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JAMES COOK UNIVERSITY AUSTRALIA

GROWTH AND NITROGEN FIXATION OF INLAND AND COASTAL TROPICAL *ACACIA* SPECIES

Thesis submitted by

Michael Siu Fung Lee
BSc (Hons). The University of Hong Kong (2005)

in January 2013

for the degree of Master of Science by Research in Tropical Plant Sciences
within the School of Marine and Tropical Biology,
James Cook University

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I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

Michael S. F. Lee January 2013

STATEMENT ON THE CONTRIBUTION OF OTHERS

This research was funded primarily by the National Climate Change Adaptation Research Facility (NCCARF). Part of the research fund also came from the Postgraduate Student Support Fund (PSSF) and the Tropical Vegetation Dynamics Laboratory (TVDL) in JCU.

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> " *It does not matter who teach you, what they teach you and why they teach you. What matters the most is what ideas you can develop from the established knowledge in a logical and coherent manner. Stay foolish and hungry, be open-minded, constantly reflect on learning outcomes and maintain the will to act. Then nothing is impossible.* "

ABSTRACT

Nitrogen is mainly lost from most Australian tropical savanna ecosystems via fire and replenished by nitrogen fixation. Fires in Australian tropical savanna woodlands may become more frequent and severe under climate change and thus nitrogen loss may be exacerbated. This research partly examines how climate change can affect the seed germination of *Acacias* (family: Mimosaceae) in terms of changing rainfall and fire regimes. The research also examines the effect of climate change on the nitrogen fixation and growth of tropical *Acacias*, in terms of changing rhizobia sources (in case of range shifts under climate change), responses to drought (which are predicted to become more frequent and severe), and factors affecting the nodulation of mature *Acacia* trees/shrubs (since the dry season may be prolonged and soil properties may change).

By comparison to coastal *Acacias,* the following key hypotheses were proposed:

- seeds of inland *Acacias* have weaker physical dormancy when no pretreatment was applied;
- inland *Acacias* display co-adaptation with sympatric rhizobia in nitrogen fixation;
- inland *Acacia* seedlings cope with drought better; and
- mature inland *Acacias* produce fewer nodules and rely proportionally less on symbiotic nitrogen fixation in obtaining nitrogen from the surrounding environment

When no pretreatment was applied, we found no significant difference between inland and coastal *Acacias* in the seed germination responses. Thus the original hypothesis was not supported. We also could not find any clear differences in the heat tolerance and sensitivity between inland and coastal species. But in another study by Congdon ***et al.*** (in prep.), which the current experiment supplements, inland *Acacias* had a lower heat tolerance level (80^{o}C dry heat for five minutes) than coastal species (100^{o}C dry heat for 5 minutes). It appears that inland *Acacia* species have adapted to fire in years of average rainfall as 80^{o}C is the soil temperature recorded at 3 mm soil depth in an early dry season fire. More intense fire can drive the temperature at 3 mm to 182^{o}C and hence can cause substantial mortality of seeds of both inland and coastal *Acacia*.

In the provenance experiment, nodulation always enhanced seedling growth. *Acacia* hosts did not necessarily grow best with sympatric soil rhizobia, implying that tropical *Acacias* can grow at least equally well in soil outside their current ranges as in sympatric soil. Thus any climate-induced or assisted range shifts of tropical *Acacia* species under climate change might not be

constrained by mutualism. Three, of the four *Acacia* species tested, established and/or grew better with inland rhizobia. Hence species expanding into inland areas might even benefit from more effective mutualistic symbiosis. Adding inland soil as a source of rhizobia might benefit the growth of tropical *Acacia* seedlings in a nursery situation.

Absence of watering for 8 weeks had a negative effect on the biomass and nitrogen contents of *Acacia* seedlings. The inland *Acacias* were more adapted to drought than coastal ones by having smaller changes in foliage water content, foliage thickness (deduced from specific foliage weight), and higher water use efficiency under drought than under the normal watering regime (deduced from foliage ^{13}C values). Secondly, nodule nitrogen contents and the relative importance of symbiotic nitrogen fixation in acquiring nitrogen under drought were lower than those under the normal watering regime. The percentage reduction was found to be more severe in inland *Acacias* than coastal *Acacias* and might be of adaptive significance. Furthermore, the decreases in foliage and nodule biomass, stem to root ratio and foliage nitrogen content in all the four *Acacia* species under drought were smaller in the presence of inland rhizobia than in the presence of coastal rhizobia, suggesting symbioses formed with the inland source of rhizobia were less affected by drought. In short, *Acacia* species restricted to inland areas or species growing on inland soil might therefore have higher resistance to a single drought event.

Using the ^{15}N natural abundance method, it was found that symbiotic nitrogen fixation and/or mycorrhizal uptake of soil nitrogen was likely to be the primary mechanism(s) of all four tropical *Acacia* species for acquiring nitrogen in the wild. Abundant nodules were found growing on the roots of coastal *A. crassicarpa* in the top 24 cm of soil while few nodules were found on the roots of coastal *A. aulacocarpa*, and inland *A. elachantha* and *A. ramiflora*. Their nodules, if any, might have developed in deeper soil (below 24 cm). Deeper soil (12-24 cm vs top 12 cm), greater crown width and root biomass of the *Acacia* hosts, the change from dry to wet season, decreasing soil moisture and soil bulk density were positively related to the development of effective nodules of *A. crassicarpa*. Total soil nitrogen and phosphorus were, however, not correlated with effective nodulation. Prolonged periods of drought under climate change can be detrimental to plant growth. Based on the field study results, it is also likely to inhibit effective nodulation in surface soil directly, or indirectly through slower tree growth and hence smaller tree size. It is possible that effective nodules can develop in deeper soil in the dry season to compensate for the decrease in surface soil. Deeper soil should be sampled in the future to verify this possibility.

ABBREVIATIONS

%Ndfa	Percentage of nitrogen derived from atmosphere
[P]	Phosphorus concentration
Δ	Isotopic discrimination
ABA	Abscissic acid
AIC	Akaike Information Criteria
AM	Vesicular-arbuscular species
$\mathbf{A_{max}}$	Photosynthetic capacity
ATP	Adenosine triphosphate
BOM	Bureau of Meteorology
CAIC	Corrected Akaike Information Criteria
CAZR	CSIRO Center of Arid Zone Research
CC	Whole plant carbon concentration
COV	Coefficient of variation
CSIRO	Commonwealth Scientific and Industrial Research Organization
DDND_A	Dead nodule density
DDND_DW	Dead nodule dry weight
DKCRC	Desert Knowledge Cooperative Research Centre
DV	Dependent variable
DW	Dry weight
ECM	Ectomycorrhizae
f	The uptake of nitrogen by hyphae
FMR	Foliage mass ratio
G3P / Triose-P	Glyceraldehyde-3-phosphate
GP	Germination percentage
GSG	Great soil group
HPO	Hughenden Post Office
HSB	Horseshoe Bend
IPCC	Intergovernmental Panel on Climate Change
JCPO	Julia Creeks Post Office
LEDND_A	Live and effective nodule density
LEDND_DW	Live and effective nodule dry weight
LIDND_A	Live and ineffective nodule density

LIDND_DW	Live and ineffective nodule dry weight
LM	Linear model
LMM	Linear mixed model
MAR	Mean annual rainfall
MAT	Mean annual temperature
MIM	Mount Isa Mine
ML	Maximum likelihood
NADPH	Nicotinamide adenine dinucleotide phosphate
NAR	Net assimilation rate
NB	Negative binomial
NBR	Needle leaf biomass ratio
NDW	Nodule dry weight
NMR	Nodule mass ratio
NodC	Nodulation condition
NPP	Net primary productivity
NPUA	Nitrogen per unit area
NPUDW	Nitrogen per unit dry weight
NT	The Northern Territory
NUE	Nitrogen use efficiency
OLS	Ordinary least square
Pa	Daytime net photosynthetic rate of shoot per unit area
PAR	Photosynthetically active radiation
PB	Plant biomass
PN	Plant nitrogen
PCR	Photosynthetic carbon reduction
PNC	Plant nitrogen concentration
PPF	Principal profile form
PV	Predictor variable
QLD	Queensland
Ra	Respiration of roots for 24 hours and of shoots at night
RBR	Root biomass ratio
REML	Restricted Maximum Likelihood
RGR	Relative growth rate in terms of plant biomass
RGR-N	Relative growth rate in terms of plant nitrogen content

RPO	Richmond Post Office
Rubisco	Ribulose-1,5-biphosphate carboxylase-oxygenase
RV	Response variable
SBR	Stem biomass ratio
SFA	Specific foliage area
SFW	Specific foliage weight
ShootTRR	Shoot to root ratio
SLA	Specific leaflet area
SNA	Specific nitrogenase activity
SOC	Soil organic matter
StemTRR	Stem to root ratio
T_{50}	Time to 50% germination
T_f	The proportion of nitrogen in the hyphae that is transferred to the host plant roots
TSVA	Townsville airport
TTCCP	Townsville Town Common Conservation Park
WA	Western Australia
WUE	Water use efficiency
WW	Wet weight
Z	Z-score
ZANB	Zero-altered negative binomial
ZAP	Zero-altered Poisson
ZINB	Zero-inflated negative binomial
ZIP	Zero-inflated Poisson
$\delta^{13}C$	Natural abundance of ^{13}C isotope
$\delta^{15}N$	Natural abundance of ^{15}N isotope
$\delta^{15}N_A$	Natural abundance of ^{15}N isotope of nitrogen fixing *Acacia* species forced to rely on atmospheric nitrogen as its sole source of nitrogen
$\delta^{15}N_F$	Natural abundance of ^{15}N isotope of nitrogen fixing *Acacia* species taking up both soil nitrogen and atmospheric nitrogen
$\delta^{15}N_R$	Natural abundance of ^{15}N isotope of non-nitrogen fixing reference plants

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1 Aims and Background to the Study

1.1 An introduction to Australian tropical savanna woodlands

The goal of the current study is to examine how climate change will affect symbiotic nitrogen fixation of tropical *Acacias*. It investigates several aspects of the ecology of tropical *Acacias* in Australian savanna woodland, ranging from the seed germination, the growth and symbiotic nitrogen fixation of seedlings to the nodulation of mature trees and shrubs. An up-to-date overview of these specific topics will be provided within individual chapters. This introductory chapter presents an overview of the Australian tropical savanna woodland, particularly the major issues related to nitrogen cycling in this important habitat. Since many *Acacia* species are adapted to this environment, the chapter starts with a discussion of the evolutionary history of tropical savanna woodland and the associated plant species. The factors affecting different components of nitrogen cycling in Australian tropical savanna, in particular the role of fire disturbance, are described. An overview of the research of climate change on plant ecology is then followed by an introduction to the approach adopted in this investigation about the effect of climate change on symbiotic nitrogen fixation. The chapter ends with a description of the study sites, the aims and the structure of the thesis.

1.1.1 General features

Tropical savanna woodlands – the focus of the current study – are distinguished from grasslands, deserts, and forests by their using projective foliage cover, structure, species composition, soil nutrition, and fire disturbance (Schmidt and Lamble 2002; Attiwill and Wilson 2003). These woodlands are an important habitat that covers one-fourth of the Australian land mass. Rainfall is highly seasonal and the annual average can range from more than 2000 mm to less than 400 mm yr^{-1} (Russell-Smith and Yates 2007). The projective foliage covers of woodlands and open woodlands are respectively 10 to 30% and less than 10%. While the dominant trees of woodlands can range from 10 to 30 m in height, those of open woodlands range from 5 to 10 m (Specht 1970). Soil available nitrogen and phosphorus concentrations in Australia tropical savannas are lower than that in Africa (Beadle 1981; Schmidt and Lamble 2002). It is not uncommon to find *Eucalyptus* dominated woodlands consisting of understorey legumes such as *Acacia spp.* as well as *Hakea, Banksia, Grevillea spp.* belonging to the Proteaceae. Mitchell

grass (e.g. *Astrebla*) or hummock grasses (e.g. *Triodia*) are common, depending on aridity (Beadle 1981; Myers *et al.* 2004).

1.1.2 Fire-prone environment

Tropical savannas in northern Australia, which have a climate of high mean annual temperature and a mean annual rainfall from less than 400 mm to over 2 000 mm yr^{-1}, are generally highly fire-prone. In Australia, fire swept through an average of 497 240 km^2 per year between 1997 and 2005. Around 67.8% of the area burnt, equivalent to 336 980 km^2, occurred in tropical savannas (Russell-Smith and Yates 2007). About 76% of the tropical savanna fires occurred in the late dry season, when fuel was dry and more combustible, giving rise to fires of higher intensity (Russell-Smith and Yates 2007). Historically, while some fires are triggered by lightning, prescribed fires are also could be initiated by land managers for conservation, pasture management and household protection purposes. Aboriginal burning in the monsoon tropics was carried out in the past to aid hunting and travelling, to clear camp sites, and to make fire breaks to protect rainforest patches (Bowman 2000). Wild fires in semi-arid and arid areas generally occur in the dry season or early wet season (winter in northern Australia and summer in southern Australia) (Williams 2002; BOM 2011). More severe wild fires often occur in the first dry season following one or several years of above-average rainfall (Leigh and Noble 1993), which promotes growth of herbs and seedlings, as well as production of leaves. As this plant material senesces, it results in large amounts of dry matter, providing increased fuel loads. Tropical eucalypt-dominated savanna woodlands in Queensland burn once every two to five years due to lightning and prescribed burning (Williams 2002; Williams *et al.* 2004). The fire frequency is higher in the tropical savannas in the Northern Territory and Kimberley, i.e. once every one to three years (Williams 2002; Rossiter-Rachor *et al.* 2008).

1.1.3 Historic interaction between climate, vegetation and fire in shaping savanna woodland

In Australia, inland sites were drier and had a higher fire frequency than the coast historically. Australia has became more arid since the early Miocene, and became warmer following the end of global cooling at the end of the Oligocene (Attiwill and Wilson 2003). Pollen records show that, during the early Miocene, temperate rainforest persisted on the eastern and southeastern Australian coasts (Kemp 1993). While rainforests also thrived inland, they were more restricted to watercourses. For the first time, significant amounts of grass pollen showed up in pollen

records. Dry sclerophyll or open forest originated between the late Miocene and early Pleistocene as climate drying continued. Savanna woodland appeared during the Pliocene-Pleistocene in inland areas (Kemp 1993). The lack of Koala fossils from the Oligo-Miocene transitional period could have been caused by the low availability of *Eucalyptus* as a food source (Attiwill and Wilson 2003). As the Miocene arrived, eucalypts proliferated with increasing aridity, and gradually expanded to the continent's margin in this period (Attiwill and Wilson 2003). Eucalypts are flammable due to their characteristics such as their high foliage oil content, spot-fire promoting bark, open crown with pendulous foliage that encourages updraught (Bowman 2000). Their woody capsules can protect seeds from fire. Fire triggers dehiscence of capsules to release seeds. Thus many *Eucalyptus* species are ecologically adapted to fire. Woodland ecosystems dominated by *Eucalyptus* trees in semi-arid and arid Australia (mainly along watercourses) have been subject to fire disturbance for a very long time. Wild fire, therefore, has been an important selective force on these ecosystems. Given the flammable characteristics, and the historic increase in grass cover in central Australia compared to coastal areas (Gill 1993), inland woodlands experience more frequent natural fire than coastal woodlands.

Australian *Acacias* evolved in the Miocene (Kemp 1993), when central Australia was becoming increasingly arid. It is a logical deduction that fire disturbance would have accompanied the spread of aridity. Since the flammable eucalypts evolved in the Palaeocene and diversified from the Oligocene (Ladiges ***et al.*** 2011), it is possible that their spread promoted fire disturbance. Fire stimulates the germination of *Acacias* from the soil seed bank, through breaking down the thick testa and promoting imbibition (Langkamp 1987). Two notable ecophysiological features of *Acacias*, as with many legumes in South America or Africa, help them adapt to low soil moisture and phosphorus in both temperate and tropical Australia. They include the synthesis and accumulation of high concentrations of osmotically compatible solutes such as pinitol in foliage, and the ability to form cluster roots and mycorrhizal associations to extract patchily distributed phosphorus from the old Australian soil (Adams ***et al.*** 2010). In fact, a global study showed that soil phosphatase activity in the presence of nitrogen-fixing plants is three times higher than that in the absence of nitrogen-fixing plants (Houlton ***et al.*** 2008).

