leq 0.05$) to the other inoculated and uninoculated treatments at G, P and Q1 (Figure 5.5). The growth of inoculated *A. ligulata* Benth. at G was two-fold greater than either inoculated or uninoculated harvested plants at P and Q1 (Figure 5.5). There was a correlation in the foliage weights of *A. ligulata* Benth. in the pits to the observed condition status of these plants at four months, particularly at P and Q1 (Figure 5.3).

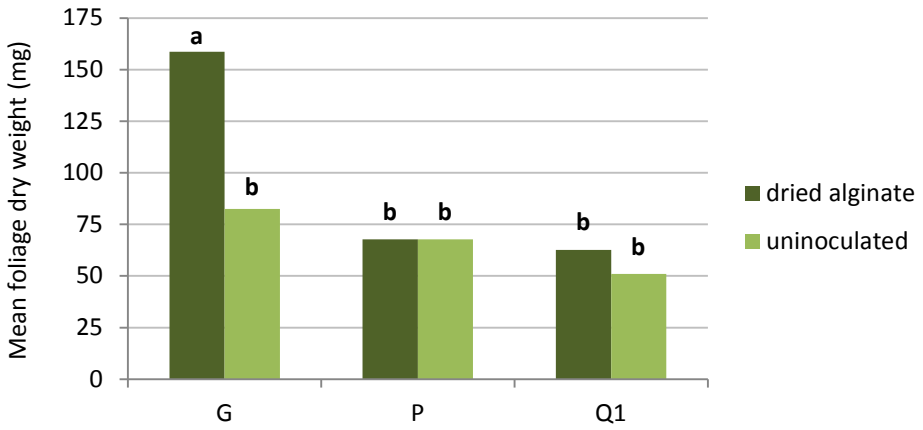


Figure 5.5: Mean dry foliage weights of *A. ligulata* Benth. sown and inoculated in 2007 and harvested at 4 months at pits G, P and Q1. Treatments that share a letter are not significantly different according to Fisher's LSD test ($P \leq 0.05$).

5.3.2.2.2 *A. tetragonophylla* F.Muell.

There was a large variance in the foliage weights of the replicates of *A. tetragonophylla* F.Muell. with no significant effect ($P \leq 0.05$) of inoculation on foliage production at G, P and Q1 detected by 2-way ANOVA (Figure 5.6). However, the LSD test did detect a difference between the treatment means, particularly at P. There was a positive effective on the foliage weights of *A. tetragonophylla* F.Muell. with peat and dissolved alginate treatments, while clay and dried alginate treatments produced a comparatively negative effect on growth (Figure 5.6). A negative effect on foliage weight was also seen with the dissolved alginate treatment at G in comparison the other sites and treatments (Figure 5.6).

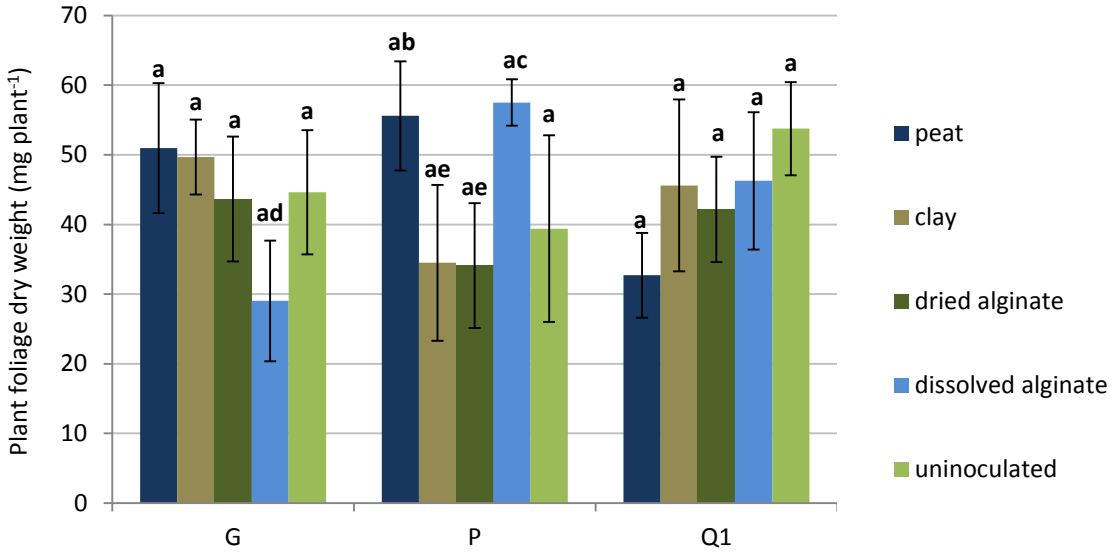


Figure 5.6: Mean dry foliage weights of *A. tetragonophylla* F.Muell. sown and inoculated in 2007 and harvested at 4 months at pits G, P and Q1. ANOVA ($P \leq 0.05$) detected no significant difference between treatments and sites. Vertical lines shown are standard error of means. Treatments that share a letter are not significantly different according to Fisher's LSD test ($P \leq 0.05$).

5.3.2.3 Nodulation response

5.3.2.3.1 *A. ligulata* Benth.

Nodulation was evident at all sites on *A. ligulata* Benth. in both the inoculated dried alginate and uninoculated treatments (Table 5.1). A greater proportion of the harvested *A. ligulata* Benth. were nodulated in the inoculated treatments with 100% at G and P and 75% at Q1, compared to the uninoculated plants with 86%, 33% and 67% for G, P and Q1 respectively (Table 5.1).

5.3.2.3.2 *A. tetragonophylla* F.Muell.

The percentage nodulation of *A. tetragonophylla* F.Muell. plants varied widely across the different inocula treatments, with no nodules on the uninoculated plants harvested at P. Dried alginate produced the lowest percentage nodulation compared to the other inocula treatments at all sites and the uninoculated plants at G and Q1 (Table 5.1). At Q1, 100% of harvested *A. tetragonophylla* F.Muell. were nodulated in the peat, dissolved alginate and uninoculated treatments (Table 5.1).

Table 5.1: Percentage nodulation in pits at 4 months of harvested *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. sown and inoculated in July 2007.

Treatment	G	P	Q1
A. ligulata Benth.			
Dried alginate	100	100	75
Uninoculated	86	33	67
A. tetragonophylla			
Peat	80	60	100
Clay	50	57	67
Dried alginate	14	37.5	57
Dissolved alginate	86	86	100
Uninoculated	25	0	100

5.3.3 Plant field establishment with inocula carrying selected provenant isolates (June 2008-February 2009)

5.3.3.1 Plant establishment and condition status

No plants survived germination in site Q1 due to the flooding of the site with hypersaline water from an adjacent evaporation pond. The following results are only for the field establishment parameters of pit sites G and P.

5.3.3.1.1 *A. ligulata* Benth.

There was no significant effect ($P \leq 0.05$) of inoculation or borrow pit on the percentage of *A. ligulata* Benth. plants germinated at four months and established at eight months from the potential number of seed sown (Figure 5.7). However, at four months the Fisher's LSD test did detect differences with the number of established dissolved alginate inoculated plants at site G being 17% lower than with clay and dissolved alginate at this site and 20% lower than with clay and dissolved alginate at site P (Figure 5.7). The number of dissolved alginate inoculated plants at site G was 26% lower than established plants at site P inoculated with peat at 4 months (Figure 5.7). By eight months, the variation in the percentage of established plants was less marked and the only difference is between the dissolved alginate plants at G and the peat plants at P Figure 5.7). A small site effect was indicated by a greater proportion of *A.*

ligulata Benth. germinates at P surviving to eight months with a loss of between 1% and 7% from the plants at four months compared to a loss of between 6% and 10% across the treatments at G.

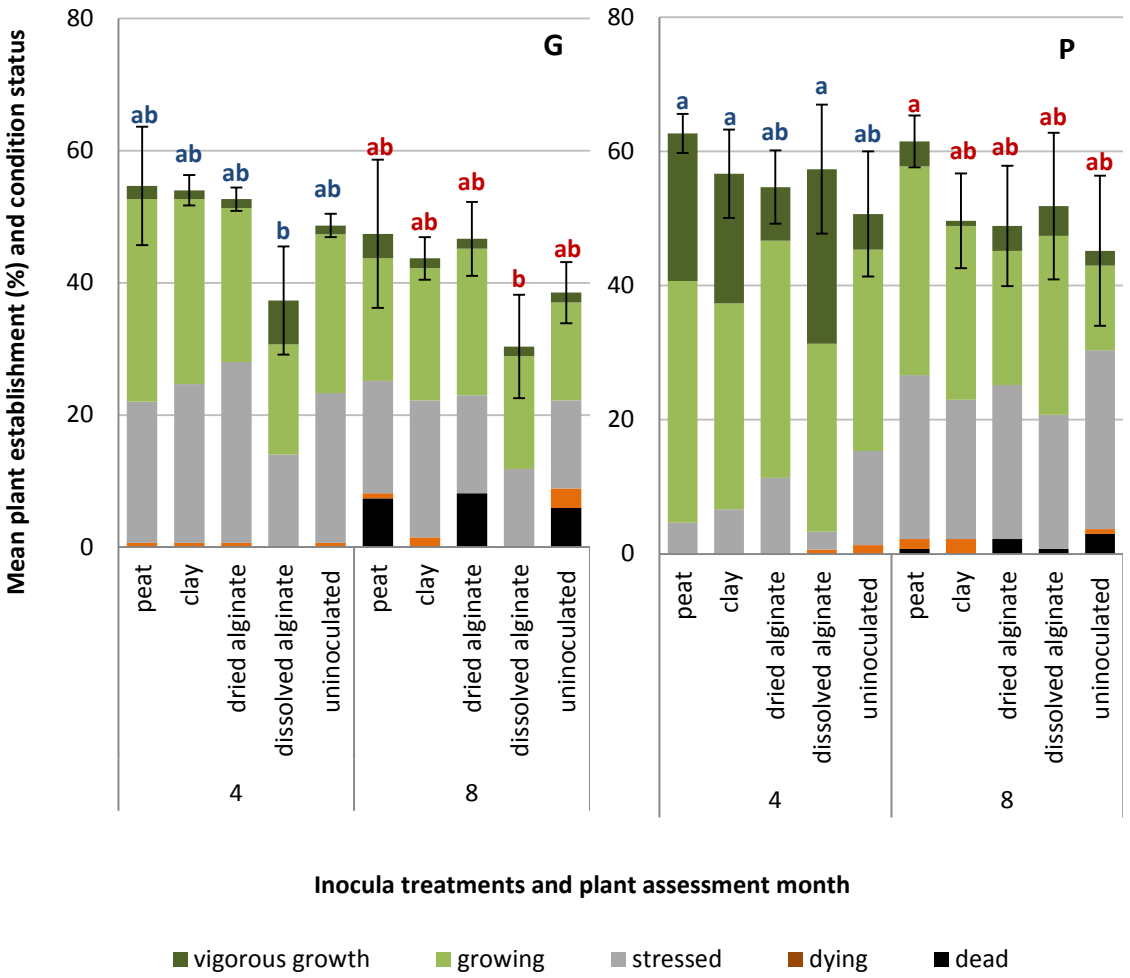


Figure 5.7: 2008 plant establishment and condition status assessment at pits G and P of *A. ligulata* Benth. at 4 and 8 months post sowing and inoculation. Mean percentage values at 8 months account for harvested plants at 4 months. ANOVA ($P \leq 0.05$) detected no difference in the means and values are shown with standard error of means (horizontal lines). Treatments at each assessment period that are not significantly different according to Fisher's LSD test ($P \leq 0.05$) share a letter at 4 (blue) and 8 (red) months.

This site effect was also evident in the condition status of the plants at P at four months. The percentage of growing and vigorous *A. ligulata* Benth. was between 2.3 to 13.5-fold greater to the stressed, dying and dead plants at P for the uninoculated and dissolved alginate treatments respectively (Figure 5.7). Whereas at G, the growing and vigorous *A. ligulata* Benth. was only 0.9 to 1.7 fold greater than the stressed, dying and dead plants for the dissolved and dried alginate treatments, respectively (Figure 5.7).

At eight months there was no site effect evident on the vigor of the established plants at G and P with a negligible difference in percentage proportion of vigorous and growing plants to the stressed, dying and dead (Figure 5.7).

5.3.3.1.2 *A. tetragonophylla* F.Muell.

There was no significant effect ($P \leq 0.05$) of inoculation and borrow pit on the percentage of *A. tetragonophylla* F.Muell. plants germinated at four months and established at eight months from the potential number of seed sown (Figure 5.8). However, at four months the Fisher's LSD test did detect differences. At site P there was 60% germination of peat inoculated *A. tetragonophylla* F.Muell., this was 21% more than dried alginate at site P and 22%, 14% and 32% greater than the clay, dried alginate and dissolved alginate treatments (Figure 5.8). Although the percentage of *A. ligulata* Benth. germinated for the treatments at site P was higher than the respective treatments at G, there was some consistency in the response of *A. tetragonophylla* F.Muell. to the different inocula (Figure 5.8). This inoculation response was no longer evident in the percentage of established plants surviving to eight months and the only discernible difference between the treatments was peat inoculated *A. tetragonophylla* F.Muell. at P and both alginate treatments at site G (Figure 5.8).

A. tetragonophylla F.Muell. growing at site G was exhibiting more signs of stress at four months in comparison to those at site P. The percentage proportion of growing and vigorous *A. tetragonophylla* F.Muell. to stressed, dying and dead at P ranged between 3.5 and 16.3-fold greater, compared to site G where the growing and vigorous *A. tetragonophylla* F.Muell. was only 1.7 to 3.7-fold greater than the stressed, dying and dead plants (Figure 5.8). At eight months, there was little difference between sites in the proportion of actively growing plants to those with a condition of stressed to dead. The vigorous and growing *A. tetragonophylla* F.Muell. were 1.1 to 1.4-fold and 1.2 to 1.8-fold greater than stressed, dying and dead plants at G and P respectively (Figure 5.8).

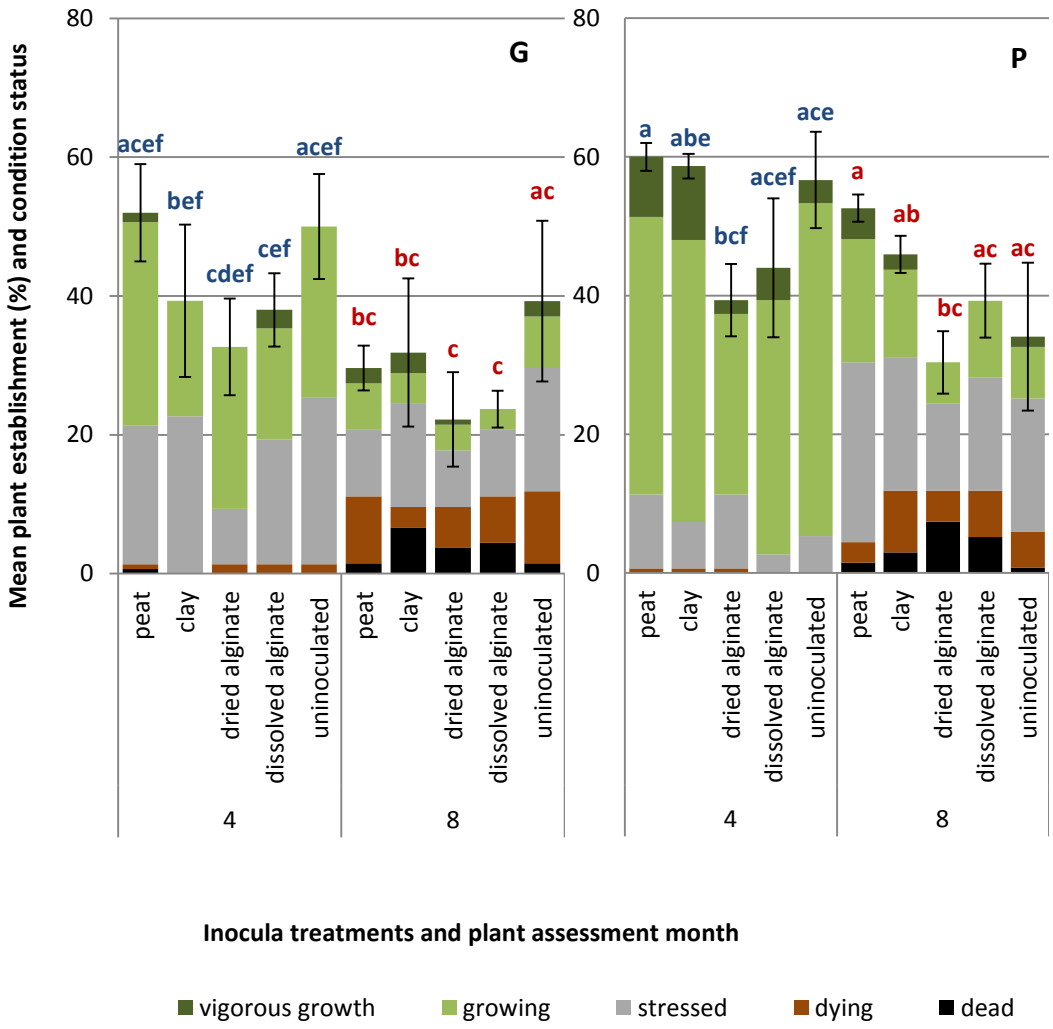


Figure 5.8: 2008 plant establishment and condition status assessment at pits G and P of *A.tetragonophylla* F.Muell. at 4 and 8 months post sowing and inoculation. Mean percentage values at 8 months account for harvested plants at 4 months. ANOVA ($P \leq 0.05$) detected no difference in the means and values are shown with standard error of means (horizontal lines). Treatments at each assessment period that are not significantly different according to Fisher's LSD test ($P \leq 0.05$) share a letter at 4 (blue) and 8 (red) months.