1.1.4 Seasonality of rainfall and evaporation

The wet season ranges from from October to April and the dry season from May to September (BOM 2012a). The ratio of rainfall to evaporation decreases with distance from the coast in tropical Queensland. For instance, in Townsville, the average rainfall in December slightly

exceeds the amount of evaporation so that the ratio is 1.06. But in Hughenden (about 200 km southwest from Townsville) in central Queensland, the ratio quickly drops to 0.54 in December. Further west, in Mount Isa, the ratio is only 0.33 in December (Table 1.1). Such a gradient of decreasing rainfall to evaporation ratio is also observed in the dry season (Table 1.1). Also, the number of days with rain decreases with increasing distance from the coast. For example, Townsville, Paluma Horseshoe Bend, Charters Tower and Torrens Creek had 91, 59, 68 and 41 days of rain per year respectively (BOM 2010). With a low rainfall to evaporation ratio and fewer rainy days, the soil may dry out quickly even in the wet season.

1.2 Nitrogen cycling in tropical savanna woodlands

Nitrogen is a key macronutrient for plants as it is a the constituent element of protein including enzymes, nucleic acids and ATP (the principal energy carrier in cells) (Perry *et al.* 2008). The forms of nitrogen commonly taken up by plants are ammonium (NH_4^+) and nitrate (NO_3^-), which are respectively fixed from atmospheric N_2 and absorbed directly from the soil (Lavelle and Spain 2001). Organic nitrogen, such as amino acids, can be taken up via ectomycorrhizal associations with fine roots (Craine *et al.* 2009) (Fig. 1.1).

Soil nitrogen availability to plants in tropical ecosystems, like other nutrients, is the result of input from external sources such as biological fixation of atmospheric N_2 to NH_4^+ or nitrogen deposition through precipitation/fertilization, losses to external sinks such as denitrification from NO_3^- to N_2O (nitrous oxide), NO (nitric oxide) and atmospheric N_2, and nitrogen cycling within the system such as conversion of NH_4^+ to NO_3^- via nitrification, or mineralization of soil organic matter (Perry *et al.* 2008).

Savanna trees are known to absorb nutrients such as nitrate in the wet season (Schmidt and Lamble 2002), when mineralization and nitrification rates increase. This has been confirmed by indirectly quantifying the respiration rate of microbes, and the change in amino acids and ammonium in the soil (Holt and Coventry 1990; Richards *et al.* 2012). As mineralization and nitrification require adequate moisture, the rates of these processes vary with rainfall and season and pulses of nutrient availability are likely to occur in the soil.

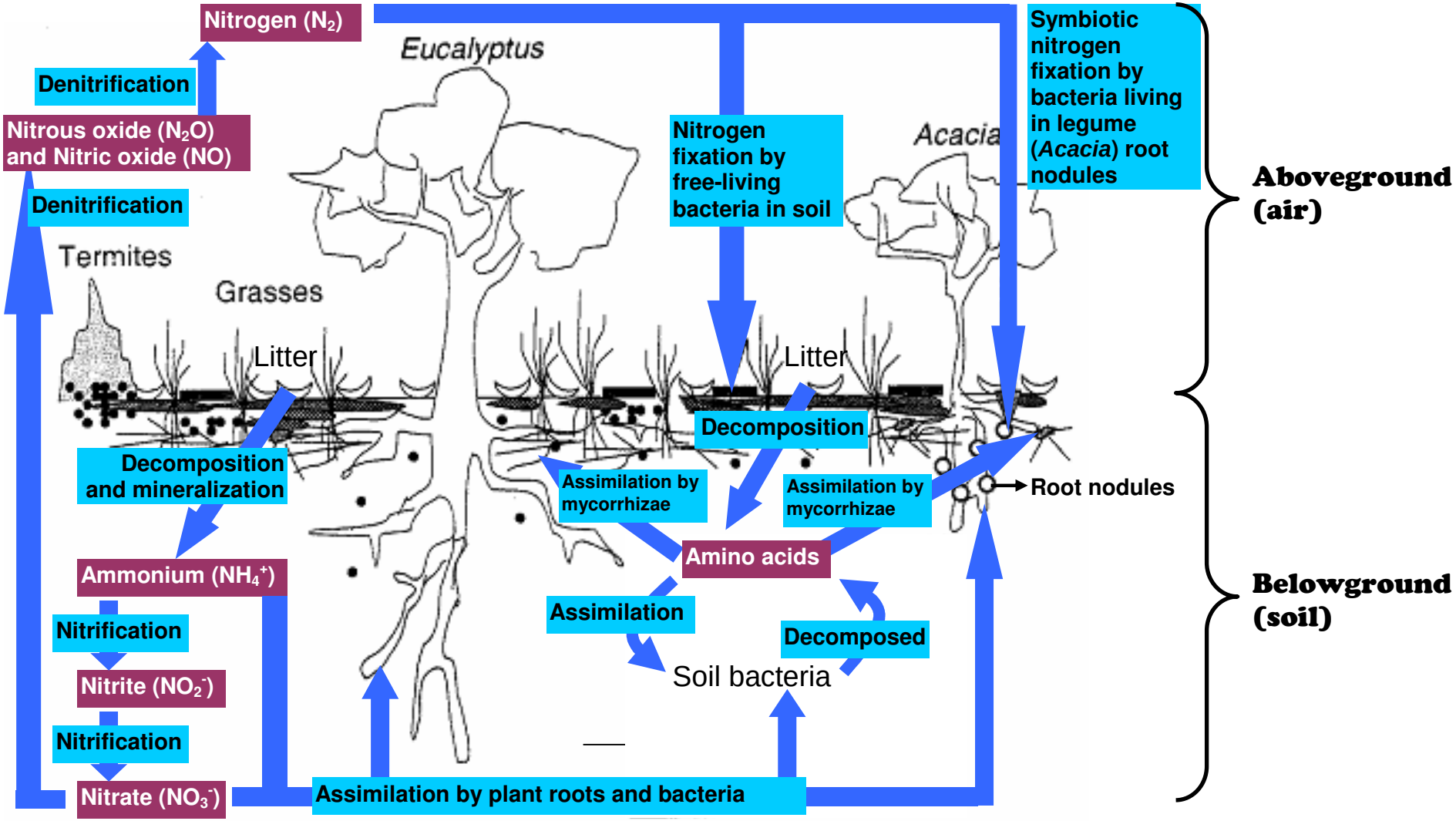


Figure 1.1. An illustration of nitrogen cycling in an Australain tropical savanna woodland. Adapted from Schmidt and Lamble (2002).

Table 1.1. Mean monthly rainfall to mean monthly evaporation ratio from the east to the west of tropical Queensland

TSVA, Townsville airport (Station no.: 032040); HPO, Hughenden Post Office (Station no.: 030024); RPO, Richmond Post Office (Station no.: 030045); JCPO, Julia Creeks Post Office (Station no.: 029025); MIM, Mount Isa Mine (Station no.: 029126). Values in bold are wet season figures. Data were downloaded from the Bureau of Meteorology website: www.bom.gov.au

Site	Jan	Feb	Mar	Apr	May	June	July	Aug	Sept	Oct	Nov	Dec	Mean	Years of average
TSVA	**1.06**	**1.39**	**0.70**	**0.34**	0.20	0.12	0.07	0.11	0.06	**0.11**	**0.25**	**0.57**	0.43	1969 – 2011
HPO	**0.54**	**0.51**	**0.26**	**0.17**	0.15	0.12	0.08	0.06	0.04	**0.11**	**0.2**	**0.3**	0.23	1970 – 2001
RPO	**0.51**	**0.45**	**0.27**	**0.13**	0.06	0.09	0.06	0.03	0.02	**0.06**	**0.11**	**0.24**	0.18	1970 – 2012
JCPO	**0.34**	**0.48**	**0.21**	**0.07**	0.07	0.07	0.05	0.02	0.01	**0.08**	**0.14**	**0.22**	0.16	1982 – 2001
MIM	**0.33**	**0.32**	**0.24**	**0.06**	0.08	0.03	0.05	0.02	0.03	**0.06**	**0.08**	**0.16**	0.13	1965 – 1992

Fire is responsible for the loss of nitrogen from savanna, together with phosphorus, calcium, magnesium, and potassium (Cook 1994; Laclau et al. 2005). Laclau et al. (2005) studied nutrient inputs and losses from a tropical savanna in the Congo, where the MAR was 1200 mm, MAT 25^{o}C and the soil was sandy (>85% sand). In the dry season, symbiotic nitrogen fixation had a low input to the savanna (2.2 kg N ha^{-1} year^{-1}) while wet deposition was the second largest source (1.1 kg N ha^{-1} year^{-1}). Wet deposition was also the major phosphorus supply to the savanna system amounting to 0.1 kg P ha^{-1} year^{-1}. Phosphorus is required for both the symbionts, the nitrogen fixing process and the host plant in the symbiosis (Binkley and Giardina 1997; O'Hara 2001). Since old Australian soil is generally depleted in nitrogen and phosphorus (Attiwill and Wilson 2003), phosphorus is likely a limiting nutrient in Australian tropical savannas. Fire was responsible for loss of nitrogen, amounting to 23.4 kg N ha^{-1} year^{-1}. About 85% N and 25% P contents in the aboveground litter were lost to the atmosphere in fire (Laclau et al. 2005).

In the wet season, symbiotic nitrogen fixation was the major nitrogen input and about 19.4 kg N ha^{-1} year^{-1} was fixed into the ecosystem. Wet deposition also contributed 3.7 kg N and 0.2 kg P ha^{-1} year^{-1}. Some leaching down the soil profile was present but was not substantial given the low amount of rainfall in this habitat compared to tropical forest. Similar patterns of input-output were reported in the Ivory Coast and Kapalga in the Northern Territory (Cook 1994; Laclau et al. 2005).

In fact, symbiotic nitrogen fixation was also estimated to be the major nitrogen input mechanism in Brazilian and Orinoco cerrado tropical savanna woodlands, as well as temperate mesquite woodland in North America (though nitrogen deposition was another major source in America) (Bustamante *et al.* 2006). However errors in various methods of estimation render precise quantification of the nitrogen input through symbiosis difficult. South American tropical savannas consist of diverse and abundant leguminous species (Bustamante *et al.* 2006). Legumes in these savannas likely contributed a significant amount of nitrogen through symbiotic nitrogen fixation, based on the natural abundance of ^{15}N and the concentration of ureids in the xylem sap of young shoots.

Fire and symbiotic nitrogen fixation likely play major roles in nitrogen cycling at the study sites. The MAR in the savannas between Townsville and the Burra Range ranges from about 1300 mm to less than 500 mm. The MAR of the current study sites thus overlap with those of the Congo, Ivory Coast and Kapalga savanna sites as mentioned above. Many parts of Queensland burn once every two to five years. High fire frequency can deplete ecosystem nitrogen by

repeatedly volatilizing nitrogen in the aboveground litter (Cook 1994), or by volatilizing a large proportion of soil nitrogen (Richards *et al.* 2012).

The role of fire in nitrogen dynamics varies from site to site. For instance, fire was responsible for 90% of the nitrogen loss in a pasture in the Northern Territory (Norman and Wetselaar 1960), whereas in some sites in Australia and Africa with *Eucalyptus* forests/savannas, regular burning in the long-term fails to alter soil nitrogen levels significantly (Holt and Coventry 1990). Sometimes areas with fast-growing and highly nutritious exotic grasses have greater fine fuel load and hence greater nitrogen loss due to fire (Rossiter-Rachor *et al.* 2008), since fuel load and N volatilization rate are positively correlated (Cook 1994).

Richards et al. (2012) demonstrated a strong effect of fire on tree-grass interaction and the consequence on soil nitrogen dynamics. Their fire experiment was run in the Territory Wildlife Park near Darwin with a MAR of 1 400 mm. Canopy and intercanopy areas were subject to early or late dry season burning at different intervals. Across all fire treatments, canopy soil had significantly higher nitrogen concentrations, in terms of amino acid (10% higher), ammonium (60% higher) and total nitrogen (30% higher), than intercanopy soil. The mean soil nitrate level of burnt plots was 60% lower than that of unburnt plots but soil ammonium and amino acid did not vary significantly Thus if catastrophic fire, which is likely to become more frequent in Queensland in terms of Fire Danger Index (OCC 2008), burns away a significant proportion of the canopy, the loss of inorganic nitrogen from the ecosystem in the long-term could be substantial. Plants may increasingly rely on mycorrhizae or symbiotic nitrogen fixation, which can be energy demanding and may become another stress. How this change in soil nutrition affects mycorrhizal or nitrogen-fixing plants should be experimentally investigated. *Eucalyptus* and *Acacia* species are known to form ectomycorrhizae and arbuscular-vesicular symbioses respectively, whereas Proteaceae such as *Hakea* and *Banksia* do not associate with mycorrhizae nor with nodulating bacteria (Schmidt and Stewart 2003).

1.3 Climate change in Queensland

Part of the aim of this thesis is to determine how climate change affects the symbiotic nitrogen fixation of tropical *Acacias*. The importance of symbiotic nitrogen fixation in replenishing nitrogen lost through recurrent fire in savanna woodlands has been discussed in Section 1.2. According to the latest modelling predictions and using 1980 to 1999 as the baseline period, the 50th percentile annual rainfall of northern Queensland will fluctuate between +2% and -2% by 2030, while that of southern Queensland will decrease by 2 to 5% by 2030. A decline by as

much as 10% may be achieved in winter and spring in some areas of Queensland (CSIRO and BOM 2010). The 50th percentile annual relative humidity will fluctuate between +0.5% and -1% by 2030 with a decrease by up to 1% in spring and winter (CSIRO and BOM 2010). The 50th percentile annual evaporation will be raised by 2 to 4% by 2030 (CSIRO and BOM 2010). Thus the air and the surface soil in Queensland will probably become drier in the future. The extent of increased dryness will likely be more severe in winter and spring than in summer and autumn. This trend is spatially variable, implying that finer scale climatic modelling should be applied to match the locations of individual habitats. Significant changes in rainfall and evaporation, as well as temeprature and fire hazards, in Queensland by 2050 are also expected (Table 1.2).

Table 1.2. Predicted climate changes in Queensland by 2050 using 1980 to 1999 as the baseline period, based on a high emission scenario

Climate variables	Queensland state-wide	Townsville-Thuringowa	North-west Queensland
Mean annual temperature (^{o}C)	+1.7 to +2.2	+1.9	+2.1
Mean annual rainfall (%)	-1 to -7	-5	-3
Evaporation (%)	+5 to +7	+7	+6
Fire hazards	• *"Decreases in relative humidity, combined with projections of increased temperature, an increase in the number of hot days and less frequent rainfall events, are likely to increase the number of high Forest Fire Danger Index (FFDI) days".* • FFDI measures the risk of forest fire and incorporates the influence of humidity, temperature, wind speed and drought.		

Source: OCC (2008)

1.4 Approaches in studying climate change effects on vegetation

1.4.1 Overview of research approaches

Global leaders are yet to reach a consensus on new legally-binding emission caps for different countries as the current cap set up under the Kyoto protocol is about to expire by 2012. Nevertheless, planning and implementing climate change adaptation activities is no less urgent. Risk evasion is effective only after the potential risks are identified and predicted. Therefore, many researchers nowadays attempt to predict the change in carbon budget and fluxes, species

distribution and population dynamics under climate change (Thomas *et al.* 2004; Fordham *et al.* 2012).

While the current study investigates the effect of climate change on *Acacias*, it employed a statistical modelling approach. To put the current study into context, it is useful to review the main themes and the associated modelling approaches in climate change research. Many studies employ mechanistic models to predict the change of greenhouse gases. The feedback between climatic parameters such as temperature and rising atmospheric CO_2, and biological processes such as photosynthesis and respiration were investigated by integrating General Circulation Models and Dynamic Vegetation Models (Beerling and Woodward 2001). Recently, scientists attempted to integrate the effect of drought, soil processes and probability of extreme events on vegetation and hence greenhouse gas flux (Heimann and Reichstein 2008; Lehmann *et al.* 2008). Secondly, to understand the potential impact of climate change on biodiversity and hence facilitate the planning of adaptation activities, the potential shift in species distribution is also subjected to spatial modelling (Thomas *et al.* 2004; Fordham *et al.* 2012).

Plant population dynamics depend on factors such as flowering, recruitment, growth rate, mortality, disturbance and stress. In a fire-prone mediterranean ecosystem in Africa, the adult and seedling populations of an *Erica* species, age at maturity, longevity, seed production rate, probability of survival of adult and seedlings, and habitat carrying capacity were modelled to determine the changes in population size of seeders and resprouters under different fire frequency and drought intensity (Ojeda *et al.* 2005). The authors found that seeders will only successfully invade and outnumber a resprouter population under a mild mediterranean climate with moderate summer drought. An average 20-year fire interval also favours domination by seeders than an average 15-year fire interval by reducing the chance of consecutive fires that are detrimental to adult seeders and seedling recruitment. Extreme events worldwide, including in Australia, are predicted to be more frequent and intense and include wild fire (CSIRO and BOM 2010; IPCC 2012). This kind of simulation modelling is species-specific and is usually undertaken at a regional or local level.