5.3.3.2 Plant growth

5.3.3.2.1 *A. ligulata* Benth.

A significant ($P \leq 0.05$) site effect and inocula carrier effect is evident in the growth of *A. ligulata* Benth. with greater foliage production of plants grown with peat at site P in comparison to all treatment plants harvested from site G (Figure 5.9). Although not significantly different ($P \leq 0.05$) with the treatments, clay, dried alginate, dissolved alginate and uninoculated dry weights at site P to those at site G, a site effect was evident with mean treatment weights at P 26 mg to 99 mg greater than the respective

treatment dry weights at G (Figure 5.9). Inoculant carrier also had an effect within both sites G and P, where the foliage weights of peat and dissolved alginate treated plants were greater compared to the clay, dried alginate and uninoculated treatments at these sites (Figure 5.9).

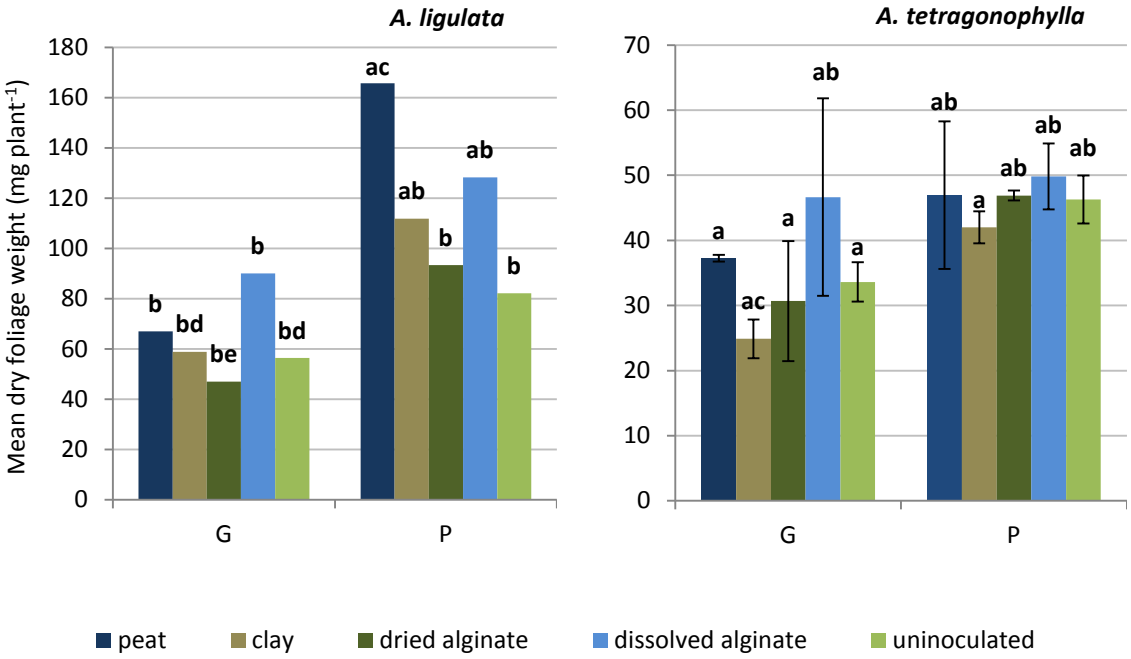


Figure 5.9: Mean dry foliage weights of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. sown and inoculated in 2008 and harvested at 4 months at pits G and P. Treatments for a host species that share a letter are not significantly different according to Fisher's LSD test ($P \leq 0.05$). ANOVA ($P \leq 0.05$) detected no significant difference between treatments and sites for *A. tetragonophylla* F.Muell. and vertical lines shown are standard error of means.

5.3.3.2.2 *A. tetragonophylla* F.Muell.

Inoculation or site had no significant effect ($P \leq 0.05$) on the mean dry foliage weights of *A. tetragonophylla* F.Muell. and for a number of treatments there was a large variance in the foliage weights of the replicates (Figure 5.9). As with *A. ligulata* Benth., there was a site effect evident with the dry weights of *A. tetragonophylla* F.Muell. at site P greater the dry weights of the respective treatments at site G (Figure 5.9). Similarly, there was an inoculation effect with peat and dissolved alginate on *A. tetragonophylla* F.Muell. within each site compared to clay, dried alginate and uninoculated plants, particularly at site G with the dry weights for peat and dissolved alginate treatments between 3.6 and 21.7 mg greater than clay, dried alginate and uninoculated plants (Figure 5.9).

5.3.3.3 Nodulation response

5.3.3.3.1 *A. ligulata* Benth.

Nodulation of *A. ligulata* Benth. was observed in all the inoculated and uninoculated treatments at both sites G and P. The inoculation treatments of peat, clay, dried and dissolved alginate had a positive effect on the percentage of nodulated *A. ligulata* Benth., with 93% or 100% of harvested plants nodulated at both sites G and P (Table 5.2). Whereas, nodulation at sites G and P of the uninoculated *A. ligulata* Benth. was only 67% and 40%, respectively (Table 5.2).

5.3.3.3.2 *A. tetragonophylla* F.Muell.

Nodulation of *A. tetragonophylla* F.Muell. was highly variable in the inoculated and uninoculated treatment and no positive nodulation effect was evident in response to inoculation. The lowest nodulation percentage of *A. tetragonophylla* F.Muell. was those inoculated with dissolved alginate, where 33% and 47% were nodulated at sites G and P, respectively (Table 5.2).

Table 5.2: Percentage nodulation in pits at 4 months of harvested *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. sown and inoculated in June 2008.

Treatment	G	P
A. ligulata Benth.		
Peat	93	100
Clay	93	93
Dried alginate	93	93
Dissolved alginate	100	100
Uninoculated	67	40
A. ***tetragonophylla*** F.Muell.		
Peat	73	53
Clay	60	87
Dried alginate	43	60
Dissolved alginate	33	47
Uninoculated	73	60

5.3.4 Competitive ability of 2008 inocula to nodulate *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. and establishment of RNB isolates in pit sites

5.3.4.1 Isolate occupancy of nodules

There were 47 isolates retrieved from desiccated nodules collected from individual *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. grown at sites G and P that had been inoculated with dissolved alginate or were uninoculated. Of these 47, 38 matched a RPO1-PCR fingerprint to 1a26, 2b35, 3b33, 3b44, 4b36, 5a11, 6a12 or 6b45 and 6a15, which were among the 16 selected isolates used in 2008 for inoculation. The remaining nine isolates produced an identical unique RPO1-PCR fingerprint, hereafter designated as type 1, which did not correspond to any of the selected isolates used in inocula in 2007 and 2008 or to those authenticated in Chapter 3. The four most frequently occurring RPO1-PCR fingerprints at 27.7%, 19.1%, 10.6% and 10.6%, corresponded to the isolates 2b35, type 1, 3b44 and 6a15, respectively.

From pit G, eight isolates were obtained from nodules of *A. ligulata* Benth. inoculated with dissolved alginate and there was a match to five RPO1-PCR fingerprints corresponding to the inoculation isolates 2b35, 6a12 or 6b45, 1a26, 3b44 and 5a11 (Figure 5.10). The seven uninoculated *A. ligulata* Benth. nodule isolates from site G had RPO1-PCR fingerprints matching isolates 3b33, 3b44 and 6a15. At site G, there were seven and eight *A. tetragonophylla* F.Muell. nodule isolates from the inoculated and uninoculated treatments respectively, with 57% of the inoculated and 63% of the uninoculated nodules occupied by 2b35 (Figure 5.10). The remaining 43% of the inoculated *A. tetragonophylla* F.Muell. nodules were occupied by isolates with a RPO1-PCR fingerprint corresponding to 3b33 and 6a15 and the remaining 37% of the uninoculated to 1a26, 3b33 and 3b44 (Figure 5.10).

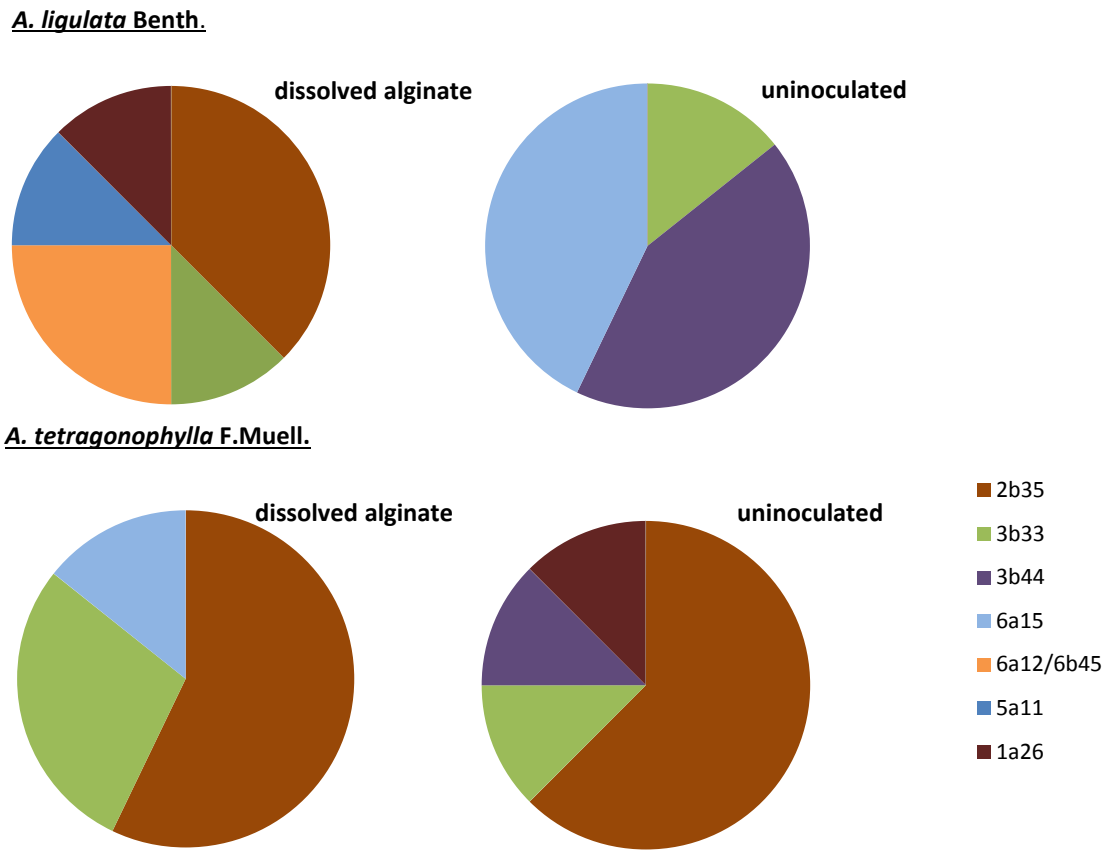


Figure 5.10: Percentage of isolate nodule occupancy of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. at pit G. Nodules recovered from uninoculated plants and plants inoculated with dissolved alginate containing selected isolates.

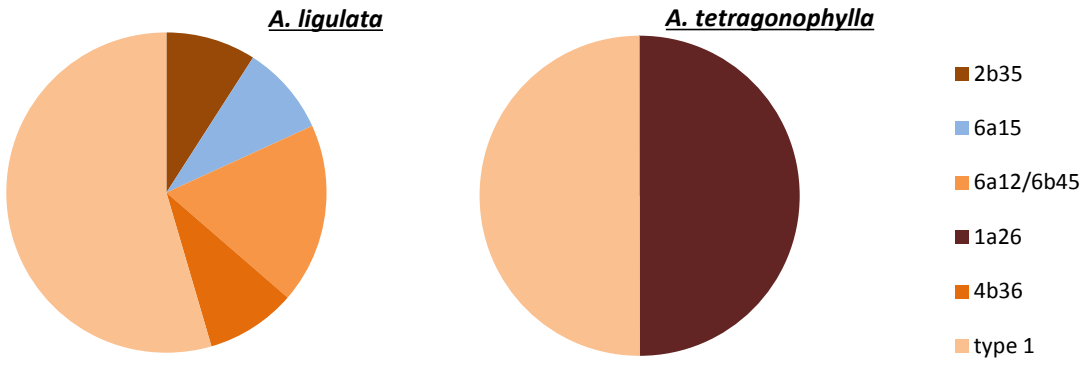


Figure 5.11: Percentage of isolate nodule occupancy of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. at pit P. Nodules recovered from plants inoculated with dissolved alginate containing selected isolates.

From pit P, there were 11 *A. ligulata* Benth. and six *A. tetragonophylla* F.Muell. isolates obtained from dissolved alginate treatment nodules. Few isolates were obtained from uninoculated treatment nodules of both *Acacia* sp. and no RPO1-PCR fingerprints were produced from these. Nodule occupancy at site P was dominated by the type 1 isolate, occurring in 54.5% and 50% of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. nodules respectively (Figure 5.11). The remaining nodule occupancy of *A. ligulata* Benth. at site P were by the isolates 2b35, 4b36, 6a15 and 6a12 or 6b45 and the remaining 50% of *A. tetragonophylla* F.Muell. nodules were occupied by 1a26 (Figure 5.11).

5.3.4.2 Most probable number of root nodule bacteria

From the soils collected in October 2008 from site G, four months post sowing and inoculating, the 95% upper and lower range of the mean estimate (P=0.05) indicated the uninoculated soil RNB numbers that nodulate *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. were significantly less than the RNB in the inoculated pit and undisturbed soils (Figure 5.12). The numbers of RNB detected in the inoculated soils on *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. were comparable to undisturbed soil RNB numbers (Figure 5.12). In the four months between June and October 2008, there was a 12 fold and four fold reduction in RNB in the undisturbed site G soils on *A. tetragonophylla* F.Muell. and *A. ligulata* Benth. (Figures 2.14 and 5.12).

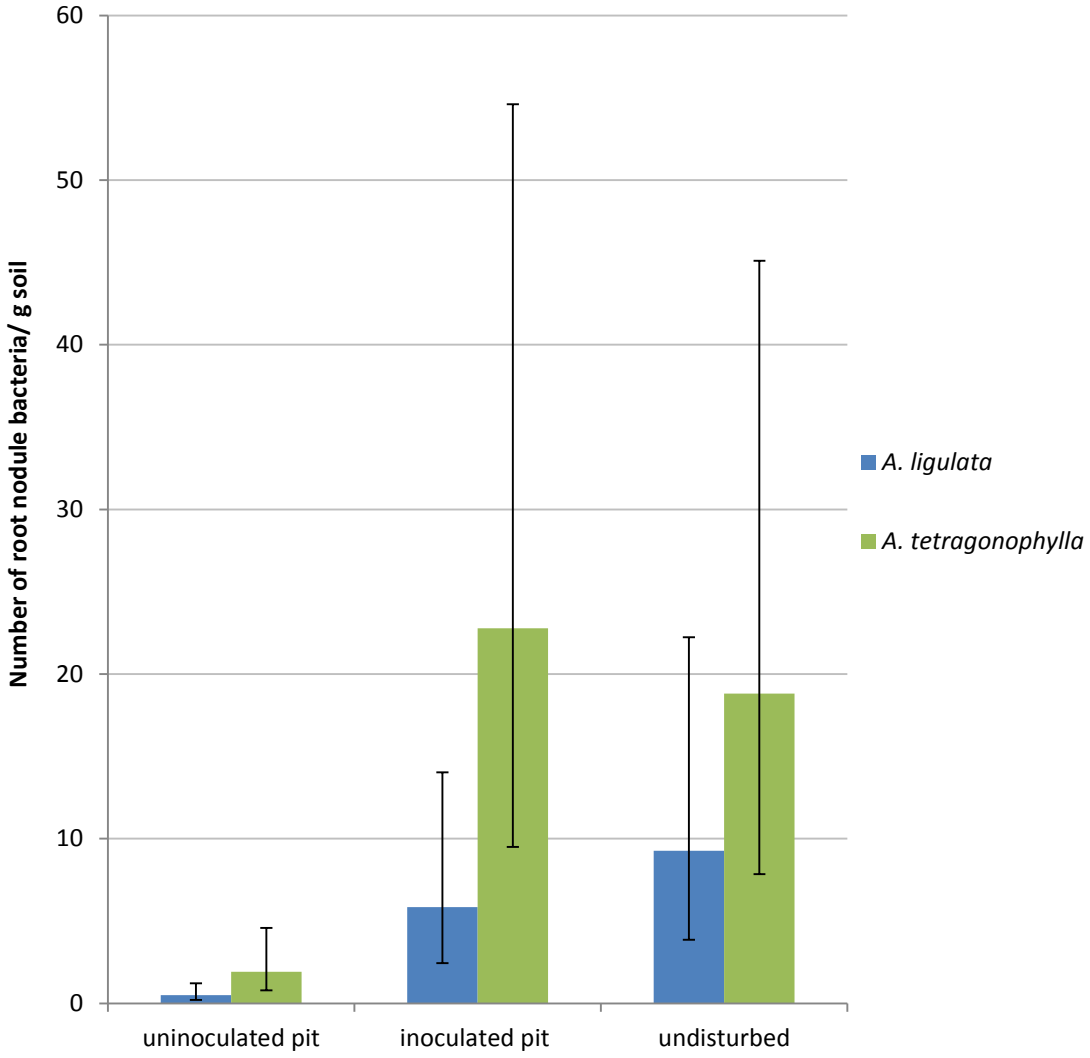


Figure 5.12: Most probable number of root nodule bacteria in soils collected from pit G in October 2008, four months post sowing and inoculation. Vertical bars represent range of confidence limit values (*P*=0.05).

5.4 Discussion

In general, inoculation of selected RNB on *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. sown in the pit sites increased nodulation in comparison to the uninoculated treatments. The nodules were occupied by the selected inoculant RNB, in addition to a novel isolate identified in nodules from plants grown in pit P. RNB inoculation into the pit soils increased the population and at 4 months post inoculation the population was at similar numbers to those in the surrounding undisturbed soils.

There are numerous challenges to re-establishing a plant community in an area that has remained in an un-vegetated state and these are amplified when the area is a

dryland zone. A number of the obstacles to rehabilitation that are applicable to the SBLA include the slow-growing nature of the vegetation, low and often sporadic rainfall, poor soil fertility and low numbers of soil microbes (Bachar *et al.*, 2012; Belnap *et al.*, 2005; EPA, 2005). Dryland areas do not regenerate spontaneously under these conditions (Bastida *et al.*, 2006) and this is certainly evident by the condition of the disturbed pit sites in this study.

When seeds were sown in the pits, the germination rates in 2007 were approximately 50-60% for *A. ligulata* Benth. and ranged from 36% to 60% in 2008 in sites G and P, with a greater number of seed germinating in site P in 2008. The germination rates of *A. tetragonophylla* F.Muell. in sites G and P were 25% to 50% in 2007 and 35% to 60% in 2008, with a greater number of seed germinating in site P in 2008. In 2007, *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. in pit Q1 inoculated with the alginate treatments had a significantly increased germination response in comparison to the uninoculated seed. This site was slightly saline with the conductivity in pit Q1 surface soils at 2.294 dS/m (Table 2.3). This level of salinity, while not toxic to *A. ligulata* Benth.*, A. tetragonophylla* F.Muell. and many other provenant plant species of the area, is high enough to be toxic to many species, particularly agricultural plants (Cook, 2006).

Unfortunately, the site pit Q1 was compromised for several reasons. In July 2007, five days after the seed had been sown; it was observed that there was predation on the seeds of both *Acacia* spp. (Figure 5.13). The predation had occurred randomly and the extent of it was unknown as it was observed immediately prior to leaving the site and more seed predation may have subsequently occurred. This affected the number of surviving germinates and there may have been a bias of the predators towards the uninoculated seed. In addition to the predation of seed, pit Q1 became an unviable site due to the flooding of the site with hypersaline water from an adjacent evaporation pond as a result of rainfall generated by tropical cyclone Ophelia in March 2008. This event killed any surviving germinates sown in 2007 in pit Q1 and of the seed sown and inoculated in June 2008 at this site, there were no surviving germinates.

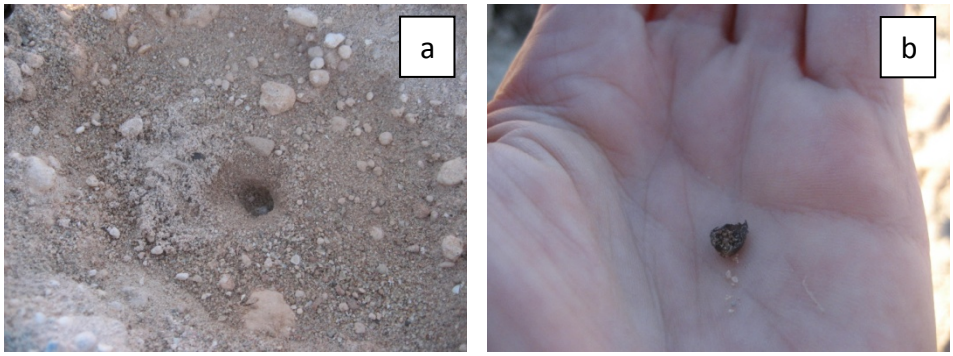


Figure 5.13: Seed predation in pit Q1 four days post sowing and inoculating in July 2007. Diggings at sown plot (a) and remains of the predated seed (b).

The germination rates of numerous Western Australian native species average 71% (Bell *et al.*, 1995). Given the aridity of the SBSLA site, the percentages of germination for both *Acacia* species in the field conditions are within acceptable limits for rehabilitation. It appears there is no impediment to seed germination at the sites and increased soil moisture levels at site P may have increased the number of surviving germinates of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell.. Many of the native species in the area rely on dispersal by ants and birds (Davidson & Morton, 1984; Standish *et al.*, 2007) and the movement of water across the landscape is an important mechanism for both seed and microbe dispersal (Belnap *et al.*, 2005; Pointing & Belnap, 2012). Presumably, some seed and various microbes from the surrounding areas would have been transported into the pit sites in these ways. However, the continued lack of vegetation signifies that factors other than germination are impeding the development of any recruits to maturity in these sites.

At the time of sowing and inoculation in July 2007 and June 2008 the MPN of RNB in the selected pit sites on *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. was much reduced in comparison to the numbers in the adjacent undisturbed area soils, with no RNB detectable in the Q1 pit soils in either year (Figure 2.13). The re-introduction of RNB into pit G appears to have successfully established a mixed RNB population as evidenced by nodule occupancy and MPN results. In 2009 at 4 months post inoculation, the inoculated soils in site G contained a RNB population that was comparable to the populations in the surrounding undisturbed area, whereas the numbers of RNB in the uninoculated pit soils were significantly lower (Figure 5.12). This is a significant finding as the death rates of RNB in inoculant media are the highest

immediately after application to the seed (Roughley & Vincent, 1967) or soil (Date, 2001). The surface soils at SBSLA can become dry rapidly, and failure of the RNB to nodulate is more likely in drought conditions after sowing and inoculating (Smith, 1992). In 2007, there was 43 mm of rainfall in the four-month period between sowing and inoculation and the assessment of germination, plant condition and nodulation (meteorological data supplied by SBS). In 2008, there was 75 mm of rainfall in this four-month period between sowing, inoculation and assessment and because of this increased rainfall, soil moisture conditions would have been more favourable to both microbial and plant growth during this time.

The RPO1- PCR fingerprints of the isolates recovered from nodules collected from the plants sown in 2008 matched the fingerprints of the inoculant isolates at time of sowing on both inoculated treatments and uninoculated treatments for both *Acacia* hosts at site G. This may indicate that these isolates were present in the soils at this site at the time of sowing. Of the isolates recovered from the *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. inoculated treatment at site P, 50% and greater were a novel RPO1-PCR fingerprint (Figure 5.11) which suggests a highly competitive RNB in the soils at site P able to nodulate both species. It is unknown whether the novel isolate was present in the soils at the other sites, nor is it known whether there was a population in site P in 2007 at the time of sowing. Tropical cyclone Ophelia in 2008 resulted in soil from the surrounding undisturbed areas being washed into the sites G and P. Prior to sowing in 2008, it was observed that a large amount of soil had been washed into the pit P site surrounding numerous plants sown in 2007, in some cases only the uppermost leaves of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. were prominent. This movement of soil into the pit may have introduced a number of RNB into the site, which could explain the presence of the novel isolate at site P. The average of the dried foliage weights across the different treatments of *A. ligulata* Benth. plants sown in site P in 2008 was 116.32 mg compared to an average of 63.88 mg for those in site G (Figure 5.9). Although only a small number of nodules were assessed, this isolate appears to be highly competitive and perhaps more effective at N_2 fixation compared to the re-introduced RNB.

The positive impact of increased rainfall improving the conditions for the plants sown in 2007 and 2008 was observed on site. The increased rainfall in 2008 in comparison to the severe drought of 2007 resulted in increased growth and vigour of the plants sown in 2008 and in the condition of the plants that had survived from the sow in 2007. Should re-introduced RNB (inoculation or washed into the areas) continue to persist in the soils and improved site conditions promote new plant and soil microbial growth, both the *Acacia* spp. could be re-infected by the resident RNB (Brockwell *et al.*, 2005) and contribute to the depleted nitrogen economy of the pit sites (Beadle, 1964).

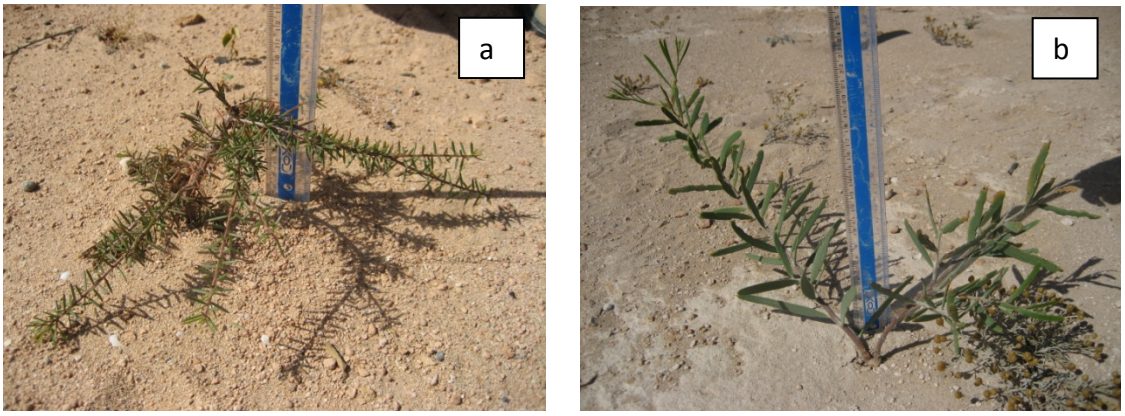


Figure 5.13: *Acacia ligulata* Benth. (a) and *Acacia tetragonophylla* F.Muell. (b) and at 19 months since seed had been sown. Plants were growing at site G. Image taken in February 2009 during high summer conditions.

The different inocula treatments may have affected not just the survival of the re-introduced RNB and the nodulation response of the two *Acacia* spp. but also the growth conditions of the developing seedlings. The glasshouse trials of the different inocula media showed no significant difference between the inoculated media compared to the uninoculated media in plant foliage weights for both *Acacia*, although the mean inoculated plants were larger for both *A. ligulata* Benth. and *A. tetragonophylla* F.Muell., with the exception of the dissolved alginate treatment for *A. tetragonophylla* F.Muell. (Figure 5.2). The bentonite clay treatment, although not significant had reduced nodule scores and the inoculated clay resulted in dried foliage weights significantly less than the other treatments for *A. ligulata* Benth. (Figures 5.1 and 5.2). The non-significant growth response of the plants to inoculation may be due to an insufficient growth period to see a response develop. The difference in the

growth rates of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. was described in section 3.4 and even though *A. ligulata* Benth. is a reputed fast grower, harvesting the plants at 42 dpi may have been too premature for even this species to demonstrate a response to the inoculants. There may have also been a growth response of both *Acacia* to the vectors themselves and not just the selected RNB. When a medium is assessed as a possible vector for inoculation, the main considerations are the survival of the isolates over time and in sufficient numbers at time of application (Deaker *et al.*, 2004; Herridge, 2008; Smith, 1992). The response of the plants to the medium itself in different environmental applications has received little attention.

Given the low nutrient levels and nature of the soils in the pits of SBSLA, the application of the inoculant media to the soil at time of sowing may have affected the properties of the rhizosphere for the plants germinating in the pits. Each of the carriers has different pH properties, water holding capacity (WHC), nutrient availability or soil ameliorating properties. None of the carriers was assessed for pH, WHC or nutrients, nor was the pH of the carriers amended. Peat, depending on the source, is usually acidic (Sparrow & Ham, 1983) and Na alginate has a neutral pH (Bashan *et al.*, 2002). Bentonite clay has been reported as neutral pH (Heijnen *et al.*, 1993) but this is highly dependent on source. Western Australian bentonite slurries have been reported at pH 9-10 whereas bentonite from Queensland had a pH of 7 (Duff, 1965).

Peat is an organic product and the addition of organic material to certain soils can dramatically increase the available water capacity (AWC) of the soil (Hudson, 1994). Sparrow and Ham (1983) analyzed the properties of Wisconsin sedge peat which was found to contain 26 ppm nitrate and 51% total carbon. Peat that has not been pH adjusted would also lower the soil pH and there would be the release of some nutrients from the bacterial culture media used to inoculate the peat. Sodium alginate has also been found to increase the WHC of inoculated soils by up to 11% through the formation of metal salts (Bashan, 1986). The solution used for the dissolved alginate treatment was a 0.2 M KH_2PO_4 (section 5.2.1.2) which would act as a fertilizer releasing essential macronutrients (Thomson *et al.*, 1993) into the soil. The WHC of bentonite sourced from Wyoming, USA has been found to hold 71% H_2O compared to

approximately 48% H_2O held in other clays from Virginia, USA (Thomas & Moody, 1962). Amending soil with bentonite can increase pH, with Heijnen *et al.* (1993) showing that the addition of bentonite to loamy sand increased the pH from 5.7 to 6.4. All the selected RNB were able to tolerate up to pH 11.0 (Table 4.3) and the site soils ranged from pH (0.01 M $CaCl_2$) 7.9 to 8.1 (section 2.3.1). An increase in pH may have not affected the inoculated RNB population. However, it may have affected either plant growth or nodulation in some way, as some effect was observed in the glasshouse results as discussed previously.

The properties of the inocula preparations and/or the growth, culture and storage conditions of the isolated RNB may have promoted changes in genetic properties, protein biosynthesis and function as has been shown in other bacteria (Björkman *et al.*, 2000; Nilsson *et al.*, 2005; Smith & Bidochka, 1998). Variable growth conditions can change the characteristics of RNB in as little as 2-5 generations and this can result in reduced infectiveness in some strains (Bergersen, 1961). Although it was difficult in the environmental conditions of the site to clearly discern the effects of inoculation on the establishment of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. in the pit sites, it is possible that the re-introduced RNB isolates had lost some of their properties, reducing their fitness to survive the conditions in the SBSLA and to form effective symbioses with the *Acacia* species.

Much of Australia's native flora, including *Acacia* spp., form associations with other beneficial microbes such as vesicular–arbuscular mycorrhizal (VAM) fungus, which assist in nutrient uptake and water acquisition for the plants (Brundrett & Abbott, 1991; Jasper, 2007). The disturbance and removal of soils and substrate at the pit sites will have further compromised low soil microbial populations and altered the microbial diversity and community function at these sites (Bastida *et al.*, 2006; Bolton *et al.*, 1993; Herrera *et al.*, 1993; Requena *et al.*, 2001). Typical soils are estimated to contain between 10^8 to 10^{11} microbes/g of soil, but these populations are reduced with increased aridity and hyperarid soils are estimated to contain 10^2 to 10^5 microbes/g of soil (Fletcher *et al.*, 2011). The number of microbes in dryland soils, such as those in SBLA, is unknown and the re-introduction of other beneficial microbes in addition to RNB would profit a more diverse floristic community in the pit sites.