Fitting data of a specific ecosystem process, such as change in flowering dates per degree of warming, to statistical models is another approach. A recent study compared the observed long-term flowering and leafing dates of 1,558 plant species and another 115 species were experimentally investigated across biomes (Wolkovich *et al.* 2012). While it is generally agreed that global warming will induce early flowering and leafing, experimental studies consistently predict a smaller shift or sometimes a shift to later flowering. Observational studies, however, conclude a greater shift to much earlier flowering times. A projected increase in the number of

warm nights and hot days in inland Queensland, lower mean annual rainfall, higher intensity of extreme rainfall and less frequent rainfall will likely alter the phenology of *A. aneura,* which is common in south-east and north-west Queensland (Hodgkinson 1979; OCC 2008). Flowering of *A. aneura* is opportunistic after rainfall while leafing is in late summer and autumn only if there is sufficient rain. Likewise, flowering of *A. peuce* was observed sometimes after rain (Luly *et al.* 2010). Changes in the rainfall frequency alone may have less effect on the leafing of inland *A. murrayana* which occurs after spring flowering irrespective of rainfall (Hodgkinson 1979).

Many species-specific studies have also investigated or reviewed the effect of elevated CO_2, changing precipitation and temperature on plants' physiological traits such as net assimilation rate, water use efficiency and stomatal conductance (Nativ *et al.* 1999; Loveys *et al.* 2002; Lewis *et al.* 2004), as well as on morphological changes and biomass allocation such as changes in leaf mass ratio (Atkin *et al.* 1999). Some studies have focused on interested in the drought stress caused by El Nino extreme events and on linking dieback to physiological causes such as *in situ* embolism within xylem (Rice *et al.* 2004). These researchers employ statistical models such as Analysis of Variance and Regression models to quantify effects of environmental changes on specific plant species. Most laboratory and glasshouse experiments, and field experiments or surveys are of local, regional or at most country level. Data from multiple studies are sometimes pooled for a meta-analysis though some careful screening and interpretation is necessary and methodological differences or temporal differences between studies should be considered. Some generalized predictions of impacts of climate change on a particular habitat type, such as tropical forest or a group of species, such as species adapted to wet or dry environments, is possible through systematic review or meta-analysis. The outputs can inform local or regional management authority. Potentially such outputs, if they involve changes in vegetation communities, can be compared with specific grid results of the global climate-vegetation simulation model.

1.4.2 A comparative approach using statistical models adopted in this study

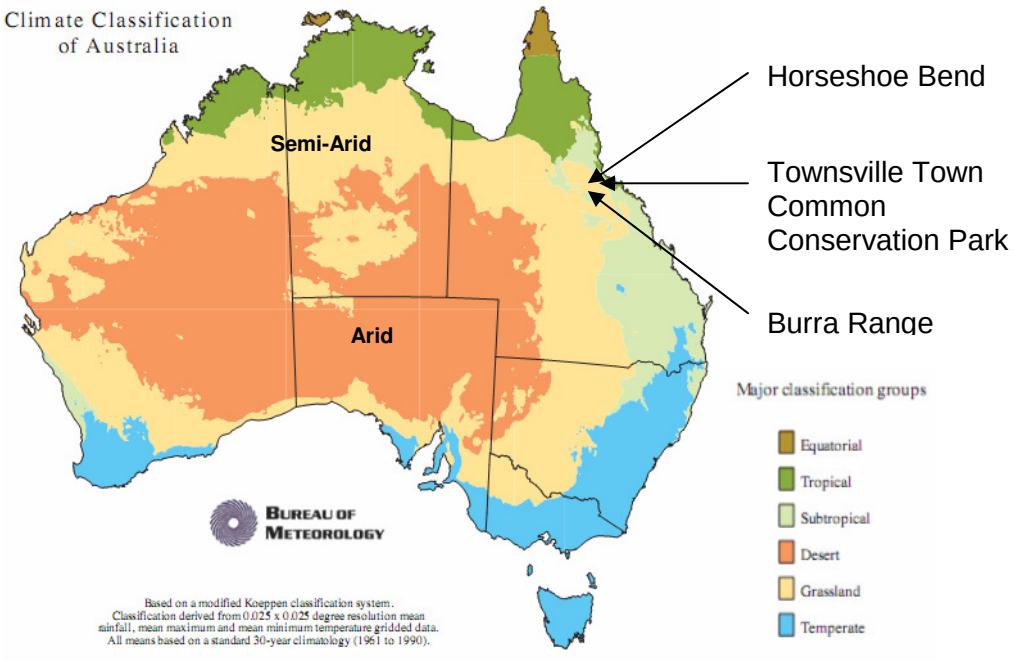
This study compared the growth and nitrogen fixation performance of inland and coastal tropical *Acacias* using traditional statistical models. Computer simulation was not undertaken. Since climate change effects can differ between species, and there are more than 300 000 described plant species worldwide (Hilton-Taylor *et al.* 2008), impact prediction is a daunting task. In Australia alone, there are more than 24 000 described plant species (Chapman 2009). It is thus useful to group species according to their associated climate or habitat in response to which they may have developed adaptive traits. For example, many *Acacia* species commonly

found along the Australian east coast have a fast growth rate while many arid or semi-arid species grow slowly (Atkin *et al.* 1999). Slower maximum growth rate could be an adaptive trait in response to decreasing precipitation. This study also follows this comparative approach because change in climate such as rainfall will likely vary spatially (Section 1.3).

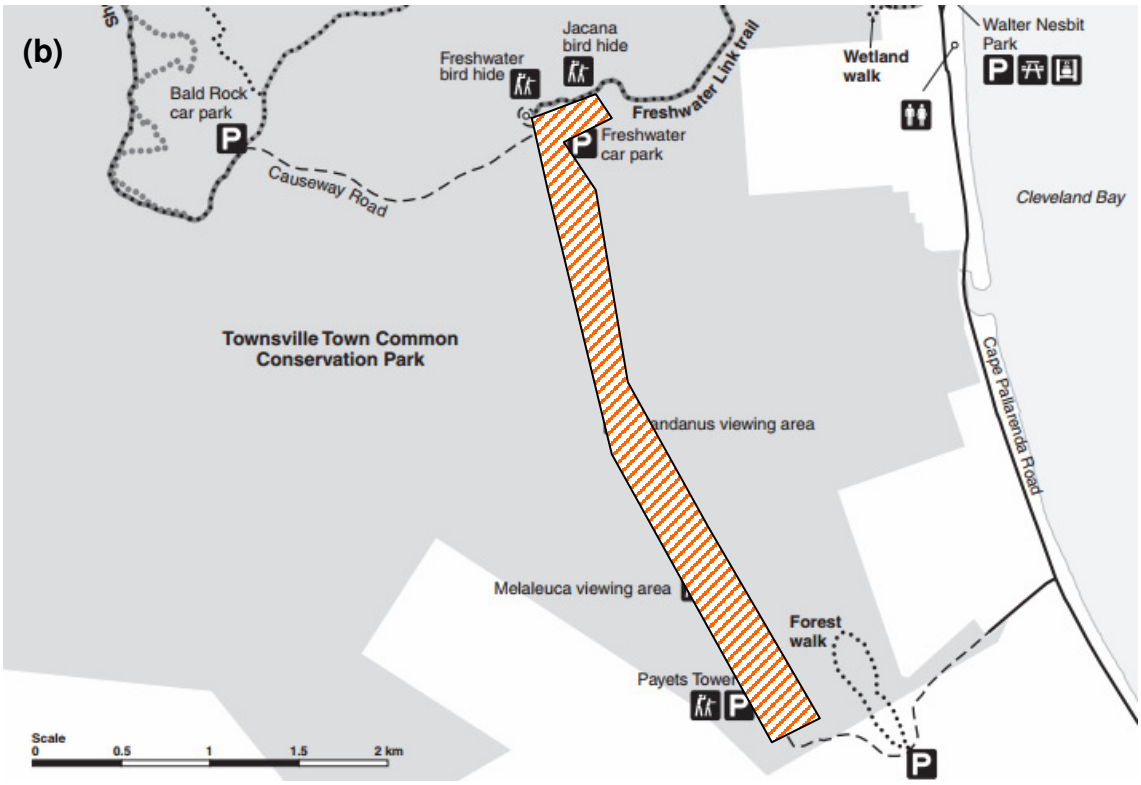
In this study, inland and coastal species were chosen in order to compare the nodulation patterns, nitrogen fixation rates and growth under different climatic conditions. Temperature, rainfall, and soil properties have important effects on these biological properties. Their distributions reflect markedly contrasting climate (especially rainfall) to which these species have adapted. Coastal species receive mean annual rainfall of 800 mm to more than 2 000 mm. Inland species occur within areas receiving 800 mm or less in the north, and 500 mm or less in the east of Queensland. Inland areas have a greater diurnal temperature range and more days with temperatures in excess of 40^{o}C in the wet season and less than 2^{o}C in the dry season. This concept of coastal / inland species is developed by reference to the concept adopted by the CSIRO Center of Arid Zone Research (CAZR). CAZR defines semi-arid species as those receiving 400 to 800 mm mean annual rainfall in the north and 300 to 500 mm in the east, while arid species only receive less than 300 mm on average each year (CAZR 2005). This division roughly follows a modified Koeppen classification (Fig. 1.2a). In the Koeppen classification, dry areas are divided into "grassland" (semi-arid areas) and "desert" (arid areas). Such "habitat" terms imply that the two climatic zones support two different assemblages of vegetation that have adapted to two different sets of mean annual rainfall, rainfall seasonality and mean annual temperature (Stern *et al.* 2000).

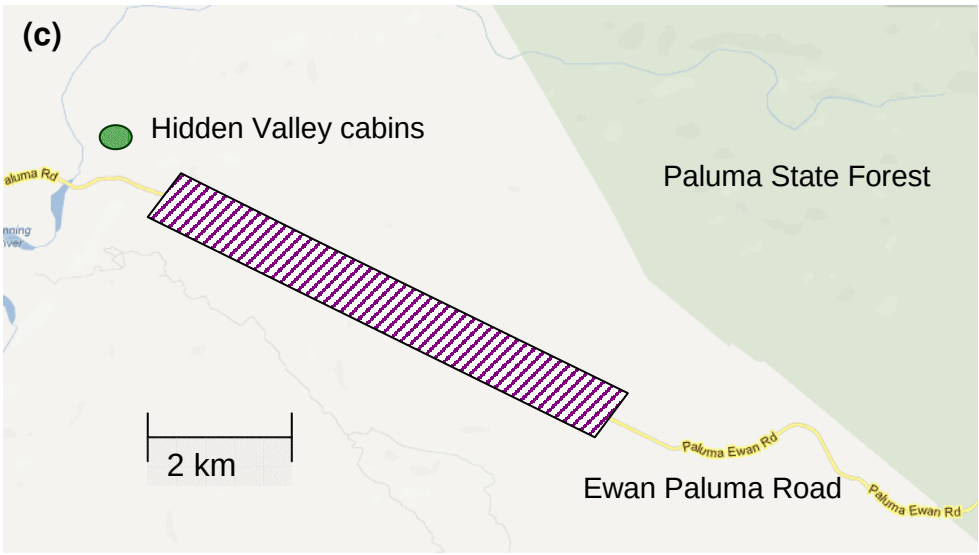
Arid zones have also been defined as having a mean annual rainfall of 250 mm or less (DKCRC 2009; BOM 2012a), while semi-arid zones only receive 250 to 350 mm every year on average (DKCRC 2009). Alternatively, the semi-arid and arid zones can be defined using a moisture index, i.e. the ratio of rainfall to evaporation rate. The moisture index of arid and semi-arid zones are respectively less than 0.2 and 0.2 to 0.4 (DKCRC 2009). Hence the Desert Knowledge Cooperative Research Centre's definitions of semi-arid and arid zones are more inland than the zones resulting from Koeppen's and CAZR's definitions.

(a)


Climate Classification of Australia
Semi-Arid
Arid
Horseshoe Bend
Townsville Town Common Conservation Park
Burra Range
Major classification groups
Equatorial
Tropical
Subtropical
Desert
Grassland
Temperate
Bureau of Meteorology
Based on a modified Koeppen classification system.
Classification derived from 0.025 x 0.025 degree resolution mean rainfall, mean maximum and mean minimum temperature gridded data.
All means based on a standard 30-year climatology (1961 to 1990).

(b)


Bald Rock car park
Causeway Road
Freshwater bird hide
Jacana bird hide
Freshwater Link trail
Freshwater car park
Wetland walk
Walter Nesbit Park
Cleveland Bay
Townsville Town Common Conservation Park
Cape Pallarenda Road
Melaleuca viewing area
Forest walk
Payets Tower
Scale
0
0.5
1
1.5
2 km



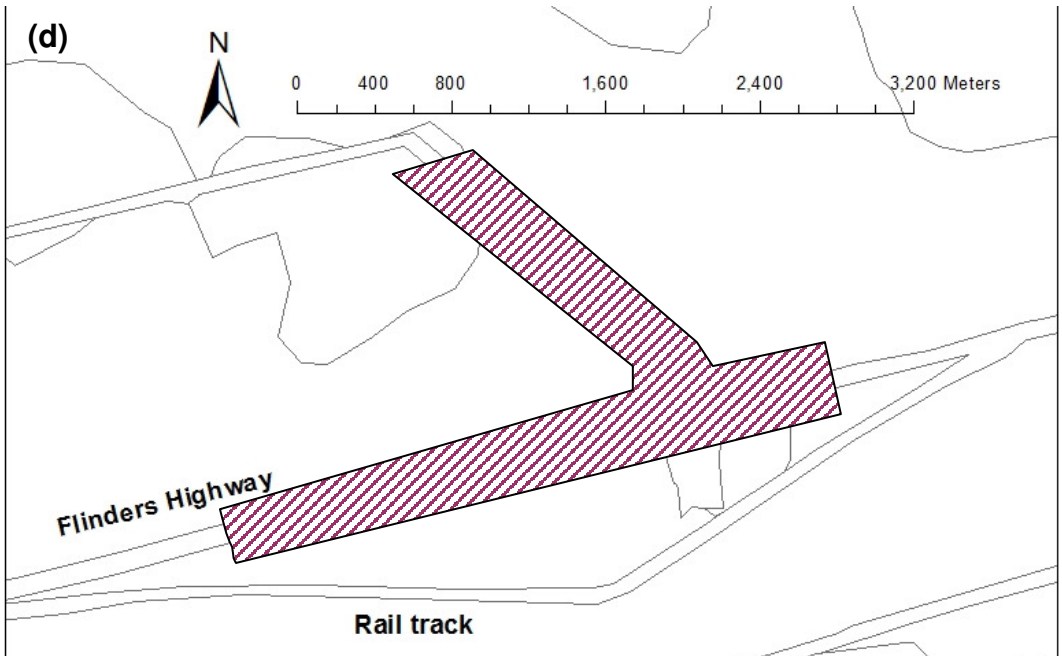


Figure 1.2. (a) Arid and semi-arid climatic zones as defined by Koeppen (Stern *et al.* 2000; DKCRC 2009) and marked with the locations of the three study sites. (b-d) Sampling area highlighted as hatched polygons: (b) Town Common Conservation Park, (c) Horse Shoe bend near Paluma and (d) Burra Range in White Mountain National Park.

1.5 Site descriptions

The roots, nodules, soil and seeds of *Acacias* were sampled from three study sties: the Townsville Town Common Conservation Park (TTCCP), Horseshoe Bend near Paluma and Burra Range within White Mountain National Park (Table 1.3) (Fig. 1.2) . All three sites display

distinct seasonality so that they have a hot wet summer and cool dry winter. However, seasonality is more pronounced with distance from the coast. The index used to demonstrate this change is the proportion of the annual rainfall falling within the wet season and inter-annual coefficient of variation of mean annual rainfall (Table 1.3).

The TTCCP was previously used for cattle but has been protected from grazing for 30 years. The western fringe is a wetland and the eastern section is a mosaic of *Eucalyptus* (open) woodland and *Eucalyptus* open forest. The wetland area has since diminished and a large area of the Town Common is now covered in para grass (*Urochloa mutica*) (Grice 2004). *A. crassicarpa* and *A. holosericea* grow in the eastern section, on siliceous sand with low water holding capacity (Table 1.4) (Fig. 1.3). The TTCCP has a mean annual temperature (MAT) of 24.6^{o}C and mean annual rainfall (MAR) of 1 150 mm. The rainfall to evaporation ratio is 0.43. The nutrient level is considered too low for agriculture (Murtha and Reid 1992) (Table 1.4). The mean total nitrogen of soil under the canopy of *A. crassicarpa* ranges from 0.69 to 0.84 mg N g^{-1} soil and is the highest among the three study sites.

Horseshoe Bend (HSB) is about 20 km west of Paluma, on the road to Hidden Valley. Mixed *Eucalypt* woodland occurs in the area. At 650 m above sea level, HSB is cooler than both the TTCCP and the Burra Range, with a MAT of 20.5^{o}C (Table 1.3). About 850 mm of rain falls annually, which is just between the MAR of TTCCP and the Burra Range. The HSB study site is more undulating than TTCCP, and is dominated by a rocky slope. Most individuals of the shrubby *A. aulacocarpa* are found near a seasonal creek that runs at the bottom of this rocky slope (Fig. 1.3). *A. aulacocarpa* sometimes grows in clusters, but more often they grow in between *Eucalypt* trees. The sandy clay loam soil in HSB contains a higher proportion of clay and silt particles compared with the siliceous sand of the TTCCP, due to its proximity to the seasonal creek (Table 1.4). The mean total nitrogen of soil under the canopy of *A. aulacocarpa* ranges from 0.52 to 0.67 mg N g^{-1} soil and is the second highest among the three study sites (Table 1.3).

The Burra Range is covered by *Eucalyptus similis* and *E. whitei* woodland, and low open forests (DERM 2010) (Table 1.3). Field sampling, as well as soil and seed collection, like that practised in the other two sites, were conducted within 200 m from the main road. The soils are distinct red and grey earths and are nutrient-depleted (Table 1.4). The Burra Range has the lowest mean annual rainfall among the sites (610 mm). Most *Acacia* species, including *A. ramiflora* and *A. elachantha*, found at the Burra Range site were shrubs or small trees, often not exceeding 6 to 7 m (Fig. 1.3). The mean total nitrogen of soil under the canopy of *A. ramiflora and elachantha* ranges from 0.23 to 0.43 mg N g^{-1} soil and is the lowest among the three study sites.

Figure 1.3. Photos showing the growth habits of *A. crassicarpa*, *A. aulacocarpa*, *A. elachantha* and *A. ramiflora* in their natural habitats (from left to right). The author's height is 1.8 m. Note that not every tree/shrub in the field is of the same height.

Table 1.3. Location, altitude, vegetation communities and climate of field sites surveyed

MAR, mean annual rainfall; MAT, mean annual temperature; COV, coefficient of variation; Moisture Index was defined as the mean annual rainfall divided by the mean annual evaporation; Total N, Total P, pH and Conductivity values are mean ± 1 S.E.M. (number of samples).

Species	Location	Altitude (m)	Vegetation community	Climate[C,D]								
				MAR (mm)	% of MAR between Dec and Mar	Inter-annual COV of MAR (%)	Moisture Index	MAT (°C)[F]	Decile 9 max. temp. at Dec (°C)	Decile 1 min. temp. in July (°C)	Mean number of days > 40 °C in Dec	Mean number of days < 2 °C in July
Acacia crassicarpa	Town Common Conservation Park in Townsville, QLD (19° 12' S, 146° 45' E)	4	Eucalypt (open) woodland[A] Open forest[A]	1 150	78.4	41.4	0.43	24.6	33.7	8.5	0	0
Acacia aulacocarpa	Horseshoe Bend near Paluma, QLD (18° 58' S, 146° 02' E)	650	Eucalypt mixed woodland[B]	848	77.0	35.8	0.38	20.5	---[E]	---[E]	---[E]	---[E]
Acacia elachantha and *Acacia ramiflora*	Burra Range within White Mountain National Park, QLD (20° 43' S, 145° 10' E)	540 - 548.6	*Eucalyptus similis* and *E. whitei* woodland[B] Low open forest[B]	607	71.0	44.3	0.25	23	38.3	5.55	2.1	0.35

[A] Murtha (1975)

[B] DERM (2010)

[C] Since no weather station has been established in the Burra Range, climate in the Burra Range was interpolated as the average of the nearest stations (rainfall from Pentland Post Office and Torrens Creek Post Office; temperature from Charters Tower Airport and Hughenden Post Office).

[D] Unless stated otherwise, all of the above climate statistics were pooled from all years of data available. *Source: Bureau of Meteorology website (www.bom.gov.au)*

[E] Only rainfall data was available from the weather station at Horseshoe Bend station.

[F] Annual mean temperatures were extracted from BIOCLIM GIS layer.

Table 1.4. Soil properties of field sites surveyed

GSG, great soil group (Stace *et al.* 1968); PPF, principal profile form (Northcote 1979); Total N, Total P, pH and Conductivity values are mean ± 1 S.E.M. Parentheses enclose the number of samples. Asterisks indicate significant differences of the associated variable between wet and dry seasons for a particular species. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS – $P > 0.05$.

Species	GSG	PPF	Field texture[D]	Total N under canopy (mg N g^{-1} soil)			Total P under canopy (µg P g^{-1} soil)			pH under canopy			Conductivity under canopy (ds m^{-1})		
				Wet season	Dry season		Wet season	Dry season		Wet season	Dry season		Wet season	Dry season	
Acacia crassicarpa	Siliceous sand[A]	Uc1.22[A]	Loamy sand	0.69 ±0.03 (46)	0.84 ± 0.05 (28)	**	107 ± 5 (46)	126 ± 12 (28)	*	5.24 ± 0.06 (42)	5.53 ± 0.07 (24)	**	0.07 ± 0.006 (42)	0.20 ± 0.07 (24)	***
Acacia aulacocarpa	---	Uc2.21[B]	Sandy clay loam	0.52 ± 0.03 (48)	0.67 ± 0.04 (24)	***	55 ± 3 (48)	67 ± 4 (24)	NS	5.74 ± 0.08 (24)	5.61 ± 0.10 (14)	NS	0.04 ± 0.003 (24)	0.08 ± 0.01 (14)	***
Acacia elachantha and *Acacia ramiflora*	Red earths and Grey earths	Gn2.11[C] Gn2.61[C]	Clay loam	*A. elachantha* 0.23 ± 0.02 (40)	*A. elachantha* 0.43 ± 0.03 (24)	***	*A. elachantha* 51 ± 4 (40)	*A. elachantha* 80 ± 4 (24)	**	*A. elachantha* 5.67 ± 0.11 (11)	*A. elachantha* 6.35 ± 0.15 (6)	***	*A. elachantha* 0.04 ± 0.01 (11)	*A. elachantha* 0.10 ± 0.03 (6)	***
				A. ramiflora 0.34 ± 0.03 (19)	*A. ramiflora* 0.33 ± 0.01 (31)	NS	*A.ramiflora* 32 ± 4 (20)	*A. ramiflora* 98 ± 15 (31)	***	*A. ramiflora* 5.72 ± 0.08 (24)	*A. ramiflora* 5.70 ± 0.15 (6)	NS	*A. ramiflora* 0.04 ± 0.003 (24)	*A. ramiflora* 0.06 ± 0.004 (6)	***

[A] Murtha and Reid (1992)

[B] Uc 1.22 and Uc2.21 refers to uniform soil profile with coarse soil texture. Soil accumulates little organic matter, is not calcareous and has a Munsell color chart value/chroma rating of 4 (Isbell and Murtha 1970). Uc2.21 refers to deep sands and sandy loams with a bleached A_2 horizon (Isbell and Murtha 1970).

[C] Gn2.11 and Gn2.61 refer to gradational soil profile. Soil of Gn2.11 is deep (5 to more than 36 m), loamy red earths with clay nodules, and often with ironstone nodules and siliceous pebbles at depth (Coventry 1982). Soil of Gn2.61 is shallow grey earths (0.8 to 3 m). Its texture is loamy or sandy and abundant in ironstone nodules and siliceous pebbles throughout the solum (Coventry 1982)

[D] Tested according to MacDonald *et al* (1990)

1.6 Aims and thesis structure

This thesis examines symbiotic nitrogen fixation of two tropical inland and two coastal *Acacia* species in relation to soil properties, tree size, source of bacteria symbionts and drought. The two-year study aims to expand the knowledge regarding the ecology of tropical *Acacias* and how climate change will affect their ecology. Such basic information is vital to planning adaptation activities. Knowledge about plant nitrogen nutrition and nitrogen cycling in an ecosystem is also important to increasing revegetation success.

This thesis is divided into six chapters. Chapter One presents an overview of the tropical savanna habitat, tropical *Acacias*, nitrogen cycling, effect of climate change on plant ecology and a description of all three study sites. Chapter Two examines the effect of abiotic factors, such as tree size, seasons, soil depth and soil nutrients, on the nodulation and symbiotic effectiveness of mature trees and shrubs of the four tropical *Acacia* species. In Chapter Three, the factors affecting seed germination of inland and coastal *Acacias* are compared. The source of soil inocula in the nodulation and symbiotic effectveness of tropical *Acacias* seedlings are examined in Chapter Four. In Chapter Five, the effect of drought on the growth, nodulation, and traits related to morphology and physiology of tropical *Acacias* seedlings are investigated. The background knowledge of individual topics is introduced in Chapters Two to Five. Chapter Six summarises the findings of all four experimental studies, discusses the implications of the results in the context of climate change impact and adaptation, and presents future research directions.

The thesis was formatted with reference to guidelines laid out in the "Notice to Authors" of the Australian Journal of Botany.

2 Abiotic factors affecting the nodulation and nitrogen fixation of tropical *Acacia* species

2.1 Introduction

Symbiotic nitrogen fixation is an important part of nitrogen cycling. It is the main source of nitrogen input into tropical savannas in which nitrogen loss through dry season fire and wet season leaching can be substantial in the short-term (Holt and Coventry 1990; Laclau *et al.* 2005; Richards *et al.* 2012). Considering that tropical savannas cover one-fifth of the World's land surface and one-fourth of Australia (Schmidt and Lamble 2002), a thorough understanding of abiotic factors controlling nodulation and nitrogen fixation of legumes and actinorhizal plants is essential. Such knowledge serves as the basis for deducing potential climate change effects. Climatic seasonality and edaphic factors can interact and provide feedback to plants' growth and nitrogen nutrition. This chapter therefore concerns environmental control of nitrogen fixation by *Acacia*, a genus with 1017 species in Australia (Thiele *et al.* 2011). I will discuss key factors affecting nodulation, followed by a review of the use of the ^{15}N isotope to quantify symbiotic nitrogen fixation. This chapter describes a field study that examined nodule production in two inland and two coastal *Acacia* species in the wet and dry seasons. It is hypothesized that inland species will be more adapted to a more hostile climate, such as lower rainfall and higher temperatures, by having fewer root nodules. Logically inland *Acacias* should also rely less on symbiotic nitrogen fixation to obtain nitrogen, compared with direct or mycorrhizal uptake from soil.

2.1.1 Temperature

It is generally agreed that nitrogen fixation by either symbiotic or free-living bacteria is sensitive to temperature because this enzymatic reaction incurs substantial carbon cost and has a close relationsip with temperature-dependent phtosynthesis (Dixon and Wheeler 1986; Sprent 2009). A recent global meta-study concluded that the activity of the nitrogenase, the nitrogen-fixing enzyme, was optimal at 25.2^{o}C (Houlton *et al.* 2008). In a controlled environment using quartz sand, a soil temperature of 35^{o}C was found to be optimal for effective nodulation in *A. mellifera* in semi-arid to arid Sudan, compared to 30^{o}C and 40^{o}C (Habish 1970). The effectiveness of nitrogen fixation of *A. pellita,* measured by acetylene reduction method, in a restored quarry site in the Northern Territory increased from 0 to about 20 moles of ethylene per

mg of dry nodule per hour when the field temperature increased from 0°C to 22°C (Langkamp *et al.* 1979). With a further temperature rise to 36°C, nitrogen fixation effectiveness increased steadily, then levelled off and remained constant. Photosynthetically active radiation (PAR) interacted with soil temperature in affecting the diurnal nitrogenase activity of root nodules. The highest amount of ethylene produced by *A. pellita*, was in the midday, when the soil temperature was 32°C and PAR was 2550 µmol m^{-2} s^{-1} (Langkamp *et al.* 1979).

In a growth-cabinet experiment, *A. senegal* seedlings were subjected to heat stress of 36, 38, 40 and 42°C for 10 hours per day for 0, 3, 7, 10 and 14 days (Rasanen and Lindstrom 1999). Infection threads were seen at day 3 and nodules started appearing at day 7 across all experimental temperatures. At 30°C (control) and 36°C soil temperature, the number of nodules at day 7 was about 10; at day 10, the number is almost doubled. At 38 °C soil temperature, the number of nodules was also about 10 at day 7 but did not increase further at day 10. The number of nodules at 40°C was less than 20% and 50% of that at 38°C at day 7 and 10 respectively. At 42°C, root hairs were deformed and infection threads disintegrated. This suggests that at soil temperatures of 36°C or above, temperature is negatively correlated with nodulation. Interestingly, the number of cultured rhizobia under 42 and 30°C was similar, although the number of dry and possibly inactive colonies increased slightly with the length of heat stress. Thus the lack of nodulation cannot be attributed to an insufficient number of rhizobia. In the same experiment, seedlings also had a lower height at 42°C than at 30°C but their shoots were of similar weights. It is uncertain if heat stress reduces seedlings' growth directly or through a negative effect on nodulation and hence nitrogen nutrition.

Apparently, the number of rhizobia (symbiotic bacteria known to infect *Acacia* roots) is high in the rhizosphere when soil temperature is optimal. In a 3-year old stand of lucerne (*Sinorhizobium meliloti*), soil at 50 cm deep had a higher temperature than soil that at 10 cm deep but was still within the optimal range (Evans *et al.* 2005). The number of rhizobia at 30 to 60 cm soil depth was three orders of magnitude higher than at 0 to 15 cm and at 15 to 30 cm soil depths.

2.1.2 Soil moisture

Adequate soil moisture is essential for nodulation and effective nitrogen fixation by *Acacia*. *A. mellifera* forced to rely on nodulation to acquire nitrogen had higher root and shoot biomass at 15% soil moisture, compared to 7.5% and 22.5% soil moisture (Habish 1970). The number and biomass of nodules at the optimum soil moisture were also found to be higher in that study.

When provided with adequate ammonium nitrate, no nodules were found and thus the nutrition strategy switched to direct uptake from soil. In this case, the biomass of roots and shoots was higher at 22.5% soil moisture, compared to 7.5% and 15%. Even at 15% soil moisture, the biomass of plants absorbing ammonium nitrate was higher than the biomass of plants forced to rely on symbiotically fixed nitrogen. Plant biomass is therefore affected by the interaction between nutrition strategy and soil moisture.

A. holosericea in the Northern Territory fixes the highest amount of nitrogen per nodule weight per hour during the hottest month with moderate rainfall or during the two to three months following the peak temperature with peak rainfall (Langkamp *et al.* 1982). *A. mearnsii*, *A. melanoxylon*, *A. paradoxa*, *A. oxycedrus* and *A. longifolia var. sophorae* near Melbourne are known to exhibit maximum acetylene reduction activities in the early and late wet season when air temperature is about five degrees lower than the maximum and when precipitation is at its sub-peak level (Lawrie 1981). These studies indicate that soil moisture and temperature interact to influence the rate of symbiotic nitrogen fixation. Nodule abundance of 0.05 to 11.981 kg nodule WW ha^{-1} is recorded from April to July among the several *Acacia* species mentioned above. Field observations suggest that this number is much lower than that in summer (Lawrie 1981). The number is also lower than that of *A. holosericea* recorded by Langkamp *et al* (1982) (19.3 kg nodule WW ha^{-1}) due to differences in the abundance of the host tree.

2.1.3 Host plant size

Larger trees have a greater absolute nitrogen demand which can sometimes drive symbiotic nitrogen fixation. For instance, a positive correlation was found between the above ground dry matter and the acetylene reduction rate per plant in *Prosopis glandulosa* seedlings (Felker and Clark 1980). However, higher nitrogen demand does not necessarily mean a higher symbiotic nitrogen fixation or direct uptake from the soil. Some of the nitrogen requirement may be met through nitrogen resorption. About 50% of the foliar nitrogen can be resorbed during sensecence (Aerts 1996). However, larger trees can have a greater nodule biomass. In a naturally regenerated stand of *Alnus hirsuta* var. *sibirica* in Japan, nodule density at 0 to 20 cm away from the trunk was negatively correlated with tree size ($P < 0.05$) (Tobita *et al.* 2010). The correlation became positive at 20 to 100 cm away from the trunk but was not statistically significant. Nodule dry weight per unit area had a similar correlation with tree size as did nodule density. However, the correlation was significant when nodules were further away from the trunk but not when nodules are close to the trunk. Total nodule biomass per tree significantly

increased with tree size. Thus as *Alnus* trees get larger, the majority of nodule biomass grows further away from the trunk.