Identifying and isolating these microbes and selecting a medium for application would be costly and time-consuming and certainly the remoteness of the SBSLA hinders certain aspects of any such rehabilitation approach and other ways of introducing these microbes onto the disturbed sites should also be considered. While top soil replacement is not possible, employing a seeding program using a slurry inoculant created from suspending soil samples in water collected from undisturbed soils may be a viable, cost effective way of introducing a suite of beneficial microbes (Smith, 1992) with minimal impact to the surrounding undisturbed areas.

Chapter 6

Competitiveness of provenant isolates and Wattle Grow™

So be sure when you step, Step with care and great tact. And remember that life's A Great Balancing Act.
-Dr. Seuss “Oh, The Places You'll Go!” 1990

6.1 Introduction

In agricultural ecosystems, symbiotic N_2 fixation is vulnerable to the nodulation of target legumes by resident RNB that are often ineffective or poorly effective (Nandasena *et al.*, 2007). The success of a highly effective selected inoculant often relies on inoculating the strain in sufficient numbers to outcompete the resident RNB (Deaker *et al.*, 2004; Howieson & Ballard, 2004; Yates *et al.*, 2011). Additionally, over time the beneficial impact of the inoculated strain on N_2 fixation declines, often due to a combination of reduced numbers in the soil or a reduced ability to compete for nodule occupancy (Crook *et al.*, 2012; Nandasena *et al.*, 2007).

Competition for nodulation can occur both in the saprophytic and symbiotic phase (Wielbo *et al.*, 2012). The genetic basis of infection and nodule development has been intensely investigated during the last three decades and more recently the microevolution of RNB populations and its effects on nodulation and N_2 fixation has received more attention (Crook *et al.*, 2012; Lindström *et al.*, 2010). Numerous examples exist of horizontal gene transfer from inoculant strains to indigenous RNB that has led to impaired N_2 fixation and increased competitiveness of the indigenous RNB (Barcellos *et al.*, 2007; Crook *et al.*, 2012; Nandasena *et al.*, 2007).

Although no longer in production, the commercialization of products such Wattle Grow™ for the inoculation of South Eastern Australian *Acacia* spp. may have the potential for the impairment of nodulation and N_2 fixation of resident RNB, with gene transfer from the inoculant similar to the effect reported in agricultural ecosystems. Wattle Grow™ was composed of four *Bradyrhizobium* strains isolated in Eastern Australia, beneficial to the establishment of native legumes for ameliorating salinity and groundwater issues on farmland and for site rehabilitation projects (Carr *et al.*, 2007; Thrall, 2011).

While the development of this and other microbial inoculant products for Australian native plants is encouraging, they have been developed from Eastern Australian isolates and at present little is still known about the microbial diversity of Western

Australia. The project, MICROBLITZ (www.microblitz.com.au) which seeks to map the soil microbial diversity of Western Australia will increase our knowledge on some levels. However, a more detailed understanding of the plant-microbe interactions of Western Australia is well overdue.

In Chapter 3, the N_2 fixation effectiveness of Wattle Grow™ was compared to isolates trapped from SBSLA soils and while it did produce effective yields particularly on *A. ligulata* Benth., there were Shark Bay isolates that were more effective for both *A. ligulata* Benth. and *A. tetragonophylla* F.Muell.. This chapter reports on the ability of selected Shark Bay isolates and Wattle Grow™ to persist in pit P soil (section 2.2.1.1) and compete with resident RNB for nodulation of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell..

6.2 Method

6.2.1 Growth tank design

Four growth tanks (1866 mm x 930 mm x 300 mm) were placed in a section of an air-conditioned glasshouse (Figure 6.1). The area was sealed off with plastic sheeting to reduce airborne contaminates. The tanks were divided with 5 mm PVC sheeting into three sub-sections, each measuring 930 x 622 mm and the base of each sub-section had a central drainage tube, 20 mm in diameter.

A mix of yellow sand and washed river sand (1:1) was prepared, this and basalt aggregate (particle size 20 mm) was placed separately into hessian bags and steamed for 2 h. The tank sub-sections were surface sterilized with 70% (v/v) ethanol and each sub-section layered with 40 mm of aggregate, 60 mm of sand mix, 25 mm of pit P homogenized soil collected in July 2007 (sections 2.2.1.1 and 2.2.3.1) and 25 mm of sand mix (Figure 6.2). Each tank was then covered with plastic sheeting to maintain aseptic conditions until sowing and inoculation. An elevated automated watering system was flushed for 15 min and programmed to operate for 5 min daily.

Figure 6.1: Growth tanks sown with *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. prior to Harvest 1. Orientation shown from west to east of the treatments Wattle Grow™, Isolates/Wattle Grow™, Isolates and Uninoculated.

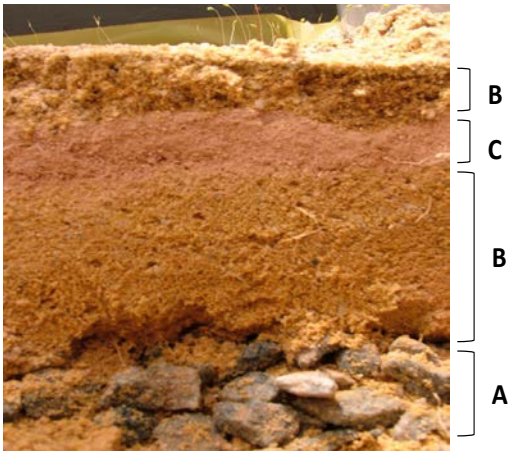


Figure 6.2: Growth tank substrate composition. Layers are composed of aggregate (A), sterile sand mix (B) and homogenized soil collected from pit P (C).

6.2.2 Experimental design

The design was to assess persistence of selected Shark bay isolates and Wattle Grow™ for nodulation of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. and their competitive ability with each other and with the resident RNB present in pit P soils. *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. were grown for three successive periods in rows as shown in Figure 6.3. There were three replicates for each treatment with each tank sub-section representing a replicate treatment. Plants were grown for 90 days and there was a 60-day lay period before the next sow. Harvest 1 plants were sown in the tanks in October 2007 and harvested in January 2008. Harvest 2 plants were sown in March 2008 and harvested in June 2008. Harvest 3 plants were sown in August 2008 and harvested in November 2008.

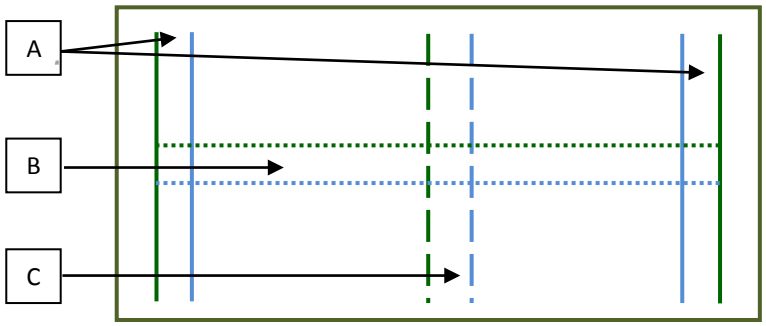


Figure 6.3: Successional sowing design of *A.ligulata* Benth. (blue) and *A.tetragonophylla* F.Muell. (green) in growth tank sub-sections. First sowing of Harvest 1 plants represented by solid lines (A), second sowing of Harvest 2 plants by dashed lines (B) and third sowing of Harvest 3 plants by dotted lines (C). Not to scale.

6.2.3 Sowing and inoculation

The four tanks were prepared for the following treatments: 1) Alginate beads inoculated with selected isolates for *A. ligulata* Benth. and *A. tetragonophylla* F.Muell.*;* 2) Wattle Grow™; 3) a mix of Wattle Grow™ and isolates and 4) uninoculated control. Mixed isolate stock suspensions were prepared with selected Shark Bay isolates for *A. ligulata* Benth. (1b36, 2b35, 3b33, 4b36, 5b42, 6b45, 7b33 and 8b3) and *A. tetragonophylla* F.Muell. (1a13, 2a11, 3a23, 4a13, 5a16, 6a15, 7a23 and 8a11) as per section 5.2.3.2 and suspended in alginate beads to contain 10^7 cfu g^{-1} (section 5.2.1.2). Wattle Grow™ was used in its commercial form and contained 10^5 cfu g^{-1}. The co-inoculation of alginate beads and Wattle Grow™ were mixed together to contain 10^7 cfu g^{-1} and 10^5 cfu g^{-1} respectively.

For the first sow, two rows that were 5 cm apart were prepared to a depth of 1.5 cm and the inoculant applied evenly along each row. Twenty pretreated seeds of *A. ligulata* Benth. (Laverton, WA) and *A. tetragonophylla* F.Muell. (Shark Bay, WA) (section 3.2.1.3) were sown into a respective row, covered with soil and watered with sterile H_2O. The alginate bead treatment was applied with 2 g of dried alginate in each row, the Wattle Grow™ at 13 g per row and the co-inoculated treatment received 2 g and 13 g per row of the dried alginate and Wattle Grow™ respectively. The successive plantings for Harvest 2 and Harvest 3 were sown in the same way without additional inoculant, in the locations as shown by Figure 6.3.

At 21 days post sowing, plants were thinned to 12 plants per row by removing all growth above the soil surface. For the duration of the growing period, plants were maintained with a weekly application to each row of 70 mL of nutrient solution (section 3.2.1.3). After 90 days, five plants in each replicate for each species were harvested carefully to cause the least disturbance to their root systems and to the substrate structure. The nodules were collected from these plants for assessment as per section 3.2.1.4 and the foliage removed at the hypocotyl and retained for analysis. The plants remaining in the tanks had their foliage removed at the soil surface and retained, leaving the root systems intact in the substrate.

6.2.4 RPO1-PCR fingerprinting of root nodules

Nodule occupancy was determined by RPO1-PCR fingerprinting of isolates obtained from macerated nodules as described in sections 3.2.1.2 and 3.2.1.4. Where possible, isolates were obtained from two nodules per plant. Banding patterns obtained from nodule isolates were compared to both the inoculant isolates and others re-authenticated in Chapter 3. Unique patterns were assigned an identification code. Due to heavy microbial contamination of the Wattle Grow™, no *Bradyrhizobium* isolates were obtained from the inoculant material to perform PCR-RPO1 fingerprinting for comparison. The percentage nodule occupancy was determined for each treatment at each harvest.

6.2.5 Assessment of N_2 fixation

The harvested foliage of each replicate was dried (section 3.2.1.3) and weights recorded. The dried foliage was then submitted to CSBP for nitrogen content analysis as per section 3.2.2.4.

6.2.6 Statistical analysis

The yield variance of the dry weight data and nitrogen content results was analyzed with 2-way ANOVA and Fisher's least significant difference (LSD) test where applicable (section 3.2.1.5).

6.3 Results

6.3.1 Competitiveness of provenant isolates and Wattle Grow™ for nodulation of *A. ligulata* Benth.

6.3.1.1 N_2 fixation effectiveness

Wattle Grow™ had a significant effect ($P \leq 0.05$) on the mean foliage weights of *A. ligulata* Benth. sown in September 2007, which yielded 63% more than the uninoculated plants (Figure 6.4a). While not significant, the Fisher's LSD test did indicate there was a response to inoculation of *A. ligulata* Benth. with the isolates and the co-inoculation of Wattle Grow™ at Harvest 1 (Figure 6.4a). At Harvest 2, ANOVA detected a significant difference ($P \leq 0.05$) in the reduced foliage weights of *A. ligulata* Benth. from the Isolates treatment compared to those of the Uninoculated and Wattle Grow™ treatments of which the dried foliage weights were up to 78.7 mg greater (Figure 6.4a). No effect between any of the treatments was evident at Harvest 3 however, the growth of the *A. ligulata* Benth. was considerably less than those of Harvest 1 and 2 with the dried foliage weights at Harvest 3 less than half of the previous harvests (Figure 6.4a).

Inoculation had a significant negative effect ($P \leq 0.05$) on the nitrogen contents of Harvest 1 plants. The uninoculated plants contained up to 0.28% more N in the dried foliage than the plants from the three inoculated treatments (Figure 6.4b). While at Harvest 2 there was no significant effect ($P \leq 0.05$) between the treatments, the uninoculated *A. ligulata* Benth. still contained the higher N concentrations (Figure 6.4b). *A. ligulata* Benth. from Harvest 3 that had been originally co-inoculated with a mix of Wattle Grow™ and isolates had significantly lower N concentrations of up to 0.42% less in the dried foliage than the N concentrations of *A. ligulata* Benth. foliage of the other treatments (Figure 6.4b). There was a negative correlation between the nitrogen concentrations and the mass of the dried *A. ligulata* Benth. foliage at Harvest 1 and Harvest 3, with increased dry foliage weight related to reduced foliage N

concentrations (Figures 6.5a and c) and though the relationship was not strong ANOVA detected a significant difference (P<0.05). There was no significant correlation between the nitrogen concentrations and the mass of the dried *A. ligulata* Benth. foliage at Harvest 2 (Figure 6.5b).

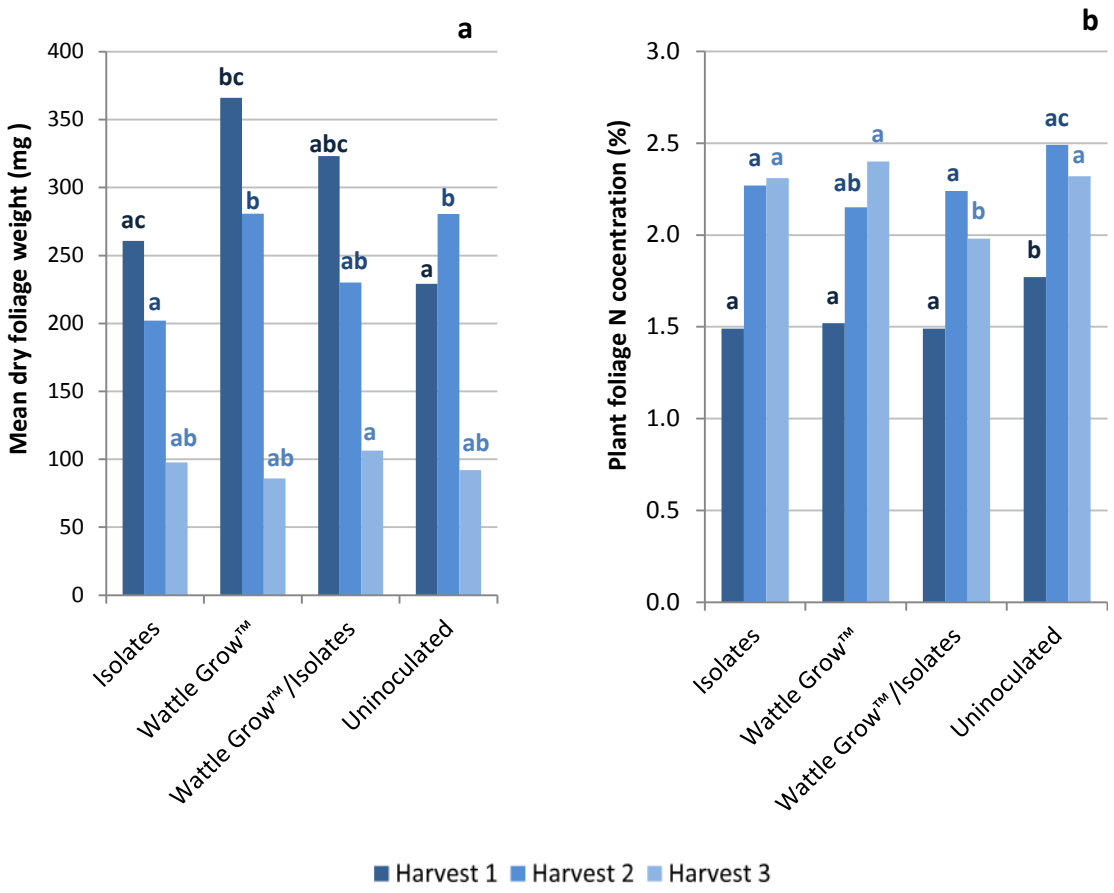


Figure 6.4: N_2 fixation effectiveness of *A. ligulata* Benth. over successive harvests: **a)** Mean dry foliage weight. ANOVA detected significant difference (P≤0.05) between the treatment means for harvest 1. **b)** Mean dried foliage nitrogen concentrations. ANOVA detected significant difference (P≤0.05) between the treatment means for Harvests 1 and 3. Means with the same letter within a harvest are not significantly different according to Fisher's LSD test (P≤0.05).