2.1.4 Soil texture and bulk density

Soil texture affects the distribution of vegetation communities in Australia as was shown by Beadle (1981) who observed that, in a landscape made up of different habitats, eucalypt communities sometimes abruptly change to tussock grassland when sandy soil is replaced by clayey soil. Clayey soil has higher soil strength and smaller pore size, thus posing high resistance to fine root penetration (Brady and Weil 2010). It should also be noted that the imbibitional forces between clay particles reduce plant-available water. Water-logging is also common with inland cracking clay after rainfall (Leeper and Uren 1993). Not many eucalypts and *Acacias* are adapted to the large seasonal fluctuations in soil moisture in clayey soil. *Eucalyptus coolabah, Acacia cambagei, A. harpophylla, A. nilotica* and *A. victoriae* are some of the most common species living on cracking clay in inland Queensland (Pers. observ. 2010). Clay's low oxygen level under water-logged conditions may present a challenge to any root nodule development.

Soil bulk density is the dry weight of a given soil volume and hence is a measure of soil compactness. Higher bulk densities may make fine root and nodule development difficult as does more clayey soil texture. Compact soil has smaller and fewer macropores so water infiltration is reduced (Congdon and Herbohn 1993). Sandy soil has higher bulk density than silty or loamy soil because it has less pores within peds and so is denser per ped (Brady and Weil 2010). Logging operations for two years in a tropical rainforest severely compacted clay soil (Congdon and Herbohn 1993). The bulk density of compact soil remained higher than surrounding undisturbed soil for 30 years.

2.1.5 Ageing

Trees that are a few years old may rely proportionally less on symbiotic nitrogen fixation than do newly planted trees but the absolute amount of symbiotically-fixed nitrogen can be similar (Danso *et al.* 1992; Isaac *et al.* 2011; Mercado *et al.* 2011). Many studies attribute this observation to the higher plant-available nitrogen in soil under older trees. This reason was suggested regarding patterns observed in *A. senegal* and *Leucaena leucocephala* (Parrotta *et al.* 1996; Isaac *et al.* 2011). The nutrient-rich foliage and dead fine roots are mineralized into NH_4^+

which can be nitrified to NO_3^-. These two plant-available minerals can be readily absorbed, thus reducing the relative need for carbon-costly symbiotic nitrogen fixation.

2.1.6 Nutrients – nitrogen and phosphorus

The effect of soil fertility on nitrogen fixation are well discussed, in particular the inhibiting effect of increasing soil nitrogen and the positive effect of more soil phosphorus (Brockwell *et al.* 2005). The physiological reasons behind this remain unclear; it is suggested that less energy should be allocated to nitrogen fixation when soil becomes the main source of ammonium and/or nitrate. But whether nitrogen fixation rate increases or decreases depends on the tested species and the forms of nitrogen available. For example, providing urea to *A. nilotica* caused a decrease in nitrogenase activity but did not affect nodule abundance (Toky et al, 1994). *A. auriculiformis* has a higher nodule abundance and growth rate when soil ammonium level increases but both are lowered by increased soil nitrate (Goi *et al.* 1993).

Higher soil phosphorus can result in higher nodule abundance and higher nitrogen fixation rate of woody nitrogen-fixing plants which generally have a high phosphorus-demanding lifestyle (Vitousek *et al.* 2002; Brockwell *et al.* 2005). There are two possible explanations – either nitrogen fixing plants have a higher demand for phosphorus, thus requiring a nitrogen-demanding lifestyle in parallel, or the nitrogen fixing (N-fixing) process requires a large amount of energy that drives up the phosphorus requirement of the species (Vitousek *et al.* 2002). A study of the effects of various levels of phosphorus on nitrogenase activity and nodule abundance of two different genotypes of *Acacia mangium*, Ma9 and Ma 11, established that that adding phosphorus does not always positively affect the growth, nodulation and nitrogenase activity. From sub-optimal to optimal soil phosphorus levels, individuals of both genotypes grown separately showed increases in the nodule number and overall nitrogenase activity per plant (Sun *et al.* 1992). From optimal to supra-optimal P, genotype Ma9 had an overall reduction in nitrogenase activity while genotype Ma11 had the opposite. As soil phosphorus was increased beyond the optimal level, genotype Ma9 experienced a decrease in overall plant dry weight possibly because of phosphorus toxicity, while the overall dry weight of genotype Ma11 remained constant (Sun *et al.* 1992). Such results indicate that increased nitrogenase activity does not necessarily result in greater shoot and root growth.

When grown in intensively aerated hydroponic solution for 36 weeks in a glasshouse, the whole plant production of *A. mangium*, in terms of total dry weight, increased with phosphorus and plateaued at 100 µmol KH_2PO_4 per plant per week (Ribet and Drevon 1996). Thus 0 to 100 and

100 to 300 µmol KH_2PO_4 per plant per week are sub-optimal and supra-optimal levels respectively for the growth of *A. mangium*. Low phosphorus levels resulted in small shoot to root ratio of N-fixing *A. mangium*. Nitrogenase activity per unit dry weight of nodules is high at low phosphorus and the shoot production per unit dry weight of nodules roughly corresponds to this. This pattern was supported by a recent review of the physiological responses of legumes under climate change (Rogers *et al.* 2009). But the total number, and hence the total weight of nodules of *A. mangium* in Ribet and Drevon's experiment are relatively low. Therefore one can conclude that the total amount of nitrogen fixed is inadequate to support high total shoot production. More resources are allocated to root production to maximise possible uptake of the limiting phosphorus supply instead.

In examining the relationship between nodule development and plant biomass, parameters such as nodule dry weight, total dry weight of the plant, and shoot and root dry weight should be measured to understand how different species respond to changing nutrient supply in terms of resource allocation to aboveground and belowground parts.

A positive effect of limiting phosphorus on nitrogen fixation and plant growth has also been recorded for other nitrogen fixing woody legumes such as *Facaltaria* (Family: Fabaceae) (Binkley *et al.* 2003).

2.1.7 The use of ^{15}N natural abundance to study nitrogen cycling

Soil has a higher ratio of ^{15}N to ^{14}N than that of air (Shearer and Kohl 1986). $\delta^{15}N$ is the excess of concentration of ^{15}N of a particular sample over the concentration of ^{15}N in the air which is 0.3663 atom %, a global constant (Shearer and Kohl 1986; Andrews *et al.* 2011). Theoretically, when plants absorb nitrogen, their $\delta^{15}N_{foliage}$ should equal or closely follow $\delta^{15}N_{source}$. For example, in western mulga soil where *A. anuera* grows, $\delta^{15}{}_{soil}$ ranges from 8.1 to 14.9‰. Those non-N-fixers growing on such soil have $\delta^{15}{}_{shoot}$ ranging from 4.62 to 15.49‰ (Table 2.1). The ranges of $\delta^{15}{}_{soil}$ and $\delta^{15}{}_{shoot}$ clearly overlap in this case. N-fixers, however, often have lower $\delta^{15}N_{foliage}$ or $\delta^{15}N_{shoot}$ than $\delta^{15}N_{soil}$ because they can take up nitrogen from both air and soil. The nitrogen fixing plants on western and soft mulga soils, for instance, have $\delta^{15}N_{shoot}$ values of 7.38 to 9.62‰ and 2.31 to 3.03‰ respectively (Table 2.1). Note that the maximum $\delta^{15}N_{shoot}$ are less positive than $\delta^{15}N_{soil}$ of their corresponding soil.

The percentage of N derived from the atmosphere can be calculated from equation 2.1 below:

$$\% \text{ Ndfa} = \frac{\delta^{15}N_R - \delta^{15}N_F}{\delta^{15}N_R - \delta^{15}N_A} \quad \text{..... (2.1)}$$

(Shearer and Kohl 1986; Kreibich 2002)

$\delta^{15}N_F$ is the natural abundance of ^{15}N of nitrogen fixing *Acacia* species taking up both soil N and N_2; $\delta^{15}N_A$ is the natural abundance of ^{15}N of nitrogen fixing *Acacia* species forced to rely on N_2 as their sole source of nitrogen; $\delta^{15}N_R$ is the natural abundance of ^{15}N of non-nitrogen fixing reference plants.

Certain assumptions have to be made when %Ndfa is quantified using this equation (Shearer and Kohl 1986):

- There is a significant difference in $\delta^{15}N$ between $\delta^{15}N_A$ and $\delta^{15}N_R$.
- Variation of $\delta^{15}N$ among reference plants is small compared to the variation between reference plants and hydroponically grown N-fixers.
- That $\delta^{15}N$ of the tissue sampled represents that of the entire plant, or that the $\delta^{15}N$ values of tissues sampled from N_2 fixing and reference plants deviate from those of the whole plant to the same degree. This is because isotopic fractionation can result from N metabolism, followed by transport of N from one tissue to another. In general, variation of $\delta^{15}N$ between plant parts is within 2‰, except nodules of certain plants.
- Isotopic fractionation associated with N soil uptake is the same in both N-fixers and reference plants.
- $\delta^{15}N$ values of soil around N-fixers are the same as that around reference plants. This means that both type of plants absorb N from a similar soil depth at similar times and need to be growing near each other, so that any variation of soil $\delta^{15}N$ will have the same effect on both plant types. They should also have similar root system type and similar phenology.

Soil nitrogen comprises different inorganic and organic forms. The discrepancy between $\delta^{15}N_{foliage}$ and $\delta^{15}N_{source}$ is affected by the type(s) of nitrogen taken up, the relative proportion(s) and the $\delta^{15}N$ of the source(s) (Shearer and Kohl 1986; Evans 2001; Houlton *et al.* 2007). Different types of nitrogen can have different $\delta^{15}N$, due to discrimination against heavier ^{15}N isotope in soil processes such as mineralization, ammonification, nitrification and denitrification

(Shearer and Kohl 1986). Should most soil ammonium be nitrified, soil ammonium will be ^{15}N-enriched and nitrate will be ^{15}N-depleted. Soil around *A. aneura* (mulga soil) is highly enriched in ^{15}N and this is likely due to high rates of soil nitrification (Pate *et al.* 1998).

Assimilation can also be a potential source of isotopic fractionation. Its effect, however, is small on $\delta^{15}N_{foliage}$ when plant-available nitrogen supply is low and less than the demand of the plant (Evans 2001). Habitats with low and potentially limiting soil nitrogen supply include mature high-latitude forests (Houlton *et al.* 2008) and Australian savannas (Schmidt and Lamble 2002; Attiwill and Wilson 2003). For plants such as rice, some ^{15}N atoms can be discriminated against during assimilation by roots but no such discrimination is found for tomatoes (Amundson *et al.* 2003). In short, $\delta^{15}N_{foliage}$ is often slightly more depleted than the $\delta^{15}N_{source.}$

Table 2.1. Some examples of soil and woody plant $\delta^{15}N$ in Australia

$\delta^{15}N$ values in bold are putative N-fixers. Other $\delta^{15}N$ values indicate non-N-fixers. Parentheses after $\delta^{15}N$ of foliage samples are standard deviations; The $\delta^{15}N$ of *Acacia* foliage without standard deviation represents one sample only. The range of $\delta^{15}N$ values was provided for shoot samples; those without a range come from one sample. ECM, ectomycorrhizae; AM, vesicular-arbuscular species. MAR, mean annual rainfall

Vegetation type	Location	Climate	MAR (mm)	Temperture range (°C)	$\delta^{15}N_{soil}$ (‰)	Sample and life form		$\delta^{15}N$ (‰)
Eastern mulga[A]	Currawinya National Park, QLD	Cold wet winter, hot dry summer	294	20-36 (max.) 5-25 (min.)	Not provided	New shoot of woody shrubs		3.24 to 14.03
						New shoot of woody trees		**7.89 to 12.09** ; 3.45 to 11.99
Western mulga[A]	Thundelarra Station, WA	Cold wet winter, hot dry summer	262	20-36 (max.) 5-25 (min.)	8.1 to 14.9 (vary with depth)	New shoot of woody shrubs		4.62 to 15.49
						New shoot of woody trees		**7.38 to 9.62** ; 6.38
Soft mulga[A]	Lake Mongers, WA	Cold wet winter, hot dry summer	262	20-36 (max.) 5-25 (min.)	17.5 to 19.7 (vary with depth)	New shoot of woody shrubs		**1.95** ; 6.43 to 10.70
						New shoot of woody trees		**2.31 to 3.03**[C] ; 8.76
Moonsoon forest[B]	Kakadu National Park, NT	Hot wet summer, cold dry winter	1 475	31-37 (max.) 18-25 (min.)	4.7 (0 to 5 cm)	Foliage	- *Acacia (ECM/AM)*	**1**
							- *Eucalyptus (ECM/AM)*	1.7 (0.8)
							- AM species	2.4 (1.1)
Escarpment woodland[B]	Kakadu National Park, NT	Hot wet summer, cold dry winter	1 475	31-37 (max.) 18-25 (min.)	2 (0 to 5 cm)	Foliage	- *Acacia (ECM/AM)*	**-0.1 (0.4)**
							- *Eucalyptus, Allosyncarpia (ECM/AM)*	-0.6 (0.3)
							- AM species	0.6 (1.1)
							- *Grevillea / Persoonia*	4.1 (1.1)
Savanna[B]	Kakadu National Park, NT	Hot wet summer, cold dry winter	1 475	31-37 (max.) 18-25 (min.)	2.5 (0 to 5 cm)	Foliage	- *Acacia (ECM/AM)*	**-0.7 (0.4)**
							- *Eucalyptus (ECM/AM)*	-0.6 (0.6)
							- AM species	0.5 (0.7)
							- *Grevillea / Persoonia*	2.9 (1.0)

[A] (Pate *et al.* 1998) [B] (Schmidt and Stewart 2003) [c] ***A. brachystachya*** is one of the putative fixers on soft mulga but its $\delta^{15}N$ is 9.76‰. Such a relatively high $\delta^{15}N$ value probably reflects that symbiotic nitrogen fixation is relatively less important to nitrogen nutrition of ***A. brachystachya*** than the nutrition of other putative nitrogen fixers.

Since putative N-fixers may obtain a significant proportion of nitrogen from atmospheric nitrogen, their $\delta^{15}N_{foliage}$ can be more negative or less positive than that of non-N-fixers at the same site. Some plants absorb nutrients indirectly via mycorrhizal symbiosis. It is precisely for this reason that a lot of plants in the subantarctic region have negative $\delta^{15}N_{foliage}$ values (Fig. 2.1). In these cold and icy places, decomposition of organic matter is slow and a large amount of such matter remains. Plants rely heavily on mycorrhizae to take up organic nitrogen and phosphorus (Stewart and Schmidt 1999). Mycorrhizal hyphae have strong discrimination power to select against ^{15}N so that most nitrogen transferred to the host plant roots is the lighter ^{14}N. Thus heavy reliance on symbiotic nitrogen fixation and/or mycorrhizal symbiosis often results in lower $\delta^{15}N_{foliage}$ than the direct soil uptake does.

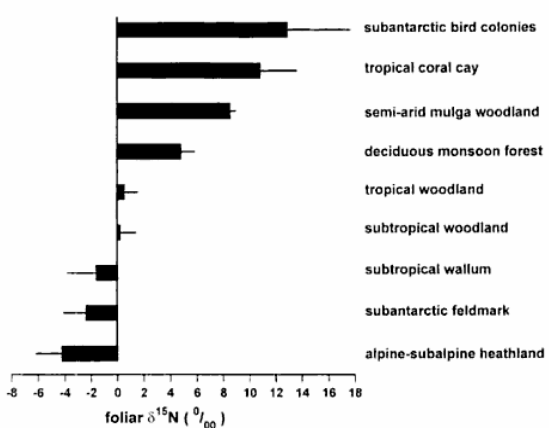


Figure 2.1. The mean foliar $\delta^{15}N$ of vegetation communities at different latitudes (Stewart and Schmidt 1999)

Mycorrhizal symbiosis is also responsible for the low $\delta^{15}N_{foliage}$ of non-N-fixing *Eucalyptus* and vesicular-arbuscular species in savannas, escarpment woodlands and moonsoon forests in Australia (Table 2.1). Their mean $\delta^{15}N_{foliage}$ value can reach as low as -0.6‰ which is similar to $\delta^{15}N_{foliage}$ of nitrogen fixing *Acacias* (-0.1 to 1‰). It should be noted that *Acacias* can either be ectomycorrhizal (ECM) or vesicular-arbuscular (AM), but the latter is more commonly observed. Thus the low $\delta^{15}N_{foliage}$ values of *Acacias* in the wild may be a result of isotopic discrimination by fungal hyphae and bacteroids in root nodules.