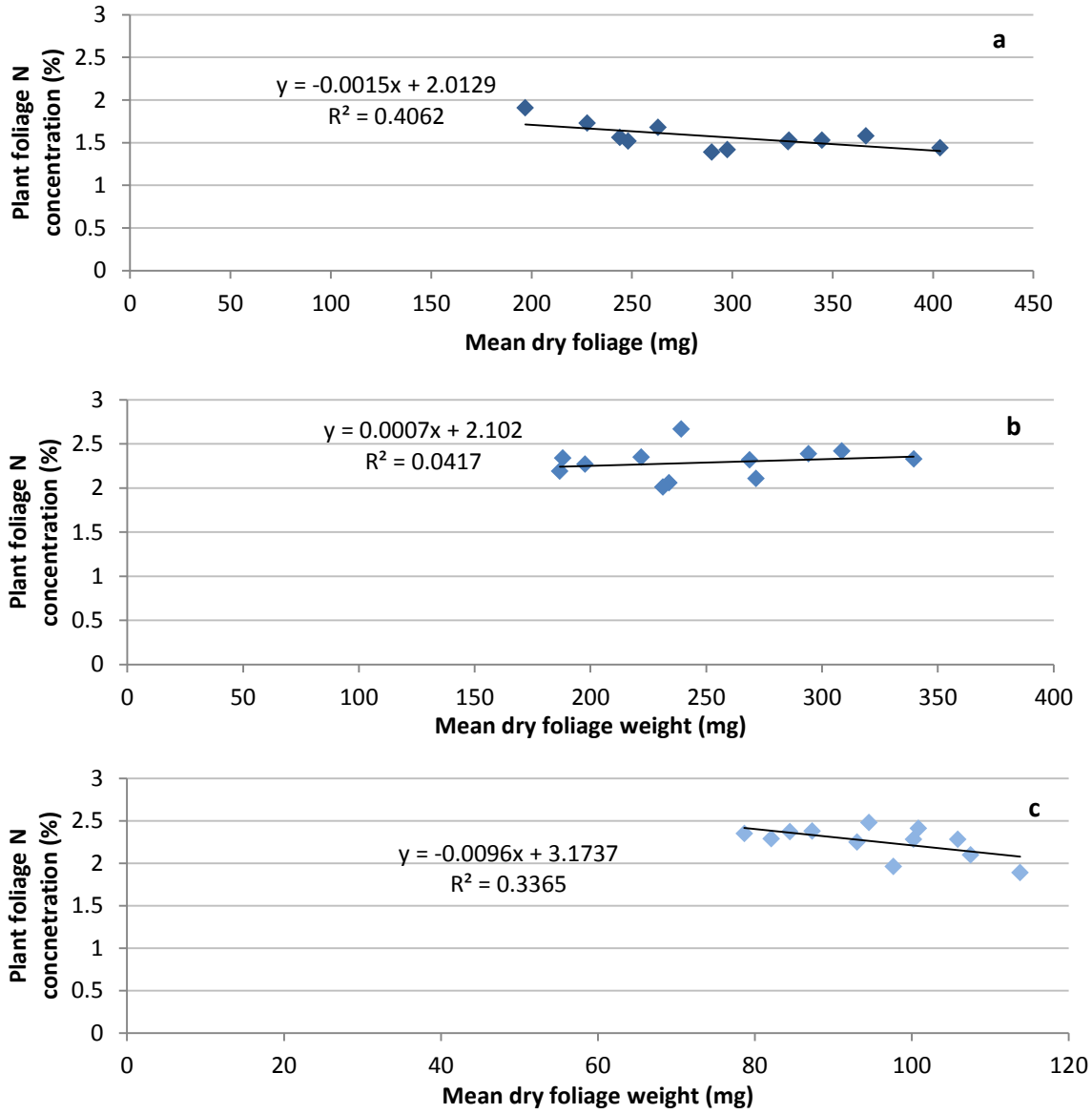


Figure 6.5: Regression graph of *A. ligulata* Benth. plant foliage dry weights (mg) and the plant percentage nitrogen concentrations at Harvest 1 (a), Harvest 2 (b) and Harvest 3 (c). Data boxes show the Isolates, Wattle Grow™, Wattle Grow™ and Isolates and uninoculated treatments. According to ANOVA there was a significant (P<0.05) correlation for Harvest 1 and 3 and no significant correlation for Harvest 2.

6.3.1.2 Nodule occupancy

All nodule isolates from *A. ligulata* Benth. produced colonies that grew to a diameter of 1-2mm in 3-4 days, implying that none of the plants including the two treatments inoculated with Wattle Grow™ were nodulated by *Bradyrhizobium* spp.. Repeated inspection of the nodule RNB isolation CRS-M1 plates showed no new colonies forming between 5 to 10 days. Inoculation in the two treatments with the selected isolates had no effect on the frequency or diversity of nodule occupancy (Figure 6.6).

Different isolates nodulated with greater frequency in each of the four treatments. At Harvest 1, Type 1 nodulated 33%, 14%, 30% and 23% and 2b35 nodulated 7%, 29%, 30% and 15% of the Isolate, Wattle Grow™, Isolate/Wattle Grow™ and uninoculated treated *A. ligulata* Benth. (Figure 6.6). At Harvest 2, 3a23 nodulated 25%, 46%, 30% and 14% and 3b44 nodulated 33%, 23% and 43% of the *A. ligulata* Benth. from the Isolate, Wattle Grow™, Isolate/Wattle Grow™ and uninoculated treatments (Figure 6.6). At Harvest 3, in the treatments of Isolate, Wattle Grow™, Isolate/Wattle Grow™ and uninoculated, there was nodule occupancy of 29%, 36%, 23% and 23%, respectively by 3a23 and 18%, 7%, 19% and 23% occupancy by 4a13, respectively (Figure 6.6).

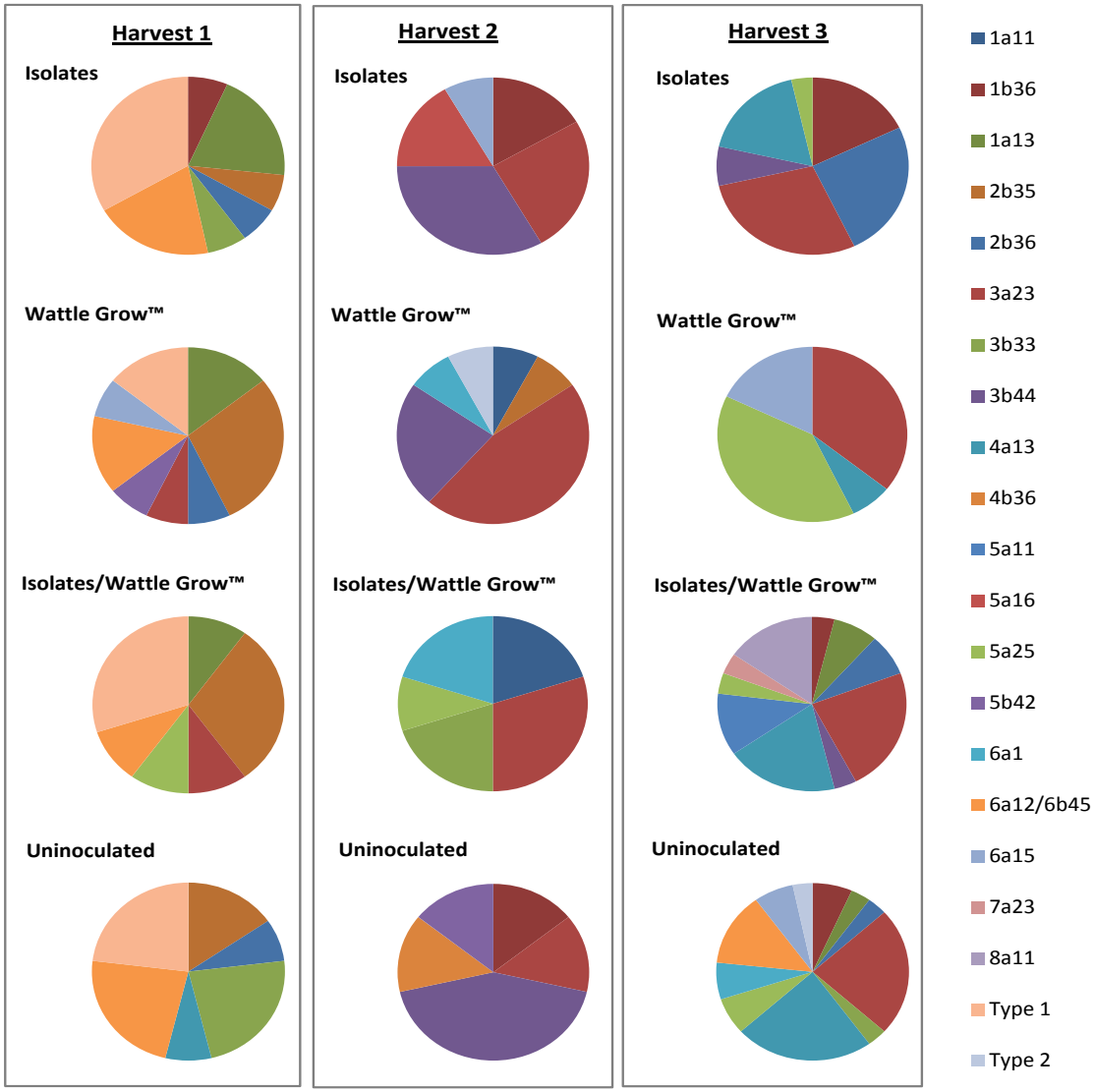


Figure 6.6: Percentage nodule occupancy by isolates of *A. ligulata* Benth. plants at Harvests 1, 2 and 3. Isolate identification labels with a or b indicates the isolates were originally isolated from *A. tetragonophylla* F.Muell. and *A. ligulata* Benth. respectively.

6.3.2 Competitiveness of provenant isolates and Wattle Grow™ for nodulation of *A. tetragonophylla* F.Muell.

6.3.2.1 N_2 fixation effectiveness

There was no significant effect ($P \leq 0.05$) of inoculation treatment in the dry foliage weights of *A. tetragonophylla* F.Muell. at any of the three harvests (Figure 6.7a) and there was a large variance in the dry foliage weights in several of the treatment replicates at Harvest 1. The mean foliage weight of the treatments containing Wattle Grow™ were 18.7% and 9.5% greater than the isolate and uninoculated treatments, respectively (Figure 6.7a). There was a considerable decrease in the foliage weights of *A. tetragonophylla* F.Muell. at Harvest 3, with yields of up to 46.7 mg less than previous harvests (Figure 6.7a).

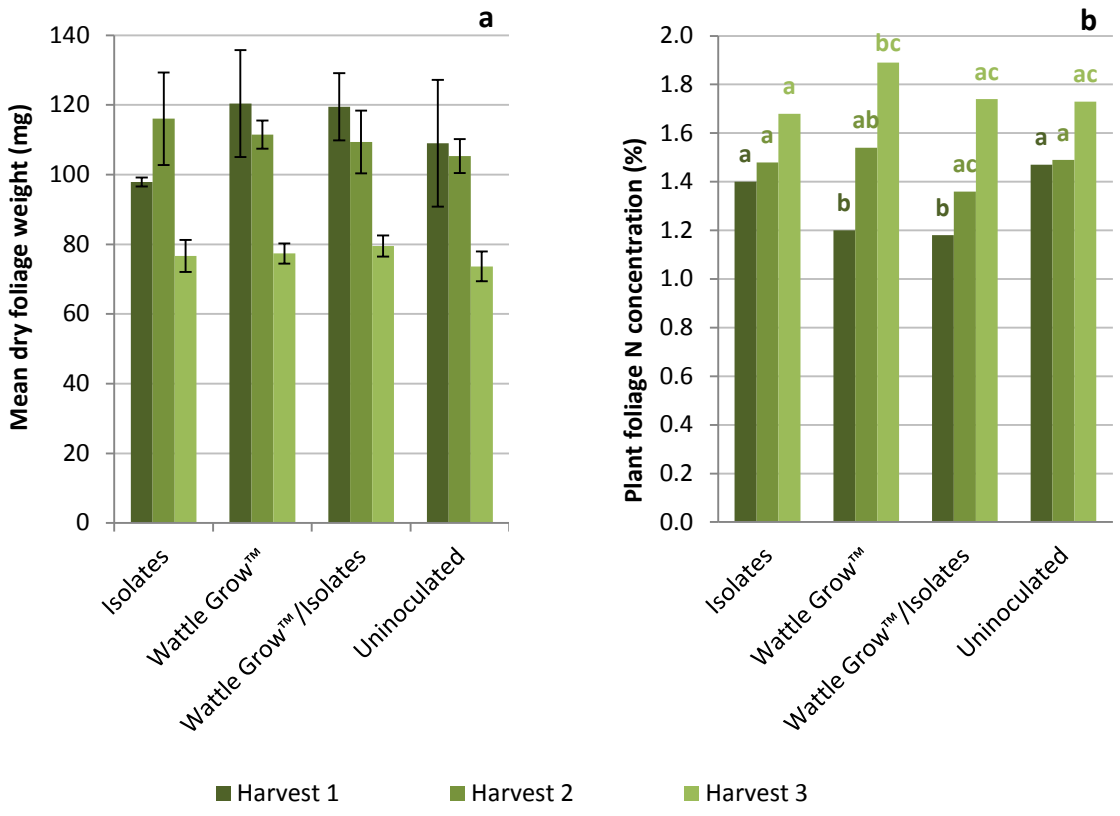


Figure 6.7: N_2 fixation effectiveness of *A. tetragonophylla* F.Muell. over successive harvests: **a)** Mean dry foliage weight. ANOVA detected no significant difference ($P \leq 0.05$) between the treatment means at each harvest. Vertical bars represent standard error of means. **b)** Mean dry foliage nitrogen concentrations. ANOVA detected significant difference ($P \leq 0.05$) between the treatment means for Harvest 1. Means with the same letter within a harvest are not significantly different according to Fisher's LSD test ($P \leq 0.05$).

The N concentrations of *A. tetragonophylla* F.Muell. dried foliage increased with each successive harvest (Figure 6.7b). At Harvest 1, ANOVA detected significantly lower ($P \leq 0.05$) percentage N foliage contents of *A. tetragonophylla* F.Muell. inoculated with Wattle Grow™ and co-inoculated with Wattle Grow™ and isolates compared to the isolate and uninoculated treatments (Figure 6.7b). Although not significant ($P \leq 0.05$), *A. tetragonophylla* F.Muell. that had been inoculated with Wattle Grow™ in the first sow had the highest concentrations of N at Harvests 2 and 3 in comparison to the other treatments (Figure 6.7b). There was a significant ($P < 0.05$) positive correlation between the dry foliage weights and the percentage N concentrations in the dried foliage of *A. tetragonophylla* F.Muell. at Harvest 1, 2 and 3 (Figure 6.8).