There is some evidence that the nitrogen per unit dry weight of foliage (foliage NPUDW) correlates with $\delta^{15}N_{foliage}$ within and among plant species that form mycorrhizal symbioses. However, a much lower degree of correlation (or not at all) exists for some nitrogen fixing species such as *Alnus, Dryas,* and *Shepherdia* (Hobbie *et al.* 2000). An investigation of $\delta^{15}N_{foliage}$ in Australian savanna and monsoon forest, Miombo woodland and lowland rainforest in Africa found that the $\delta^{15}N_{foliage}$ values of ECM species in these sites are positively correlated

with foliage NPUDW values. However, no such correlation was observed for N-fixing species (Schmidt and Stewart 2003). A global review of 9 757 and 1 604 geo-referenced observations of non-N-fixing and potentially N-fixing plants, however, reveals significant correlations between Log (foliage N concentrations) and $\delta^{15}N_{foliage}$ values in both types of plants (Craine ***et al.*** 2009). Both correlations have p-values less than 0.001. However, the total variations in $\delta^{15}N_{foliage}$ values explained by the logarithms of foliage nitrogen concentrations are respectively 25% and 8% only.

Low or negative $\delta^{15}N_{foliage}$ can be due to mycorrhizae and/or symbiotic nitrogen fixation. If $\delta^{15}N_{foliage}$ is strongly correlated with foliage NPUDW, mycorrhizae uptake of nitrogen may play an important role. If there is no such correlation, symbiotic nitrogen fixation is said to be largely responsible. Mycorrhiza is widely recognised to be enriched in ^{15}N compared to the roots of the host plant because it discriminates against ^{15}N during nitrogen transfer from its tissue to plant roots (Hobbie ***et al.*** 2000; Schmidt and Stewart 2003; Schmidt ***et al.*** 2006).

A conceptual model has been proposed to explain the relationship between foliage NPUDW and $\delta^{15}N_{foliage}$ under the influence of mycorrhizae, using Alaska Glacier Bay ***Picea, Populus*** and ***Salix*** (Fig. 2.2) (Hobbie ***et al.*** 2000). At low nitrogen availability, less nitrogen is absorbed directly by roots and more nitrogen is taken up by plants via fungi. But compared with high nitrogen availability, a greater percentage of nitrogen taken up by fungal hyphae will be retained in the hyphae themselves to satisfy its own requirement. Hence only a small percentage of the nitrogen and thus ^{15}N taken up by fungal hyphae will be transferred to the roots of the host plant. The result of these competing processes is that the foliage NPUDW and $\delta^{15}N_{foliage}$ values of host plants are lower and more negative respectively.

At high nitrogen availability, fungal uptake of nitrogen is proportionally less than direct root uptake. But compared to low nitrogen availability, only a small percentage of nitrogen assimilated by fungal hyphae will be retained in the hyphae themselves. This is because while the percentage is small, the absolute amount of nitrogen retained is already sufficient to satisfy the nitrogen requirement of fungi. A greater proportion of the nitrogen absorbed into fungal hyphae is thus transferred to the plant. The foliage NPUDW and $\delta^{15}N_{foliage}$ values of the host plant therefore become higher and less negative respectively.

On the other hand, when plants are not associated with mycorrhizae and when symbiotic nitrogen fixation consistently discriminates against heavy ^{15}N, no such relationship between foliage NPUDW and $\delta^{15}N_{foliage}$ values is observed. Another study that examined the growth of subtropical ***Eucalyptus amanita*** and ***E. gymnogaster*** seedlings, inoculated with fungi in a

glasshouse verified that the $\delta^{15}N_{fungi}$ value was negatively correlated with root and shoot NPUDW values (Schmidt *et al.*, 2006).

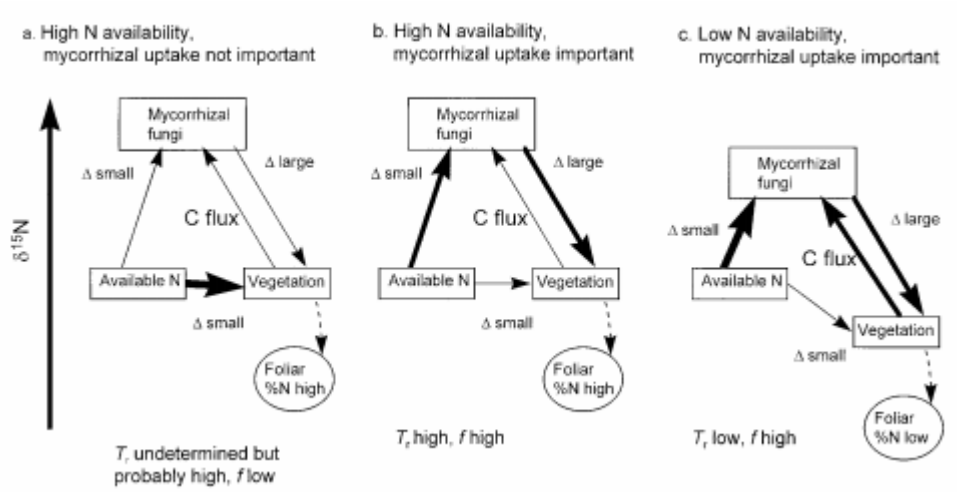


Figure 2.2. The different scenarios to which plants adjust their nitrogen uptake methods and how that affects $\delta^{15}N_{foliage}$ value (Hobbie *et al.* 2000). T_f represents the proportion of nitrogen in the hyphae that is transferred to the host plant roots. *f* is the uptake by hyphae and thus 1-f is the direct uptake by root. Δ is the discrimination factor. Large Δ means a great amount of ^{15}N is discriminated against by the sink so that most N getting into the sink belongs to ^{14}N instead of ^{15}N. Detailed explanation is given in text.

In a global study of the correlation between $\delta^{15}N_{foliage}$ and foliage NPUDW, Craine *et al* (2009) examined at the correlation in putative N-fixing and non-N-fixing plants separately. Ericoid plants were the most $^{15}N_{foliage}$ depleted (reaching an average of -5‰), followed by ectomycorrhizal plants (about -2.3‰ in average), then arbuscular vesicular foliage tissue (about -1.1‰) and finally foliage tissue of non-mycorrhizal plants (about -0.9‰). The presence of the correlation in putative N-fixing plants probably indicates that they can often form ectomycorrhizal or arbuscular-vesicular associations too (Schmidt and Stewart 2003), which would have masked the effect of symbiotic nitrogen fixation.

Would the presence of mycorrhizae render the %Ndfa estimation by ^{15}N natural abundance method inaccurate? Theoretically the inaccuracy can be made minimal by finding a reference species that has a similar type of mycorrhizae (Andrews *et al.* 2011).

2.1.8 Relevance to revegetation and climate change

Finding out what and how environmental factors affect nodulation, and hence nitrogen fixation of *Acacia* can benefit plantation and revegetation projects. *Acacia* spp. are useful forestry intercropping species in *Eucalyptus* plantations because they facilitate underground transfer of nitrogen nutrients to *Eucalyptus* trees (Brockwell *et al.* 2005). *Acacia* spp. are also commonly planted to reclaim sites severely degraded by mining and landfill activities (Brockwell *et al.* 2005). Apart from erosion control in revegetation projects, planting *Acacia* spp. can also help to replenish nitrogen in the soil because of microbial decomposition of dead roots or litter that are generally high in nitrogen content (Dommergues 1995).

While Free Air Carbon Enrichment, glasshouse and simulation studies examine the effect of elevated CO_2 on the growth and nitrogen fixation of plants including legumes (Tissue *et al.* 1997; Schortemeyer *et al.* 2002; Hungate *et al.* 2003; Hungate *et al.* 2004; Rogers *et al.* 2009), the effect is largely uncertain because sufficient long-term experiments are lacking and simulation models have yet to adequately take into account the effect of various nutrients such as molybdenum, and iron. Surprisingly, there is little discussion on the effect of climate on nodulation largely because overall nitrogen fixation is of greater interest. But symbiotic nitrogen fixation rate is closely related to nodule size, weight and number (Ribet and Drevon 1996). For rapid assessment of the importance of symbiotic nitrogen fixation to plants, examining changes in nodule type (effective vs ineffective; branched vs globose), size, weight and number is worthy of investigation. If a correlation between environmental control, nodulation, and symbiotic nitrogen fixation can be established, management authorities with time and budget constraints can benefit when assessing ecosystem health. Variations in, and interactions between, climatic factors can be expected to affect nodulation.

In this chapter, I hypothesize that inland *Acacia* species will have fewer nodules, more ineffective nodules and a lower proportion of nitrogen obtained from fixation than coastal *Acacia* species. Such a trend is related to decreasing rainfall and increasing temperature extremes further inland.

It is also predicted that nodulation patterns and the proportions of air nitrogen uptake in tropical *Acacias* are correlated with changes in soil properties, in particular total nitrogen and total phosphorus concentrations, soil moisture, salinity, pH and soil bulk density. Seasonal changes in nodule number are expected – with more nodules being produced in the wet season than dry season.

2.2 Materials, methods and data analysis

2.2.1 Species tested

One population of the following species is sampled:

- coastal *Acacia crassicarpa* Cunn ex. Benth 1842
- coastal *A. aulacocarpa* Cunn ex. Benth 1842
- inland *A. ramiflora* Domin 1926, and;
- inland *A. elachantha* M.W. McDonald and Maslin 1997

2.2.2 Sampling methods

The nodulation pattern was examined once in the dry season 2010 and once in the wet season 2011 (Table 2.2).

Table 2.2. Sampling dates for field sampling of four *Acacia* species

Parentheses after sampling dates are the number of trees sampled

Species	Sampling dates	
	Dry season 2010	Wet season 2011
A. crassicarpa	8 Aug (3)	17 Apr (6)
	22 Aug (4)	26 Apr (5)
	25 Aug (4)	
	12 Oct (3)	
A. aulacocarpa	6 Oct (1)	26-27 Mar (6)
	24 Oct (4)	3 Apr (3)
	29 Oct (7)	1 May (3)
A. ramiflora	28-29 Aug (10)	9-10 Feb (10)
	30 Oct (6)	
A. elachantha	16-17 Oct (12)	5-6 Mar (12)

Since root and nodule densities can decrease with distances from actinorhizal plants such as *Alnus* (Rytter 1989; Tobita *et al.* 2010), there may be a similar pattern in the study species. A pilot test with a mature *A. auriculiformis* tree in Townsville confirmed this was the case. It was decided that, using the soil core method, at least three samples should be taken at each of the distances 20, 40, 60 and 80 cm away from the trunk for reliable representation of each distance.

The results of a pilot trial, also using the soil core method, indicated that *Acacia* root and nodule biomass could decrease with distance (Fig. 2.3).

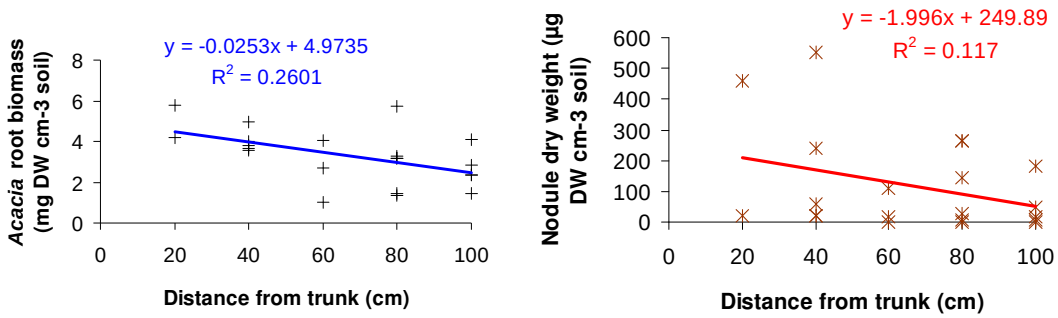


Figure 2.3. The root (left) and nodule biomass (right) of a *A. crassicarpa* tree sampled in the JCU campus, Townsville. Each mark represents a core sample out of 26 in total.

2.2.2.1 Soil sampling procedures

Due to strong spatial variability of soil chemical properties (Bengough *et al.* 2000), stratified sampling was undertaken by dividing each site into three to four plots. These plots were randomly located through the use of a compass. The distances between plots were at least 500 m to ensure their independence. Beginning with the separation of 500 m, extra distances were added to ensure maximum degree of independence and such distances were randomly determined. Each plot was therefore a random grouping variable for running a linear mixed model, specifically an hierachical or partly nested linear model (Section 2.2.3). The dimensions of each plot were 100 to 150 m long and 50 to 100 m wide. Plot size was large because target individuals were sparsely distributed. All four species occurred within 200 m from main road (Fig. 2.4). Within each plot, three to five individuals were sampled. From a random starting point, a three-digit compass direction was randomly chosen and this direction was paced for 100 m until an individual was encountered within a 20-to-40m-wide band. The next individual was chosen from the preceding individual using the aforementioned compass technique. When no individuals were found within 100 m of walking distance, another random compass direction was chosen at the stopping point. Searching continued towards the newly specified direction. Chosen individuals had to be mature and within the dominant height range of that species at the site. These heights were 4 to 12 m for *A. crassicarpa*, and 2 to 7 m for *A. aulacocarpa*, *A. ramiflora* and *A. elachantha*.

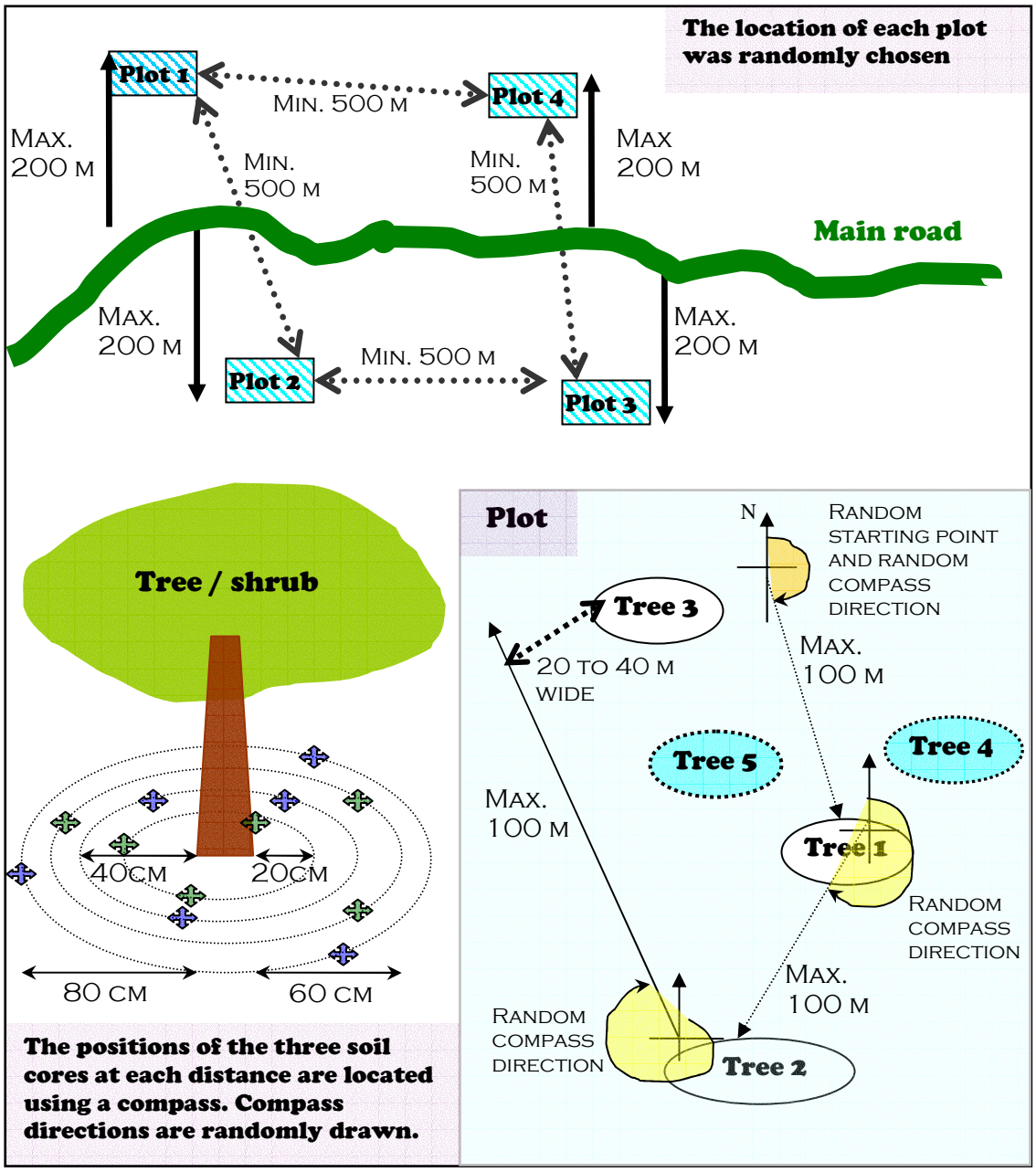


Figure 2.4. The sampling design used to collect roots and nodules of the target species at the four study sites. Details are also given of tree sampling within plot 3 as well as of soil core sampling under every host tree.

Soil core sampling was undertaken around each tree at 20, 40, 60 and 80 cm away from the trunk rim. At each distance, three locations were randomly chosen using a compass. Soil at each location at each distance was dug up using a shovel and an open-ended metal tube of 5.3 cm in diameter and 6 cm in height. Two soil cores were taken to represent 0 to 12 cm depth; another two lower cores represented 12 to 24 cm depth (wet season only). The two soil cores collected from each depth were stored in a plastic bag which was tightly sealed. As the two cores were be processed together, there was no attempt to keep them separate from each other in each plastic

bag. Soil cores were stored in a fridge at 4°C. The height of each individual tree was visually estimated. The basal circumference and circumference at breast height (1.3 m above ground) were determined with a measuring tape. Where there were multiple stems, all stems were measured separately and their diameters were summed up to a composite that represented the individual. The crown width was measured using the logger's tape method (Bechtold *et al.* 2002). The largest crown width was measured, followed by the width perpendicular to it. An average of the two measurements was taken as the crown width of the individual.

2.2.2.2 Phyllode collection for $\delta^{15}N$ testing

At least five mature phyllodes of all target individuals were collected in both seasons. Only healthy phyllodes were collected as recommended by Shearer and Kohl (1986). This was judged by a normal green color (not pale), absence of fungal hyphae, and with as few rusty or dark spots as possible.

2.2.2.3 Laboratory processing

In the laboratory, both soil cores from each plastic bag were weighed together. The soil cores collected at the upper depth from the three locations at 20 cm distance were then mixed thoroughly to form a composite. All upper depth soil cores sampled between 40 and 80 cm distances were then mixed together to form another composite. For the wet season only, soil cores from the lower depth were also weighed and mixed in the same way as the upper depth ones. Soil was passed through a stack of 4 cm, 2 cm and 1 cm and 2 mm sieves.

Roots and nodules were separated from the soil and sorted. Roots were separated into live and dead roots. Living roots could be distinguished as belonging to *Acacia* or grass but dead roots could not. Living roots were more elastic, had intact cortex and often displayed branching (Schuurman and Goedewaagen 1965; Böhm 1979). Their colors were either whitish (grass) or reddish (*Acacia*). *Acacia* also has a distinctly different branching pattern from grass roots. *Acacia*, grass and dead roots were weighed when dry. Soil of the same weight was sometimes retrieved more than once and the roots or nodules from multiple batches were averaged. Repeated measurements reduced any bias resulting from incomplete mixing so that measurements more adequately represent the rooting and nodulation status at that distance(s).

Nodules were examined under the microscope, and their shapes were recorded as globular, semi-globose, elongated or branched (Sprent 2001). The diameter of globular nodules, and the

width and length of semi-globose, elongated and branched nodules were measured. The thickness of branched nodules was also measured. Each nodule was then halved once or twice to reveal the colour. Orange, pink or red nodules contained leghaemoglobin and suggested active or effective nitrogen fixation (Sprent 2001). Green, brown, black or yellow color suggested absence of leg-haemoglobin and hence inactive or ineffective nitrogen fixation (Sprent 2009). Hollow or dry nodules were classified as dead. Effective, ineffective and dead nodules were weighed when dry. The abundance of these three types was also counted. It took about 1 to 4 month(s) to process the large number of samples of each species.

The fine soil fraction (particles less than 2 mm) was analysed for their total nitrogen, phosphorus and organic carbon, pH and conductivity (Rayment and Higginson 1992). The gravimetric moisture content and bulk density of soil were also determined.

The phyllodes of some seedlings of the four *Acacia* species grown in the provenance experiment were also subject to $\delta^{15}N$ analyses. These seedlings were either forced to rely on symbiotic nitrogen fixation or direct uptake from their native soil as their only source of nitrogen. Their $\delta^{15}N$ values were deduced to represent two extremes (highly negative values in the former and highly positive in the latter). The $\delta^{15}N$ values of the field-collected phyllodes were expected to fall in between as the natural populations of the four *Acacia* species might obtain nitrogen via both means.

Phyllodes were oven dried at 70^{o}C for 24 to 48 hours to prevent microbial transformation/assimilation of nitrogen during storage which can discriminate against $\delta^{15}N$ and hence enrich $^{15}N_{phyllode}$. They were then stored at room temperature. After six months to a year, phyllodes of each individual were separately ground using a laboratory mill and subjected to isotope analysis. The $\delta^{13}C$ and $\delta^{15}N$ values, and nitrogen weight percent (%N,) were determined using a Costech Elemental Analyzer (ECS 4010). The ECS 4010 was fitted with a zero-blank auto-sampler coupled via a ConFloIV to a ThermoFinnigan DeltaV^{PLUS} using Continuous-Flow Isotope Ratio Mass Spectrometry (EA-IRMS). The analysis was undertaken at James Cook University's Cairns Analytical Unit. Stable isotope results were reported as per mil (‰) deviations from the VPDB and AIR reference standard scales for $\delta^{13}C$ and $\delta^{15}N$ values, respectively. Precision (S.D.) on internal standards and samples were ±0.2‰ and ±0.3‰ respectively.

2.2.3 Statistical analysis

2.2.3.1 Nodule density and dry weight of A. crassicarpa between seasons and soil depths

The distribution of nodule dry weight and densities, i.e. the dry weight or number of nodules per unit soil volume, were heavily skewed to the right because most soil cores had a low number of nodules, yielding many zeros (Fig. 2.5). The data followed a Poisson or negative binomial (NB) probability distribution which requires the response variables to be real non-negative integers (Bolker 2008; SPSS 2011).

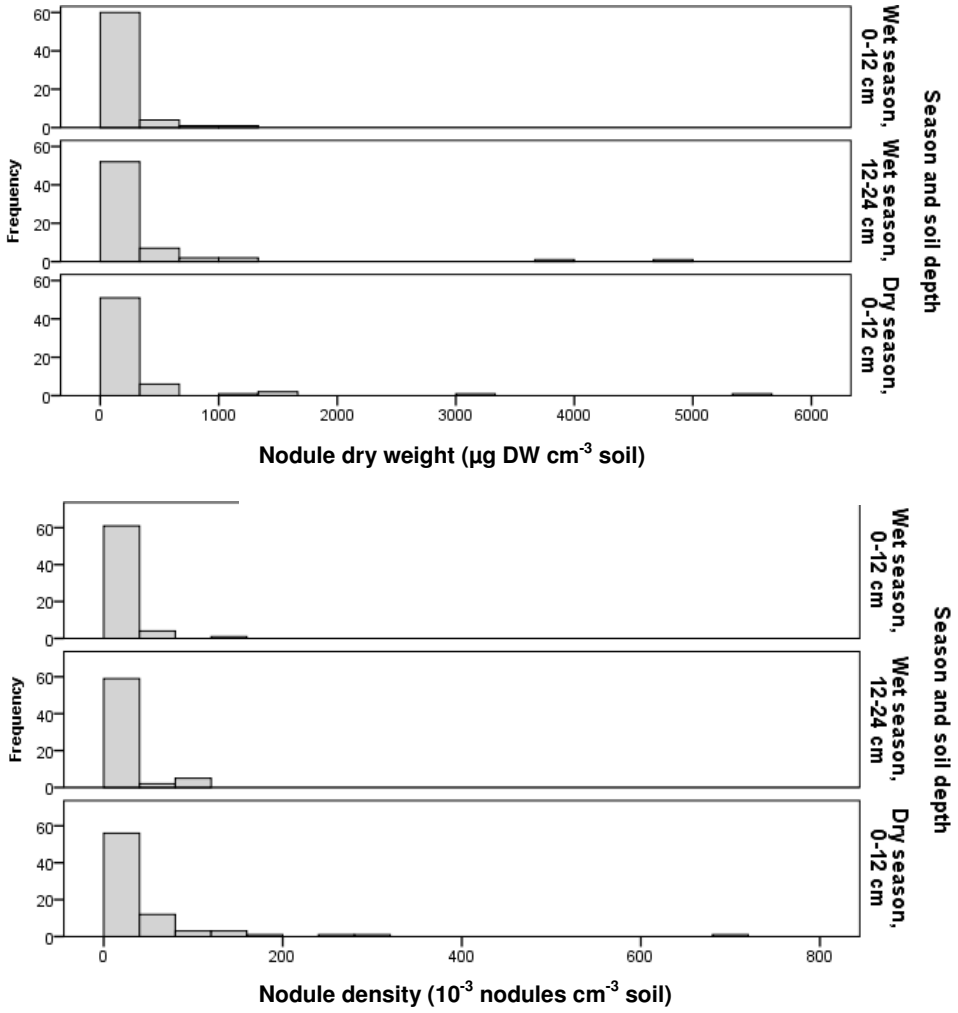


Figure 2.5. The histograms of nodule dry weights and densities of *A. crassicarpa* on the Townsville Town Common.

Considering the hierachical structure of data with plot as the random grouping variable, a generalized linear mixed model was applied (Bolker 2008; Zuur *et al.* 2009). Several sub-models were tried to obtain the best model according to Akaike Information Criteria (AIC) (Fridley 2010). These models included zero-inflated negative binomial (ZINB), zero-altered

negative binomials (ZANB), zero-inflated Poisson (ZIP), zero-altered Poisson (ZAP) and NB, with or without random effects using SPSS or the "pscl" or "glmmADMB" packages of the software R (Zuur *et al.* 2009).

Eventually, a NB model with random intercept was applied to the data using SPSS. A logarithmic link function was chosen. Degress of freedom option "Satterthwaite approximation" was selected. "Variance component" covariance structure was chosen so that all random effects were assumed to be independent of other variables, thus giving a zero covariance and homogenous variances of all random effects (Field 2009). Fixed effects included three combinations of season and soil depths (wet season and 0-12cm, wet season and 12-24 cm, dry season and 0-12 cm), three nodulation types (effective, ineffective, dead) and their interactions. Simple main effects of estimated means were examined and the significance level was set at α = 0.05. Pearson's residuals were plotted against predictor variables to ensure no observable pattern which is an important assumption of linear model.

Four two-way ANOVAs were run to examine potential seasonal and inter-specific differences in $\delta^{15}N_{foliage}$, foliage NPUDW, soil NPUDW and $[P]_{soil}$ sampled in the field. When an interaction between species and season was found to be significant, simple main effects between levels of each factor were compared when the other factor was allowed for. The simple main effects were examined using the least significant difference method when alpha-level is not adjusted and remained at 0.05.

2.2.3.2 Regression analysis of abiotic factors affecting the nodulation of A. crassicarpa

There were abundant nodules around *A. crassicarpa* while very few nodules were found for the other three species in both seasons. Therefore, for *A. crassicarpa* only, linear mixed models that estimate parameters using (Restricted) Maximum Likelihood (ML or REML) were fitted on nodule dry weights and densities with SPSS version 20. The goal was to find out which sets of soil and tree variables can best predict these two groups of variable.

Data used in this study is hierarchical because "plot" is the random group variable, soil parameters and tree sizes are the random variables, and season, soil depth and distance from the trunk are fixed factors.

The mixed model adopted in the current regression analysis omitted the group variable because nodule densities or dry weights, which were the response variables, were found to vary simiarly

within each of the 3-4 plots in each popuation of *Acacia*. By employing AIC, models without groups also performed better than those with groups. Results were based on such a model.

As many predictor variables (PVs) may affect nodule densities or dry weights, ideally the best subset should be obtained using Corrected Alkaike Information Criteria (CAIC) instead of using the stepwise method (Quinn and Keough 2002). Such a procedure can substantially improve a model's predictive ability. I first employed a linear multiple regression model to find the best subset of PVs using CAIC. The best subset of PVs was then input into a linear mixed (multilevel) model with "plot" as the group variable. Further comparison between multilevel models, with and without group variables, indicated that excluding a group variable always resulted in a lower AIC or AICC. Such a comparison was undertaken using ML as recommended (Field 2009; Zuur *et al.* 2009). For accurate prediction, the model parameters were re-estimated with REML and formed the final output.

CAIC tends to penalise very complicated models and favours simpler ones (Quinn and Keough 2002). In running a linear regression with SPSS, the automatic data preparation option was not selected because outliers which would be automatically removed might make biological sense. Data was manually transformed to ensure homogeneity and normal distribution of residuals. These two assumptions were graphically verified and the former was supplemented by a Shapiro-Wilk normality test. There were two categories of response variables:

- nodule dry weight per unit volume (µg DW cm^{-3} soil) which was further divided into live and effective, live and ineffective, and dead nodules

- density or number of nodules per unit volume (cm^{-3}) which was again further divided into live and effective, live and ineffective, and dead nodules.

The above nodule densities or dry weights were tested separately, each time with all the PVs input into the system for best subset selection. These PVs included categorical and continuous variables. The continuous PVs have been grand-mean centered into Z-score (Z) to reduce multicollinearity. The Z-score was calculated using equation 2.2 below:

$$Z_{PV} = (PV - PV_{mean}) / PV_{standard\ deviation} \quad ..... (2.2)$$

The PVs and their original units were:

Fixed categorical predictors

- season (dry vs wet);
- soil depth (0-12 cm vs 12-24 cm);
- distance from trunk (20 cm vs 40 to 80 cm);

Random continuous predictors

- *Acacia* root biomass (mg DW cm^{-3} soil);
- crown width (m); and
- soil parameters including:
    - bulk density (g DW cm^{-3} soil);
    - moisture (%);
    - total organic carbon (%-Heanes)
    - total nitrogen (mg N g^{-1} soil);
    - total phosphorus (µg N g^{-1} soil)
    - pH; and
    - conductivity (ds m^{-1}).

Crown width was found to have much better correlation with nodule density than with basal diameter, diameter at breast height or with height. Since these four parameters should be correlated from an allometric perspective, only crown width was selected to represent tree size to reduce the multicollinearity problem in multiple regression analysis.

In fitting the final linear multilevel model, the residual was verified to be normally distributed and multicollinearity between predictor variables was reduced by grand mean centering (Bickel 2007; Field 2009). Each value was divided by the mean of that variable to derive a Z-score. The intercept of the fitted model was thus the nodule density or dry weight when every PV has a mean value, i.e. when Z-score was zero. Parameters were estimated using REML which correct the bias of ML estimation (Quinn and Keough 2002). REML could also more accurately estimate variances of random parameters (Field 2009). "Variance component" covariance structure was selected as explained in Section 2.2.3.1.

Some studies suggest that grass competition can indirectly stimulate nitrogen fixation of African *Acacia* seedlings because grass efficiently takes nitrogen up from the soil, lowering soil nitrogen concentrations to a limiting level (Cramer ***et al.*** 2007). However, since grasses below target individuals in the current study were removed beforehand to facilitate soil digging, the grass roots obtained from soil cores would have been severely under-estimated. Grass root biomass was therefore not input into the current regression analysis. Grass may still compete for nutrients with *Acacia* near the surface, but nodulation may not be induced if mature *Acacia* individuals can obtain nutrients from deeper soil through a tap root system which grasses do not

have. Such a difference between trees and grass in exploiting nutrients and/or water was reported in savannas in other countries (Scholes and Archer 1997).

A mixed model does not have R^2 but R_l^2 which is the proportional reduction in errors of prediction when a full model is compared with a null model (Bickel 2007). Regression with the best subset of PVs forms the full model, while one with only the intercept and error variance was considered a null model. When all PVs were dropped from a full model, the intercept and error variance accounted for all the variances in the data, and hence substantially increased. This change indirectly reflected the variance explained by the PVs. R_l^2 was obtained by:

$$R_l^2 = [\ 1- (\ Residual_{fixed} + Intercept_{fixed}\ ) / (\ Residual_{null} + Intercept_{null}\ )\ ] * 100 \qquad \text{….. (2.3)}$$

2.3 Results

2.3.1 Nodule dynamics in different seasons and soil depths

A. crassicarpa produced abundant root nodules; their densities ranged from 5.5 to 101.4 x 10^{-3} nodules cm^{-3} soil. On the other hand, *A. aulacocarpa* produced only as much as 6.6 x 10^{-3} nodules cm^{-3} soil. *A. elachantha* and *A. ramiflora* often did not produce any nodules and, where they did, the mean density only amounted to 0.9 x 10^{-3} nodules cm^{-3} soil (Table 2.3). For *A. elachantha* and *A. ramiflora,* nodules were found in the wet season but not in the dry season. None of their nodules were effective.