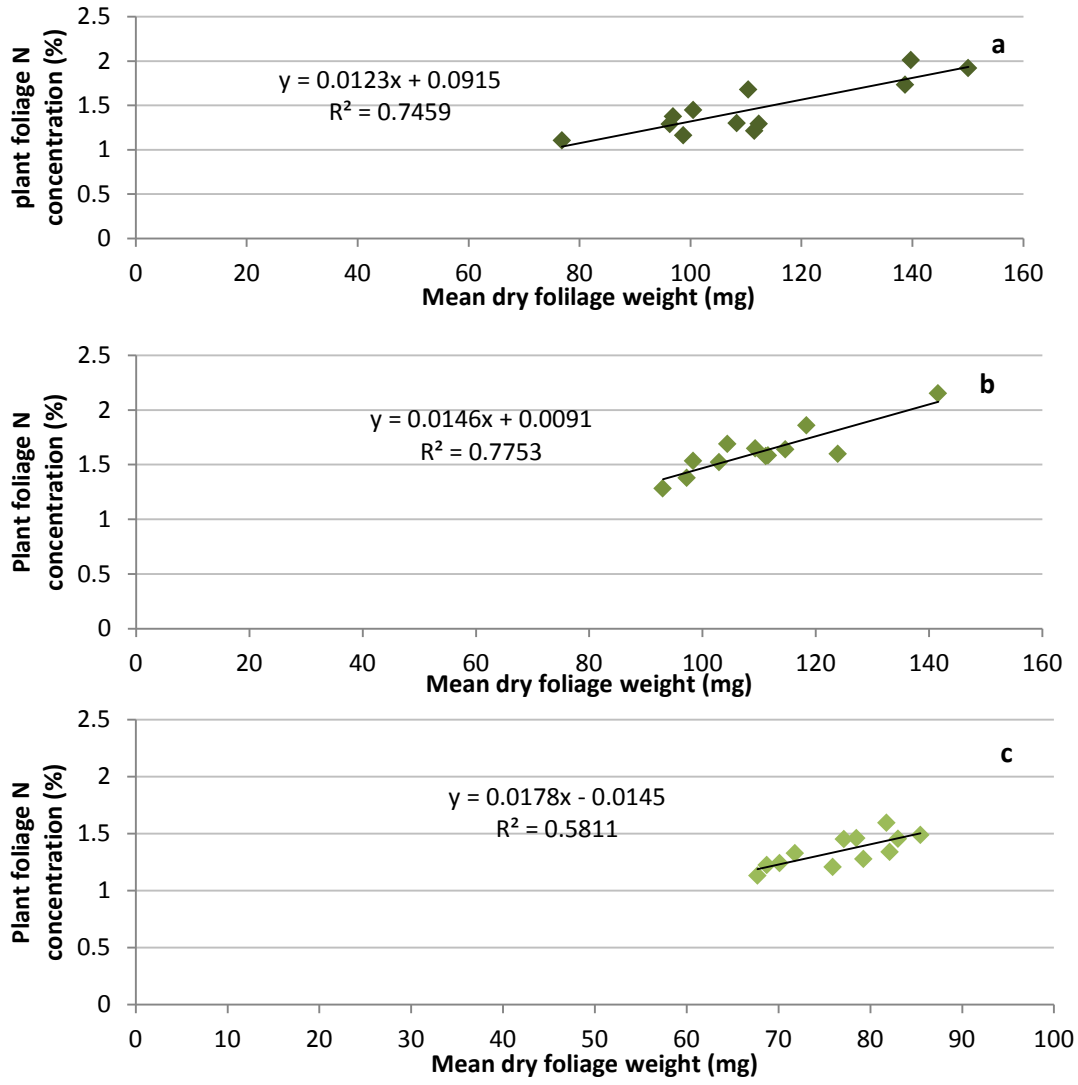


Figure 6.8: Regression graph of *A. tetragonophylla* F.Muell. plant foliage dry weights (mg) and the plant percentage nitrogen concentrations at Harvest 1 (a), Harvest 2 (b) and Harvest 3 (c). Data boxes show the Isolates, Wattle Grow™, Wattle Grow™ and Isolates and uninoculated treatments. According to ANOVA there was a significant ($P < 0.05$) correlation for Harvest 1, 2 and 3.

6.3.2.2 Nodule occupancy

All nodule isolates from *A. tetragonophylla* F.Muell. produced colonies that grew to a diameter of 1-2 mm in 3-4 days, implying that none of the plants including the two treatments inoculated with Wattle Grow™ were nodulated by *Bradyrhizobium* spp.. Inoculation in the two treatments with the selected isolates had no effect on the frequency or diversity of nodule occupancy (Figure 6.9). There was an increase in diversity of isolates nodulating *A. tetragonophylla* F.Muell. at Harvest 3, approximately 2-fold greater than at Harvest 1 and Harvest 2 (Figure 6.9).

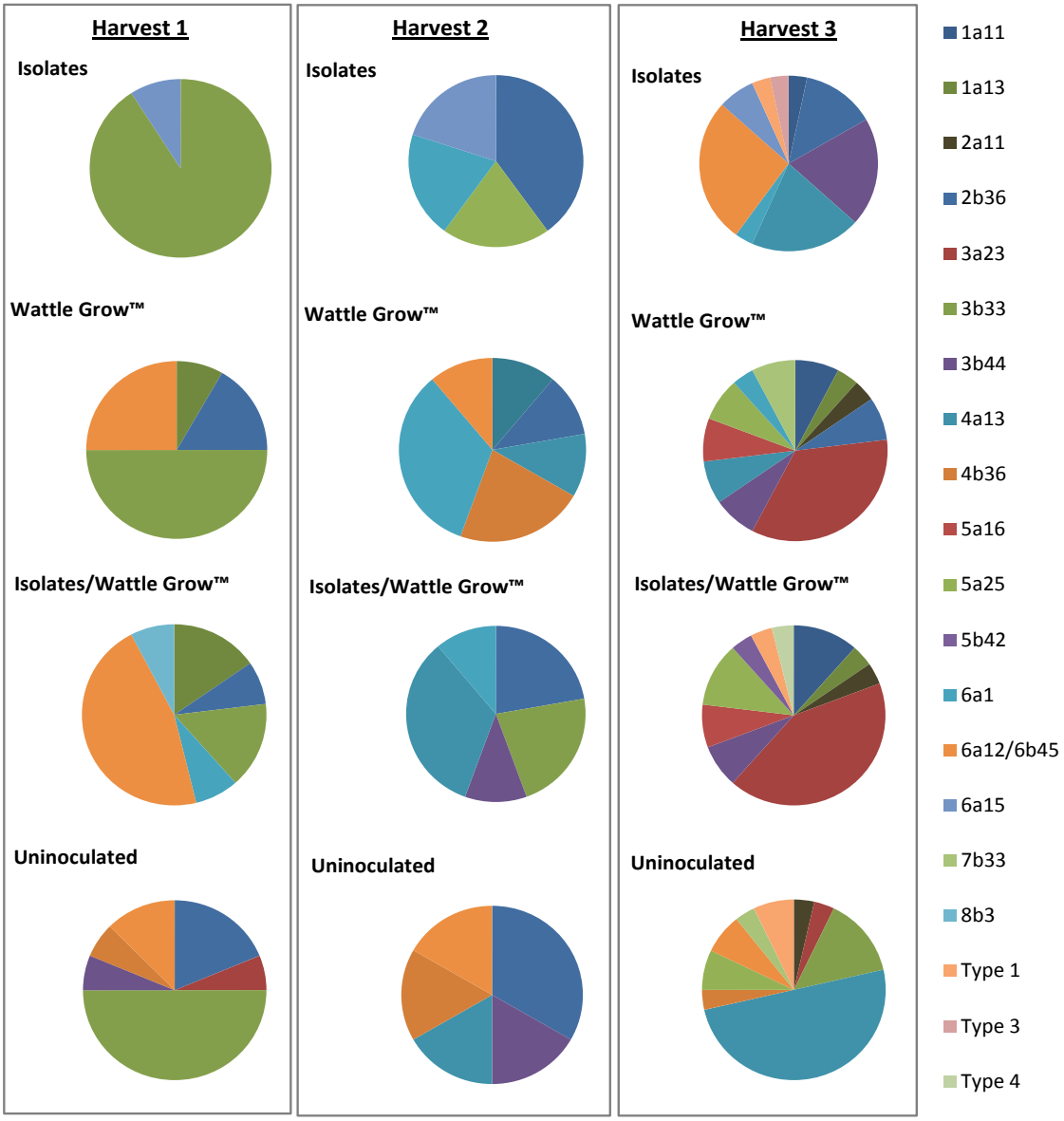


Figure 6.9: Percentage nodule occupancy by isolates of *A. tetragonophylla* F.Muell. plants at Harvests 1, 2 and 3. Isolate identification labels with a or b indicates the isolates were originally isolated from *A. tetragonophylla* F.Muell. and *A. ligulata* Benth. respectively.

At each separate harvest there were isolates that nodulated with greater frequency in each of the four treatments. At Harvest 1, 3b33 nodulated 91%, 50%, 15 and 50% of the Isolate, Wattle Grow™, Isolate/Wattle Grow™ and uninoculated treated *A. tetragonophylla* F.Muell. respectively and 6a12/6b45 was identified in 25%, 46% and 13% of the Wattle Grow™, Isolate/Wattle Grow™ and uninoculated plant nodules respectively (Figure 6.9). At Harvest 2, *A. tetragonophylla* F.Muell. was nodulated by 2b36 in Isolate, Wattle Grow™, Isolate/Wattle Grow™ and uninoculated treatments at 40%, 11%, 22% and 33% respectively (Figure 6.9). At Harvest 3 in the treatments of Wattle Grow™, Isolate/Wattle Grow™ and uninoculated, *A. tetragonophylla* F.Muell. was nodulated at 35%, 42% and 4% by 3a23 and 4a13 percentage nodulation was 20%, 8% and 50% with the Isolate, Wattle Grow™ and uninoculated treatments (Figure 6.9).

6.4 Discussion

This chapter investigated the competitive ability of RNB populations in SBSLA soil from pit P, previously selected provenant RNB isolates (Chapter 5) and the *Bradyrhizobium* spp. inoculant Wattle Grow™ to nodulate *A. ligulata* Benth. and *A. tetragonophylla* F.Muell.. No *Bradyrhizobium* bacteria were isolated from nodules of either *A. ligulata* Benth. and *A*. *tetragonophylla* F.Muell. grown in the pit P soil from any of the treatments, including the Wattle Grow™ and Wattle Grow™ and Isolate treatments over the three successive harvests. A total of 26 different RPO1-PCR fingerprints were identified from the nodule isolates obtained from all three harvest periods and both *Acacia* hosts. Of these, 23 were the selected inoculant isolates or isolates identified in previous chapters and there were three isolates with unique RPO1-PCR fingerprints. Up to 41.7% of the isolates identified as nodule occupants of both *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. were similar for each of the successional growth periods, with the nodule occupancy of isolates changing over these successional sowings to the final harvest. While there was little or no significant difference in the foliage production between the treatments for both species, there was a difference between the different harvest periods. Foliage mass of both species decreased with each successive growing period and for *A. ligulata* Benth. there was a weak negative correlation to plant foliage mass and the N concentration of the foliage. In contrast,

there was a significant positive correlation to foliage production and the N concentration of *A. tetragonophylla* F.Muell. foliage.

The *Bradyrhizobium* spp. contained in the Wattle Grow™ product does nodulate both *Acacia* hosts and the symbiosis was significantly effective with *A. ligulata* Benth. (section 3.3.3). Despite this symbiotic relationship, no slow-growing RNB were isolated from the harvested nodules of either host. The growth tank substrate for plant growth contained a mix of yellow sand and washed river sand of neutral pH and the pH(H_2O) of pit P soil was 9.2 (section 2.3.1). There was also a base layer of basalt aggregate and Western Australian basalt dust has an alkaline pH (Oldfield, 1996). Although the volume of the pit soil was small relative the sand mix layers, the seed and inoculant was sown in this layer and the alkaline pH may have reduced the viable number of *Bradyrhizobium* spp. in the treatments containing Wattle Grow™. The *Bradyrhizobium* spp. in the Wattle Grow™ were isolated from the south eastern region of Australia, where the soils are predominantly acidic, ranging between pH 4 to 5.5 (de Caritat *et al.*, 2011) and it is unknown at what range of pH these Wattle Grow™ RNB will grow. As previously discussed in Chapter 4, *Bradyrhizobium* species are reportedly sensitive to alkaline pH, with reduced nodulation and soil populations in these conditions (Amarger *et al.*, 1984; Graham, 1992; Tang *et al.*, 1995).

Should the population of *Bradyrhizobium* have been affected by the substrate pH, the RNB resident in the pit P soils may have been able to increase in sufficient numbers to compete for nodulation of the *Acacia* spp.. The response to inoculation by a range of agricultural legumes is inversely related to the resident RNB population (Thies *et al.*, 1991). Inoculants are by industry and regulatory standards, prepared in such numbers as to overwhelm the resident soil RNB to be competitive for nodulation of the target legume (Deaker *et al.*, 2004; Smith, 1992). Yates *et al.* (2008) inoculated *Trifolium purpureum* with an effective RNB which was shown to preferentially select for nodulation, until this RNB was outnumbered 1000-fold by an ineffective RNB. The trapping of RNB isolates from SBSLA soils indicate that with ideal conditions, the RNB that are present in the pit soils increase to sufficient numbers to effectively nodulate selected host legumes (section 3.3.2). Therefore, there could be a dual effect of

competition by numbers in the pit soils of resident RNB and of selection of RNB for nodulation by *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. to account for the lack of *Bradyrhizobium* identified in the harvested nodules.

The successional sowings of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. over the 13 months show a gradual change in the RNB that nodulate these legumes. For both *A. ligulata* Benth. and *A. tetragonophylla* F.Muell., there was a 65% similarity in nodule occupants between Harvest 1 and 2 across the four treatments. The nodule occupancy for both hosts between Harvest 2 and 3 were 64% similar across the four treatments. With the subtle change in nodule occupants over the harvests for both hosts, the similarity between Harvest 1 and 3 was reduced to 54%. The decrease in similarity is due to six of the isolates being identified only in Harvest 3 nodules of both *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. (Figures 6.6 and 6.9). The establishment of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. in the tank soils may have altered the population of RNB nodulating these species in a similar way to the effect seen in RNB populations at sites invaded or re-vegetated with *Acacia* spp.. At two sites in Portugal, one where *A. longifolia* (Andrews) Willd. had long been established and another site where invasion of the site was in progress, the genetic diversity of the nodulating RNB was significantly greater at the established site (Rodríguez-Echeverría *et al.*, 2007). In the comparison of two dune systems in eastern Australia, Barnet *et al.* (1985) found distinct differences in the RNB composition between the two sites and a significantly higher proportion of nodulation at the more floristically diverse site compared to the previously unvegetated site. At the time of collection, the soils from the disturbed area at pit P contained no detectable RNB that nodulated *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. (section 2.3.4) and the change in RNB nodule occupants over the successive harvests may also represent a succession in the RNB population influenced by the presence of the *Acacia* hosts.

There was a degree of similarity between the isolates which nodulated both *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. in the different treatments with 37.5%, 35.3% and 41.7% of the nodule occupants, common to both hosts for the Harvests 1, 2 and 3 respectively. However, over the course of each successive growth period there was a

difference in the variability of nodule occupants between the treatments for *A. ligulata* Benth. and *A. tetragonophylla* F.Muell.. The nodule occupant variability of *A. ligulata* Benth. increased between treatments with each growth period whereas the variability between treatments of *A. tetragonophylla* F.Muell. nodule occupants decreased. There were also differences in the diversity of isolates that nodulated *A. ligulata* Benth. and *A. tetragonophylla* F.Muell., with approximately 12% and 18% greater diversity of nodule isolates of *A. ligulata* Benth. compared to *A. tetragonophylla* F.Muell. at Harvest 1 and 2. In contrast, at Harvest 3 there was a 17% greater diversity of isolates nodulating *A. tetragonophylla* F.Muell. comparised to *A. ligulata* Benth.. Are the two host species influencing the RNB population in the tank soils? With the decreased variability between the treatments of nodule occupants of *A. tetragonophylla* F.Muell. and the increased diversity of these isolates, perhaps *A. tetragonophylla* F.Muell. is exerting a selection effect on the RNB for preferential nodulation. If this were the case and given that both *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. are grown together in such close proximity in the growth tanks, it would likely affect the population of RNB nodulating *A. ligulata* Benth.. Thrall *et al.* (2007) investigated the RNB populations of *A. stenophylla* A.Cunn and *A. salicina* Lindley., species with overlapping geographic distribution and the data suggested significant differences in host specificity with *A. stenophylla* A.Cunn the more selective of the two species. Further investigation of these isolates by Hoque *et al.* (2011) revealed that while many were common to both *A. stenophylla* A.Cunn and *A. salicina* Lindley., many isolates were unique to each species. The adaptive ability of *A. tetragonophylla* F.Muell. to select effective RNB is reflected in the positive correlation between foliage production and N_2 fixation (Figure 6.8). Whereas, the promiscuous nature of *A. ligulata* Benth. and the lack of a positive correlation between foliage production and N_2 fixation indicate that this species is not preferentially selecting effective RNB (Figure 6.5).

Although there was no significant difference between the treatments for the foliage mass of *A. tetragonophylla* F.Muell., the response to the treatments was similar to that shown by *A. ligulata* Benth.. This suggests that in these glasshouse conditions, the resident population of RNB in the pit P soils was more competitive than the inoculant isolates for the nodulation of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell.. The

warm moist conditions may have promoted faster growth of the resident soil RNB compared to those of the treatments. While it was possible to compare the RPO1-PCR fingerprints of the nodule isolates to the inoculant, it is not possible to determine whether the isolates resulting in nodulation were resident in the soil at the time or due to inoculation.

For the first sowing period both the treatments, Wattle Grow™, Wattle Grow™ and Isolate resulted in greater dry foliage weights than the Isolate and uninoculated treatments. The growth variability might be due to the position of the growth tanks in the glasshouse. The tanks, once installed were not able to be moved and randomized to reduce or eliminate positional effects for the duration of the experiment. The treatment growth tanks were positioned from west to east as Wattle Grow™, Wattle Grow™ and Isolate, Isolate and the uninoculated treatment with a single air-conditioner located adjacent to the Isolate and uninioculated treatment tanks. With this arrangement in the glasshouse, the Wattle Grow™ and Wattle Grow™ and Isolate treatment tanks were not as exposed to as much sudden variability in temperature as the other two treatments. Lower daytime temperatures have been shown to reduce photosynthesis and growth in the annual *Arabidopsis thaliana* (L.) Heynh. (Pyl *et al.*, 2012). Similarly, the perennial rye grass (*Lolium perenne* L.) invests more carbon into respiration rather than growth under cooler conditions (Barthel *et al.*, 2014). While daytime temperatures in the glasshouse ranged between 25°C and 31°C, it is not known what the temperatures of the growth tanks adjacent to the air conditioner were exposed to when it was operational. Presumably, the area of the these tanks was considerably cooler than the Wattle Grow™, Wattle Grow™ and Isolate tanks given the size of the glasshouse area.

There is also a progressive reduction in the foliage weights of both species with each successive growth period, with a reduction of foliage mass of approximately 68% and 31% for *A. ligulata* Benth. and *A. tetragonophylla* F.Muell*.,* respectively at Harvest 3 compared to Harvest 1 (Figures 6.4a and 6.7a). This may be due to a seasonal effect of different temperatures and photoperiods. In a study of various tree species of ash, beech, black locust, yellow poplar, sweet gum, oak and loblolly pine, a seasonal

reduction of the photoperiod by 5 h significantly reduced plant growth and exposure to a continuous light source resulted in significant plant growth (Kramer, 1936). There was a 4 h difference in the photoperiod prior to harvest between the three successional growth periods. For the first growth period, the days became increasingly warmer and daylight increased from approximately 12.5 h to 14 h. The daylight decreased from 13 h to 10 h over the duration of the second growth period and temperatures were cooler. The conditions of the third growth period were similar to the initial growth period with increasing temperatures and daylight increasing from 11 h to 14 h. However, Harvest 3 foliage production of both *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. was significantly reduced from that of Harvest 1 and 2. At the third successional sowing, the air conditioner failed and temperatures reached a maximum of 42°C before repairs were made.

Viable nodules and RNB isolates were obtained from the plants harvested from the third successional sowing, so nodulation was not affected by the high temperatures. However, the nodules seen may be from a secondary period of infection and nodulation, if the initial nodules formed were affected by the heat and became senescent. These species have been reported as nodulating readily in ideal conditions, as previously stated in chapter 5. Although *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. grow in an environment where they are exposed to high temperatures (section 2.3.2.2), the growth of these species are likely to have been affected by the high temperatures experienced during the third growth period. The carbon assimilation of *Eucalyptus tetrodonta* F. Muell. was shown to decrease significantly when the temperature exceeded 35°C (Prior *et al.*, 1997). In a study comparing the relative growth rate (RGR) of 16 plant species, an increase of just 5°C from 23°C to 28°C reduced the RGR for 12 of the species (Loveys *et al.*, 2002).

While the growth tank experiment did not closely replicate the conditions of the degraded sites at SBSLA, it did further illustrate the differences between *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. in growth response, N accumulation in the foliage and symbiotic relationships. This chapter further supported that both species are promiscuous, nodulating with a wide range of phylotypes within different genera

(section 4.3.2). The positive correlation between plant mass and N accumulation suggests *A. tetragonophylla* F.Muell. may be better able to form effective symbiosis with the RNB in the SBSLA soils. This may be a possible adaptation to being a more long-lived species. The selection process of these *Acacia* spp. for entering into a symbiosis with available isolates can depend on the size of the population in comparison to other RNB but this is no guarantee that they form a beneficial symbiotic relationship. Genetic studies have shown that host genetic variations (ecotypes) across geographic ranges influences the distribution of RNB lineages (Parker & Spoerke, 1998). With the ineffective response to inoculation by Wattle Grow shown by this chapter, it is clear that a 'one size fits all' approach to RNB restoration ecology would not be suitable in the degraded sites of the SBSLA or possibly other degraded dryland areas in Australia.

Chapter 7

General Discussion

Now this is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning.

-Winston Churchill

There is an increasing demand for resources and land use in the dryland areas of Western Australia. Mining and pastoral activities have disturbed large tracts of dryland areas in Western Australia, with mining alone in the Pilbara region exceeding 20 000 ha (Merritt & Dixon, 2011). With the legislative requirements for the operators in these areas to manage and rehabilitate these sites, the low success rate of rehabilitation of drylands is problematic (Carrick & Kruger, 2007; James *et al.*, 2013; Mitchell & Wilcox, 1994). There are numerous challenges for the rehabilitation of disturbed dryland areas, including rainfall and seasonal variability, slow growing perennial vegetation and low levels of nutrients in the soil (as detailed in Section 1.4). For the rehabilitation of the borrow pits within the SBSLA to be successful, these challenges are among those to be considered. It is evident that the removal of soil, subsoil and regolith at these sites has altered the topography and soil chemical properties in comparison to adjacent representative undisturbed areas (Table 2.3) and the local *Acacia* spp. and other plants provenant to the area have failed to establish in the excavated pit sites (Section 2.3.3.1 and Table 2.4). The main rehabilitation gain to be made is to improve the success of seedling establishment for the eventual persistence of a complex floristic community capable of supporting an ecosystem diversity that is representative of that which preceded the disturbance.

The loss of beneficial plant-associated microbes in disturbed dryland areas could be the fundamental issue in the failure to establish native plants (de-Bashan *et al.*, 2012). With *Acacia* spp. dominant in many of the Australian dryland floristic communities (Ladiges *et al.*, 2006; Simmons, 1988), understanding the complex interactions of dryland legumes should be an essential component of the restoration management approaches of operators responsible for the areas to be rehabilitated. No previous study has comprehensively examined the impacts of long-term disturbance on the soil microbial populations in the Shark Bay area. It was found in this study, that the disturbance at the pit sites had affected soil biota populations, specifically the RNB that associate with *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. (Figure 2.13). The work presented here investigated the RNB associations of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. and the potential of RNB inoculation for the establishment of these perennial over-story plant species in the disturbed sites of the SBSLA.

At least eight RNB isolates from the soils of SBSLA significantly increased the plant foliage N concentrations of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. in glasshouse conditions compared to uninoculated plants and those that had been inoculated with the *Bradyrhizobium* spp. contained in Wattle Grow™ (Figures 3.7 and 3.10). All of the 58 authenticated RNB were fast growing and genotyping indicated a diverse population of symbionts of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. in the SBSLA soils. The 16S rDNA sequence of a number of these isolates were homologous to those of strains of *E. meliloti, E. fredii, Neorhizobium huautlense* and *R. sullae.* The RNB, *E. meliloti, E. fredii* and *R. sullae* have previously been reported as symbionts of various *Acacia* spp. (Amrani *et al.*, 2010; Boukhatem *et al.*, 2012; Hoque *et al.*, 2011; Mnasri *et al.*, 2009). This is the first report of these species forming effective N_2 fixation symbioses with *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. and of an isolate homologous with *N. huautlense* as a symbiont of any *Acacia* spp.. The *Bradyrhizobium* spp. in Wattle Grow™ did nodulate both *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. as well as *A. rostellifera* Benth. and *T. retusa* (Vent.)R.Br., resulting in *A. ligulata* Benth. and *A. rostellifera* Benth. with significantly greater foliage weights than the uninoculated plants (Figures 3.6 and 3.9). However, the Wattle Grow™ strains were not able to compete for nodulation against selected provenant RNB and other RNB resident in pit P soils as indicated by the RNB nodule occupants of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. in the glasshouse competition study (Figures 6.6 and 6.9). In Chapter 5, it was shown that inoculation of these *Acacia* spp. with the selected provenant RNB when sown in pit sites within the SBSLA generally increased the number of germinated and established plants, the percentage nodulation and the foliage weights compared to the uninoculated plants. At four months post sowing and inoculation, the population of RNB symbionts of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. in the soils of a pit site and the surrounding undisturbed soils had declined significantly from that at the time of inoculation (Figures 2.13 and 5.12). However, the introduction of the selected RNB had established a stable population in the inoculated soils of the pit and the RNB population in the inoculated area of the pit site was comparable to that of the surrounding undisturbed soils, in contrast to the significantly reduced RNB population in the uninoculated soils of the pit.

7.1 Response to inoculation with provenant RNB

Both *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. were established at two of the three pit sites in years of highly variable rainfall, with 2007 being one of the lowest annual rainfall years recorded and 2008 having the highest recorded annual rainfall (Figure 2.3). Inoculation with RNB had a positive effect on the germination and persistence of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell.. From seed sown in 2008, 22% and 35% of *A. ligulata* Benth. treated with the peat inoculant were persisting at 8 months in sites G and P. This represents a considerable loss of potential plants from the seed sown. However, only 16% and 15% of uninoculated *A. ligulata* Benth. had persisted in the sites G and P. The costs of obtaining provenant seed is considerable for rehabilitation projects (Merritt & Dixon, 2011) and an improvement of 20% with respect to plant establishment is a very positive outcome.

It was unclear from the field data whether this increased rate of established germinates was due to the selected RNB inoculated onto the seed or the carrier itself. Further analysis of the nodule occupants for the different treatments would be required to ascertain whether the carrier medium had an effect on the legume-RNB associations formed in the pit soils and the individual carriers assessed with uninoculated treatments of each of the various inoculant carriers *in-situ*. A more extensive seeding program at SBSLA with the individual selected isolates would also determine the competitive nature of these RNB compared with the RNB resident in the soils at the time of sowing. There are indications that RNB other than the provenant isolates selected are present in the soils and initially competitive for the nodulation of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell.. There was a high frequency of a novel isolate in the nodules of both these *Acacia* grown *in-situ* at site P (Figure 5.11) and in the nodules of the initial sow of *A. ligulata* Benth. in the growth tanks containing soil from pit P (Figure 6.6). However, it is unknown whether this RNB or the other novel RNB identified in the competition study of Chapter 6 form effective symbioses with *A. ligulata* Benth. and *A. tetragonophylla* F.Muell..

The selection of the RNB for inocula development was primarily based on the foliage production of the inoculated host plants. This was undertaken in glasshouse conditions that were not representative of those at SBSLA. There may have been a bias in the RNB selected on *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. as those that were best suited to the glasshouse growth conditions may not have shown the same response in promoting the growth of the *Acacia* spp. at the pit sites. The collection and storage of the soils for the trapping and the growth conditions may have altered the abundance of certain RNB in the soils of SBSLA and affected which symbiosis occurred on these *Acacia* hosts. With soils in both field and glasshouse conditions, Duodu *et al.* (2006) assessed the PCR fingerprints of RNB that nodulated *Trifolium* spp. with the plants being nodulated by a different suite of RNB in the glasshouse compared to those grown *in-situ.* In the work reported in this thesis, there were differences in the response to RNB inoculation in different growth conditions evident in the foliage mass and foliage N concentrations of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell.. For both *Acacia* spp. there was no significant positive correlation of inoculation between foliage N concentrations and foliage mass when grown for 56 dpi (Figures 3.8 and 3.11). For the RNB competition study when *A. ligulata* Benth. was sown in the growth tanks containing pit P soil for 90 dpi there was a significant negative correlation between the foliage N concentrations and foliage mass (Figure 6.5). In contrast, *A. tetragonophylla* F.Muell. had a significant positive correlation between the foliage N concentrations and foliage mass in this study (Figure 6.8).

The difference in growth response by the two *Acacia* may be due to the suite of RNB that the species selected in the competition study or because of the longer growing period. As discussed in Chapter 3, the two species exhibit different life strategies and may have different requirements for N throughout their life. *A. ligulata* Benth. may be a disturbance specialist and possibly ideal for initiating soil stabilization as a faster growing species. However, *A. tetragonophylla* F.Muell. appears to have more promiscuous RNB associations illustrated by the nodule occupancy of the competition study (Figure 6.9) and the MPN results of the SBSLA soils (Figures 2.13 and 5.12). In the longer term, this promiscuous nature of *A. tetragonophylla* F.Muell. may prove to be

more beneficial in addition to it being a longer lived and more drought tolerant species (Section 3.4) in recreating nutrient island sites within the pit areas at SBSLA.

7.2 Inoculant application: Implications

Numerous studies have been conducted in glasshouse conditions with sufficiently available nutrients and water to assess the symbioses of Australian native legumes with RNB (Barnet & Catt, 1991; Burdon *et al.*, 1999; Lawrie, 1983). In these conditions, it difficult to assess the extent of the possible contribution of N to their ecosystem communities by these plants as *in-situ* variable environmental conditions also influence N_2 fixation and N-cycling processes. As previously discussed, the selection of a suitable RNB for a legume may be biased by different growth conditions and this is one impediment in the development of a site specific inoculant. RNB selection can also be influenced by the root depth at which nodules are collected from plants grown *in-situ* and the depth of the soils collected for the trapping of RNB. Attempts were made to trap RNB *in-situ* from the undisturbed soils of SBSLA in 2006 from planted 3 week old seedlings and from sown seed. However, this was unsuccessful as there were no surviving plants from either the seedling stock or seed due the lack of rainfall both prior to and after planting and sowing and the lowest annual recorded rainfall for SBSLA was in 2006.

The RNB populations described of dryland soils are typically from the surface strata (Bala *et al.*, 2002; Odee *et al.*, 1995; Venkateswarlu *et al.*, 1997). Soil depth has been shown to influence both the phenotypic and genotypic characteristics of RNB with the RNB at depths of up 0.75 m different from the surface soils of 0-0.25 m in the soils associated with *A. senegal* growing *in-situ* in Senegal (Fall *et al.*, 2008). None of the isolates collected from the SBSLA soils were *Bradyrhizobium* spp. and this could be because the soils collected were from a depth of 0-0.40 m. Different RNB populations may be stratified through the soil profile at SBSLA. The RNB of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. isolated are possibly those that associate with these plants in more ideal conditions or when the plants are juvenile and the RNB that they generally associate with are those existing at greater depths. In the dry Sahelian province of

West Africa, RNB populations that nodulate *A. albida* Del. were greater near the water table than in the surface soils and *Bradyrhizobium* were detected at depths of up to 34 m (Dupuy & Dreyfus, 1992). In the Sonoron desert of California, root nodules containing RNB have been found at depths of up to 7 m on the roots of *P. glandulosa* Torr. (Jenkins *et al.*, 1988a). The RNB of this species as well as *Psorothamnus spinosus* (A.Gray) Barneby., and *Acacia constricta* Benth. were distributed through the root depth environments according to host species as well as soil depth (Waldon *et al.*, 1989). The phenotype studies of Fall *et al.*(2008) and Waldon *et al.* (1989) demonstrated the contrasting adaptations between RNB at the surface and at depths with different growth responses to temperature and carbon source requirements.

The diversity of the RNB populations through the soil strata and their adaptations to the different conditions may be vital to the persistence of native legumes in dryland areas. For this reason, it would be preferable to use provenant RNB as well as provenant seed for the rehabilitation of degraded areas. The introduction of non-provenant RNB that are shown to nodulate native legumes in rehabilitation projects may over time compromise the ability of the indigenous RNB to form effective symbioses with their hosts through the transfer of genetic material. The transfer of genetic material between RNB has leading to competition for nodulation and compromised N_2 fixation has been reported in a number of agricultural species. Sullivan & Ronson (1998) demonstrated that there was a transfer of a portion of genes, termed symbiotic islands from a single *Mesorhizobium loti* inoculant strain to a diverse range of *Mesorhizbium* species that gained the ability to nodulate *Lotus* species. This genetic transfer of symbiotic islands from inoculant *Mesorhizobium* strains has led to the evolution of competitive indigenous *Mesorhizobium* strains with reduced N_2 fixation on *Biserrula pelecinus* L. (Nandasena *et al.*, 2007). Gene transfer has also been reported from *Bradyrhizobium japonicum* inoculant to indigenous *Ensifer* and *Bradyrhizobium* spp. that nodulate *Glycine max* (L.) Merr. (Barcellos *et al.*, 2007).

The introduction of RNB that are effective on a host as was shown by the product Wattle Grow™ inoculated on *A. ligulata* Benth. and *A. rostellifera* Benth. (Figures 3.6 and 3.9) may in the long term prove to be detrimental to the provenant plant-RNB

symbioses in *in-situ* situations through gene transfer processes. Any reduction in symbiotic RNB-legume interactions in dryland ecosystems could lead to reduced seedling fitness and fewer plants surviving to maturity. This would obviously have impacts on the floristic structure and soil stability of the area especially if the plants are key over story species such as *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. are in SBSLA. Future studies of the dryland ecosystems of Western Australia could determine the specificity of the relationships of different plant species with the soil microbial community. This knowledge could contribute to the different land operators in these regions developing more effective rehabilitation management strategies on a site-by-site basis.

7.3 Impediments in the establishment of *Acacia* spp. at SBSLA

There would be numerous benefits for the establishment of *Acacia* spp. in the degraded pit sites at SBSLA. They are keystone plants in the nutrient islands associated with the landscape of Australian dryland ecosystems (Ladiges *et al.*, 2006), providing a microhabitat for other floral spp., and shade and protection for fauna (Brockwell *et al.*, 2005; Manning *et al.*, 2006). The soils associated with *Acacia* spp. have increased levels of N and P due to plant-RNB symbioses and from the accumulation of vegetative material on the soil surface as well an increased localized nutrient enrichment of the soils from dung deposition of animals such as kangaroos and small marsupials (New, 1984). The plant abundance and diversity at these nutrient enriched sites is consequently greater. The selection of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. for the focus of this study was primarily due to their prominence in the different floristic communities identified at SBSLA (SBSJV, 1998) as the major over-story plants in the area.

The disturbance activities at the pit sites removed the surface soils, which contained the seed bank for these areas. Given the period of time that has elapsed since a number of the pits within the SBSLA were active, the lack of recruitment of plants from the surrounding areas demonstrates that there are inherent impediments to plant establishment at these sites. In the duration of the time spent within the SBSLA, there

was no ant activity observed at the selected pit sites and bird activity appeared to be restricted to the perimeter of the pit sites and the surrounding undisturbed areas. The seed of many Australian plants have evolved to be dispersed by myrmecochory and/or ornithochory (Davidson & Morton, 1984; Standish *et al.*, 2007) including *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. (Davidson & Morton, 1984). In sclerophyllous vegetation of the southern hemisphere the reported seed dispersal distance by ant spp. ranges from 0.06 to 77 m (Gómez & Espadaler, 1998). However, Whitney (2002) found that seed of *A. ligulata* Benth. was dispersed as far as 180 m over a 3000 m^2 area surrounding the ant nest sites of *Iridomyrmex viridiaeneus.* While seed may not have been dispersed into the pit sites by myrmecochory and/or ornithochory, seed would have been transported into these sites from the surrounding vegetation by other means. After the cyclone in 2008, it was observed that there had been the movement of soil and water into the pit sites and this would have presumably occurred at other extreme rainfall events. Seed would have also been transported, along with the soil and water (Aguiar & Sala, 1999) into the pit areas.

A major impediment to plant establishment in dryland restoration is the low germination rates of some plant species (Merritt & Dixon, 2011). Poor techniques in the storage, handling and pretreatments of the seed for restoration can result in less than 10% becoming established plants (Merritt & Dixon, 2011). Successful establishment in restoration involves the understanding of germination and sowing requirements of the different species (Commander *et al.*, 2009; Commander *et al.*, 2013). Commander *et al.* (2009) demonstrated that *A. tetragonophylla* F.Muell. was among a number of plant species of the SBSLA that required specific cues for germination including scarification. All the seed used in the glasshouse and field experiments (Chapters 2, 3, 5 and 6) were pretreated with hot water for scarification (Section 2.2.3.2) to circumvent seed dormancy. Seed dormancy is an adaptation that many *Acacia* spp. have adopted allowing a sufficient period of time for seed scarification and for the plants to not rely on a single factor such as soil moisture for germination (Letnic *et al.*, 2000). It has been shown that dispersal of seed by ants and birds alters seed dormancy, possibly reducing the response time of the seed to environmental germination triggers while seed that has remained in the seed bank

undergoes differing degrees of scarification (Letnic *et al.*, 2000). The lack of ant and bird activity in the pit sites might be a contributory factor to the failure of the seed washed into the pit sites to germinate and benefit from the improved soil moisture conditions or increased populations of beneficial soil microbes such as RNB (Figure 2.13).

Future work could investigate the mechanisms of seed germination in relation to the soil microbial community of the rhizosphere should a clear correlation be established that the plant-microbe association between the soil microbial community be beneficial to successful seed germination and plant establishment. The methods adopted for agricultural ecosystems to inoculate legumes, while not wholly applicable may be adapted for use in rehabilitation projects. However, there needs to be a greater collaboration with site operators and the scientific community to develop the means to produce, deliver and apply site-specific inoculums that are cost effective for the provenant flora species of a project area.

7.4 Concluding remarks

Given the diversity of the ecosystems of the Australian continent, there has been a limited number of studies of Australian native legumes and their micro-symbionts and these studies have been largely restricted to the more temperate or tropical areas of Australia (Barnet *et al.*, 1985; Barnet & Catt, 1991; Barrett *et al.*, 2012; Beadle, 1964; Bever *et al.*, 2013; Hoque *et al.*, 2011; Lafay & Burdon, 1998; Lafay & Burdon, 2007; Lawrie, 1981; Lawrie, 1983; Marsudi *et al.*, 1999; Murray *et al.*, 2001; Murray *et al.*, 2002; Thrall *et al.*, 2000; Thrall *et al.*, 2001; Thrall *et al.*, 2005; Thrall *et al.*, 2007; Thrall *et al.*, 2008; Thrall, 2011; Thrall *et al.*, 2011). This work contributes to the knowledge of the RNB associations of two *Acacia* spp. with widespread distribution throughout the drylands of Australia and the potential for the use of these species and associated RNB for the rehabilitation of degraded sites within the Shark Bay area. This work also highlights the importance of conducting in-situ studies of native legumes-RNB symbiotic relationships and that relying on synthetically produced growth conditions may not ensure that the associations reported on are those reflected in the natural

environmental conditions. The main gains are to improve the success of seedling establishment for the eventual persistence of a complex floristic community capable of supporting a community diversity that is representative of that which preceded the disturbance.

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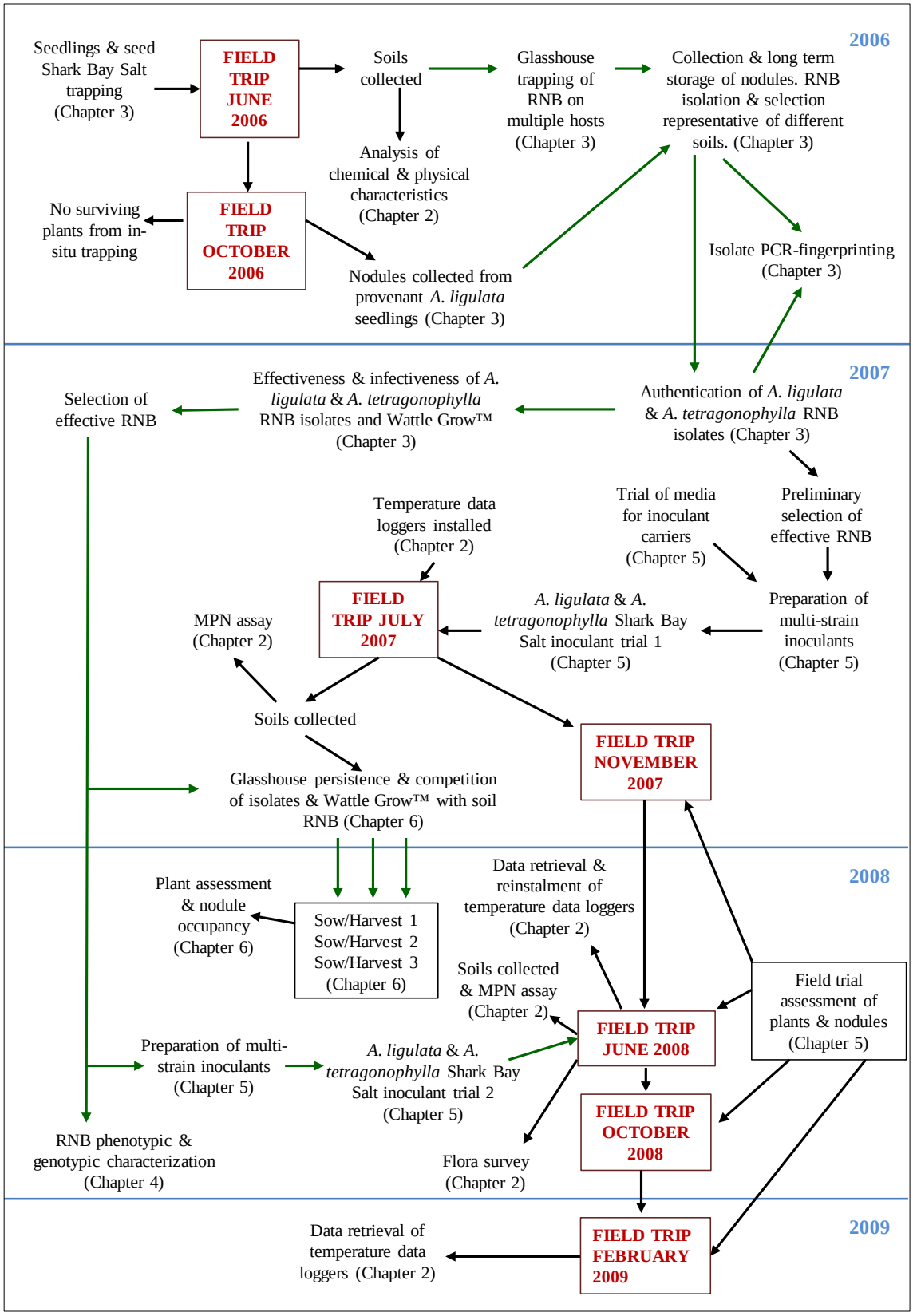
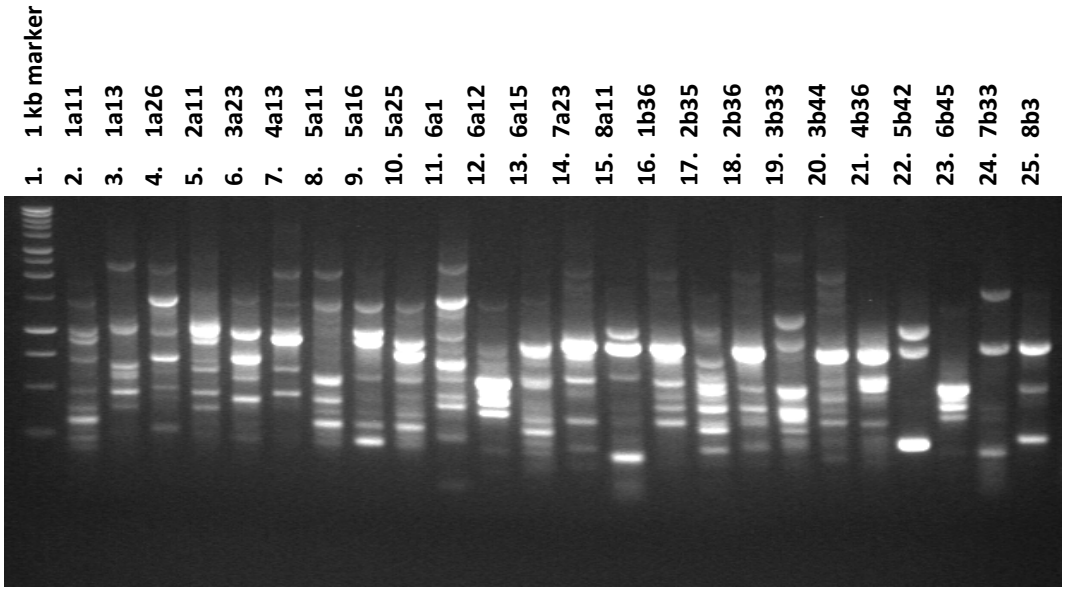


Appendix 1: Timeline schematic of experiments and activities for the duration of the thesis project.

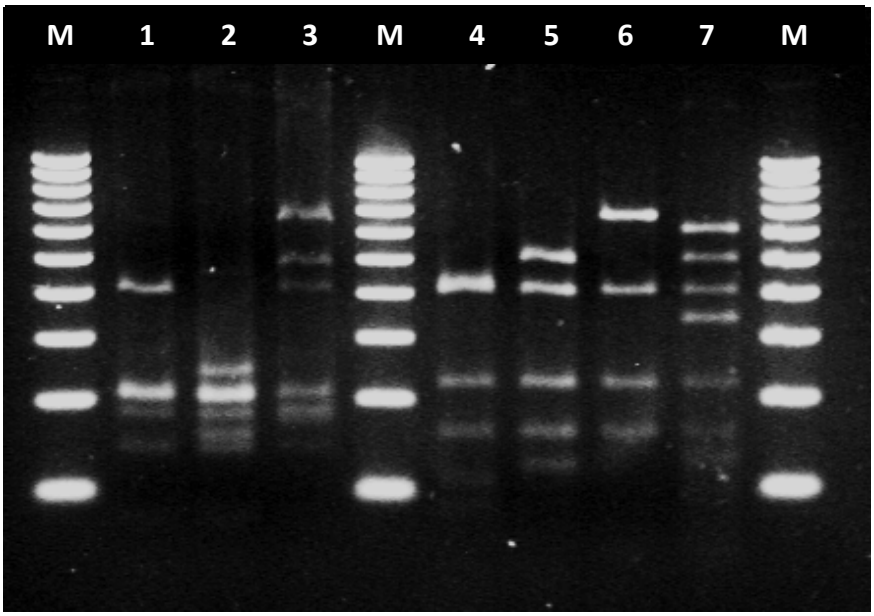


Appendix 2: PCR-RPO1 fingerprints of selected *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. isolates. The sub-set of isolates is those treatments for which the plant foliage N concentrations were assayed. Lane 1 is a Promega 1 kb DNA ladder marker and lanes 2 to 25 are the RNB isolates as labelled.

Appendix 3: 16S rDNA **GenBank®** sequences of RNB strains from the National Center for Biotechnology Information (NCBI) database which closely aligned to the sequences of *A. ligulata* Benth. and *A. tetragonophylla* F.Muell. isolates. Type strains are indicated by ^{T} adjacent to strain identification. Number of nucleotide mismatches in parenthesis adjacent to percentage sequence similarity.

RNB isolate	Close matched RNB strain and type strain Identification and accession number	Sequence similarity
1a26	*Rhizobium huautlense* SO2 (NR_ 024863)	99.9% (4)
	Rhizobium huautlense USDA 4900^{T} (AF025852)	99.7% (4)
1b36*	*Ensifer meliloti* SEMIA 6162 (FJ025127)	99.5% (8)
	Ensifer arboris HAMBI 1552^{T} (Z78204)	99.4% (9)
3a23*	*Ensifer meliloti* SEMIA 6162 (FJ025127)	99.6% (6)
	Ensifer arboris HAMBI 1552^{T} (Z78204)	99.8% (3)
3b33*	*Ensifer meliloti* SEMIA 6162 (FJ025127)	99.6% (6)
	Ensifer arboris HAMBI 1552^{T} (Z78204)	99.4% (9)
4a13*	*Ensifer meliloti* SEMIA 6162 (FJ025127)	99.5% (6)
	Ensifer arboris HAMBI 1552^{T} (Z78204)	99.3% (9)
4b36*	*Ensifer meliloti* SEMIA 6162 (FJ025127)	99.6% (6)
	Ensifer arboris HAMBI 1552^{T} (Z78204)	99.4% (9)
5a16	Ensifer fredii USDA257 (CP003563)	99.9% (2)
	Ensifer xinjiangense LMG17930^{T} (AM181732)	99.9% (1)
6a12	*Rhizobium. sp.* ORS 1457 (AY500263)	99.8% (3)
	Rhizobium sullae IS 123^{T} (Y10170)	98.7% (18)
7a23*	*Ensifer meliloti* SEMIA 6162 (FJ025127)	99.5% (7)
	Ensifer arboris HAMBI 1552^{T} (Z78204)	99.4% (9)
7b33	*Ensifer sp.* LILM2009 (FJ792814)	99.8% (3)
	Ensifer meliloti LMG 6133^{T} (X67222)	99.8% (3)

* Isolates that formed a cluster group



Appendix 4: Restriction patterns of 16s rRNA after digestion of *Alu*I and *Msp*I. Lanes with a 100bp DNA marker are labeled 'M'. *Alu*I digests assigned group A, B and C in lanes 1, 2 and 3 respectively. *Msp*I digests assigned group A, B, C and D in lanes 4, 5, 6 and 7 respectively.