On average, the effective, ineffective and dead nodules of *A. crassicarpa* in deeper soil (12-24 cm) were respectively 5.4, 4.6 and 2.1 times heavier than those in the top 12 cm of soil during the wet season. But only the difference for effective nodules was significant. The mean dry weight in deeper soil were respectively 312.1, 71 and 457.4 µg DW cm^{-3} soil. However, the densities of all three types of nodules were similar between depths in the wet season. Thus individual nodules became bigger and heavier in the deeper soil.

Most nodules of *A. crassicarpa* were hollow, or dried out and hardened, and hence were dead. Depending on seasons and depths, the percentage of dead nodules ranged from 54% to 76.4% while the percentage of dry weight contributed by the dead nodules ranged from 54% to 79%. The densities and dry weights of dead nodules almost always exceeded those of effective and

ineffective nodules, irrespective of seasons. The densities and dry weights of dead and ineffective nodules were higher in the top 12 cm of soil in the dry season than those in the wet season. Dead and ineffective nodules that are elongated and branched were smaller in the dry season while globular and semi-globular nodules of similar nodule types were not (Fig. 2.6).

In the wet season, the density of ineffective nodules in the top 12 cm of soil (5.5×10^{-3} nodules cm^{-3} soil) was similar to that of effective nodules (8.1×10^{-3} nodules cm^{-3} soil). In the dry season, the density increased substantially to more than two times that of the effective nodules (effective: 9.7×10^{-3} nodules cm^{-3} soil vs ineffective 22.6×10^{-3} nodules cm^{-3} soil). In contrast, the dry weight of effective nodules and ineffective nodules were statistically similar: the mean dry weight of effective nodules was three to four times that of ineffective nodules but the difference was not significant. There was a tendency for effective nodules to grow bigger in the wet season.

In the wet season, effective nodules of *A. crassicarpa* that are branched and elongated were larger in deeper soil, increasing from a mean size of about 4 mm to 6-8 mm (Fig. 2.6) In contrast, dead nodules that are branched and elongated, as well as branched ineffective nodules were smaller in the deeper soil; their mean nodule sizes decreased from 7-10 mm in the upper soil to 5-6 mm in the deeper soil. Thus it seems that the top 12 cm of soil was unsuitable for nodules to function effectively, possibly due to greater maximum daytime temperature and lower moisture level. Other combinations of nodule shapes and nodule types had similar sizes between depths.

For *A. aulacocarpa*, more nodules were found in the top 12 cm than 12-24 cm in the wet season. In the dry season, both the density and dry weight of dead nodules increased substantially, with density increasing from 0.1-0.4 to 6.6×10^{-3} cm^{-3} soil while the ineffective and effective nodules became smaller and too light to be weighed (Table 2.3). The average size of effective nodules always exceeded that of dead nodules in the wet season (Fig. 2.6). The density of ineffective nodules was similar between seasons but the dry weight was higher in the wet season than dry season. Thus ineffective nodules of *A. aulacocarpa* became bigger and heavier in the wet season.

Table 2.3. Nodule densities and dry weights of *Acacias* in the wet and dry seasons in the field study

Data are means ± 1 S.E.M. The S.E.M. of *A. crassicarpa* was calculated via linear mixed modelling while the S.E.M. of other species were calculated via the ordinary least square method. Different uppercase letters (A and B) represent significant differences in nodule dry weights or densities between treatment combinations (seasons x depths) for each of the three nodule types of *A. crassicarpa*. Different lowercase letters (x, y and z) indicate significant differences in nodule dry weights or densities between nodule types for each of the three seasons x depths combinations of *A. crassicarpa*. Sample sizes are shown in parentheses. All multiple comparisons are planned contrasts and their significance levels were not adjusted and remained at $\alpha = 0.05$.

Species	Season	Depth (cm)	Nodule dry weight (µg DW cm^{-3} soil)			Nodule density (10^{-3} nodules cm^{-3} soil)		
			Live and effective	Live and ineffective	Dead	Live and effective	Live and ineffective	Dead
A. crassicarpa	Wet	0-12	58.3 ± 18 $^{A\ x}$ (22)	15.2 ± 11.1 $^{A\ x}$ (22)	219.3 ± 206.3 $^{A\ y}$ (22)	8.1 ± 3.2 $^{A\ x}$ (22)	5.5 ± 2.2 $^{A\ x}$ (22)	26.7 ± 8.5 $^{A\ y}$ (22)
		12-24	312.1 ± 98.2 $^{B\ x/y}$ (22)	71.0 ± 30.4 $^{A\ x}$ (21)	457.4 ± 130.2 $^{A\ y}$ (22)	16.2 ± 5.3 $^{A\ x}$ (22)	5.5 ± 2.1 $^{A\ y}$ (22)	25.3 ± 7.2 $^{A\ x}$ (22)
	Dry	0-12	129.8 ± 109 $^{A/B\ x}$ (19)	41.8 ± 58.7 $^{A\ x}$ (19)	665.2 ± 349.1 $^{A\ y}$ (24)	9.7 ± 4.0 $^{A\ x}$ (26)	22.6 ± 8.0 $^{B\ y}$ (26)	101.4 ± 46.1 $^{B\ z}$ (26)
A. aulacocarpa	Wet	0-12	0.2 ± 0.2 (24)	4.1 ± 3.9 (24)	0.6 ± 0.4 (24)	0.2 ± 0.2 (24)	0.6 ± 0.4 (24)	0.4 ± 0.2 (24)
		12-24	0 (23)	0 (23)	0.1 ± 0.1 (23)	0 (24)	0 (24)	0.1 ± 0.1 (24)
	Dry	0-12	0 (24)	0 (24)	2.6 ± 1.6 (24)	0.2 ± 0.2 (24)	0.6 ± 0.4 (24)	6.6 ± 2 (24)
A. elachantha	Wet	0-12	0 (24)	0.3 ± 0.3 (24)	0 (24)	0 (24)	0.2 ± 0.2 (24)	0 (24)
		12-24	0 (24)	0 (24)	0 (24)	0 (24)	0 (24)	0 (24)
	Dry	0-12	0 (24)	0 (24)	0 (24)	0 (24)	0 (24)	0 (24)
A. ramiflora	Wet	0-12	0 (20)	0.9 ± 0.7 (20)	0.3 ± 0.3 (20)	0 (20)	0.6 ± 0.4 (20)	0.3 ± 0.2 (20)
		12-24	0 (20)	0.3 ± 0.2 (20)	0 (19)	0 (20)	0.7 ± 0.6 (20)	0.1 ± 0.1 (20)
	Dry	0-12	0 (32)	0 (32)	0 (32)	0 (32)	0 (32)	0 (32)

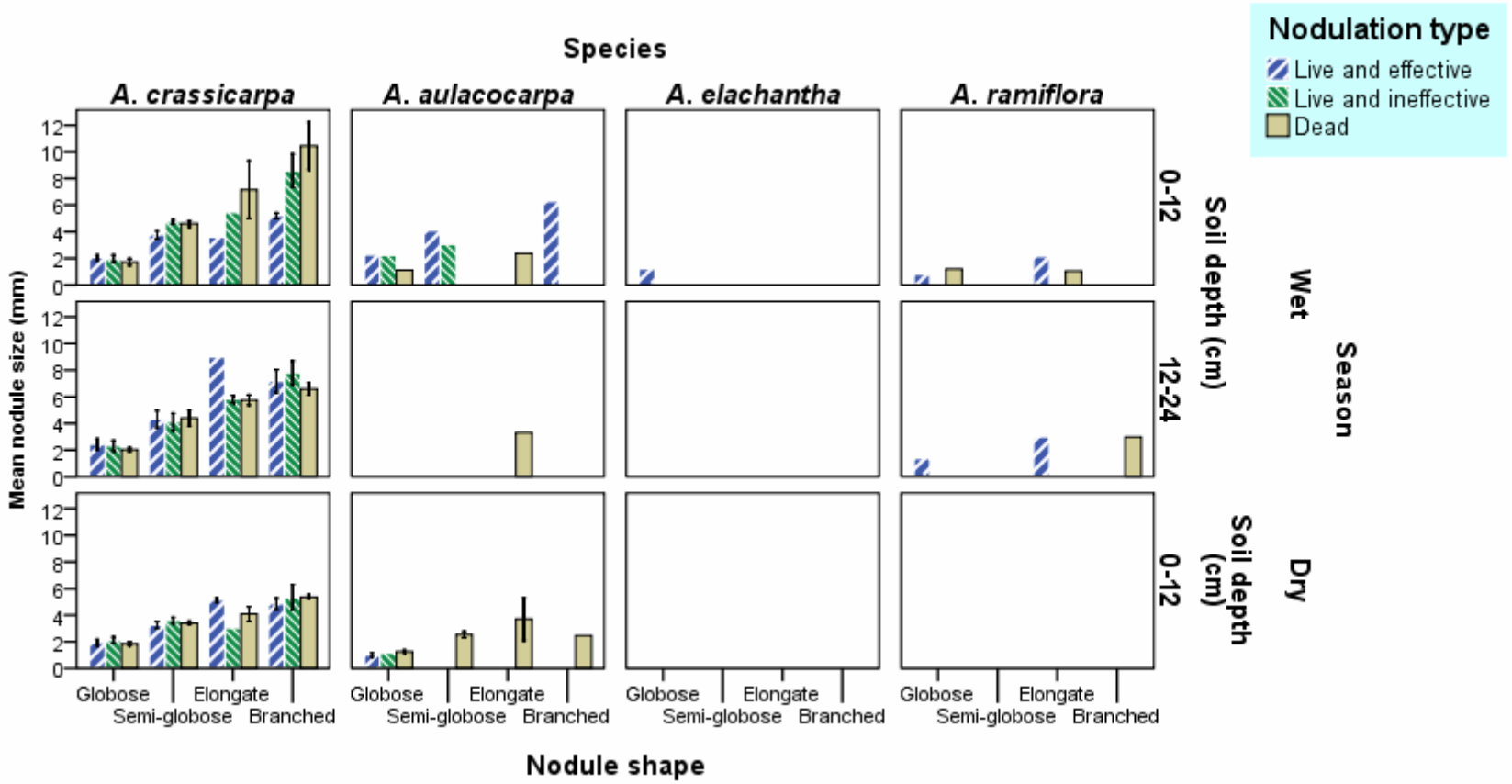


Figure 2.6. Nodule size grouped according to nodule shape, nodulation type, soil depth, and season, of the four species in the field study. Data are means (± 1 S.E.M.) of plots. Only the 0-12 cm soil depth was sampled in the dry season while both 0-12 and 12-24 cm soil depths were sampled in the wet season.

2.3.2 Factors affecting the nodulation of A. crassicarpa

The relationship between each response variable and its corresponding set of PVs was subject to linear mixed modelling (Table 2.4). The significance of predictors in influencing the response variables are indicated by the standardized coefficients of predictors. For instance, *Acacia* root biomass positively predicted the dry weights and/or densities of effective, ineffective and dead nodules. The standardized coefficient of *Acacia* root biomass ranged from 0.12 to 0.30, which indicated a relatively weak relationship with nodule densities or dry weights compared with other predictors (Table 2.4).

The continuous PVs were standardized into Z-scores using equation 2.2 and response variables were log-transformed. Hence back-transformation of both x- and y-variables is required to examine the absolute values of the response variables and PVs. For example, the live and ineffective nodule density (LIDND_A) is the response variable for which the fitted predictive model is:

$$\begin{aligned} \text{Log}_{10}(\text{LIDND\_A}*100+0.5) = \quad & \text{Intercept} + 0.43*\text{Season} + 0.14*Z_{\text{Log10(Acacia root biomass)}} - \\ & 0.14*Z_{\text{Total nitrogen}} + 0.11*Z_{\text{Soil moisture}} + 0.08*Z_{\text{Conductivity}} \end{aligned}$$

One unit of $Z_{\text{total nitrogen}}$ is equal to one standard deviation away from the mean value of total nitrogen. When all variables are zero except $Z_{\text{total nitrogen}}$, the resulting equation models how $\text{Log}_{10}(\text{LIDND\_A}*100+0.5)$ changes with $Z_{\text{total nitrogen}}$ in the top 12 cm of soil during the wet season when all other continuous variables are at the mean values. Therefore, we have:

$$\text{Log}_{10}(\text{LIDND\_A}*100+0.5) = \quad 0.36 - 0.14*(Z_{\text{total nitrogen}})$$

If the season or depth variable is allocated a value of one, instead of zero, the model of $\text{Log}_{10}(\text{LIDND\_A}*100+0.5)$ against $Z_{\text{total nitrogen}}$ would be indicative of changes in the dry season or 12 to 24 cm soil depth. Back-transformation of $Z_{\text{total nitrogen}}$ can be undertaken using equation 2.2, and requires the mean values and the standard deviation of $Z_{\text{total nitrogen}}$, which can be found in Table 2.4. The back-transformed "LIDND_A" and "total nitrogen" are shown in Table 2.5.

Table 2.4. Six types of nodule densities and dry weights predicted by categorical and continuous factors based on the field data

Each response variable was modelled with a separate set of predictors. Coefficients (±1 S.E.M) of predictor variables estimated using REML and the *P*-value are shown. All random variables were grand mean centered as z-scores and hence their coefficients were standardized. DND_DW, nodule dry weight (μg cm^{-3} soil); DND_A, nodule density (nodules cm^{-3} soil); LE, live and effective nodule; LI, live and ineffective nodules; D, dead nodules. AIC suggested that the best subset of PVs should include specific statistically non-significant predictors to achieve the highest predictive power.

Predictor variable	Mean value of each predictor	Equivalent of one standard deviation	Response variable											
			Log_{10}(LEDND_DW +3)		Log_{10}(LIDND_DW +2)		Log_{10}(DDND_DW +4)		Log_{10}(LEDND_A *100+0.15)		Log_{10}(LIDND_A *100 +0.5)		Log_{10}(DDND_A *100 + 0.1)	
			Estimate	*P*	Estimate	*P*	Estimate	*P*	Estimate	*P*	Estimate	*P*	Estimate	*P*
Fixed categorical factors														
Season (0: wet; 1: dry)			-0.53 ± 0.22	0.021	0.45 ± 0.21	0.034					0.43 ± 0.09	< 0.001	0.32 ± 0.15	0.039
Soil depth (0: 0-12 cm; 1: 12-24 cm)			0.38 ± 0.19	0.052	0.56 ± 0.20	0.008			0.20 ± 0.08	0.051				
Random continuous factors – tree-related														
Crown width	7.99 m	2.84 m	0.40 ± 0.09	< 0.001			0.42 ± 0.07	< 0.001	0.10 ± 0.06	0.083			0.30 ± 0.07	< 0.001
Log_{10}(*Acacia* root biomass)	-0.347 mg DW cm^{-3} soil	0.268 mg DW cm^{-3} soil			0.30 ± 0.09	0.002	0.23 ± 0.06	0.001	0.12 ± 0.05	0.027	0.14 ± 0.03	< 0.001	0.20 ± 0.07	< 0.003

Table 2.4 Cont'd.

Random continuous factors – soil related												
Total nitrogen	0.75 mg N g^{-1} soil	0.23 mg N g^{-1} soil								-0.14 ± 0.04	0.002		
Log_{10}(Soil moisture)	0.85%	0.39%	-0.53 ± 0.11	< 0.001								-0.27 ± 0.08	0.001
Soil moisture	10.16%	8.47%					-0.43 ± 0.07	< 0.001	-0.12 ± 0.06	0.055	0.11 ± 0.04	0.015		
Soil bulk density	1.07 g DW cm^{-3} soil	0.16 g DW cm^{-3} soil							-0.15 ± 0.06	0.008				
Conductivity	0.12 ds m^{-1}	0.23 ds m^{-1}	0.21 ± 0.10	0.036			0.19 ± 0.06	0.003			0.08 ± 0.04	0.063	0.30 ± 0.07	< 0.001
Soil organic carbon	2.64% C-Heanes	1.29% C-Heanes					-0.18 ± 0.06	0.003					-0.16 ± 0.07	0.018
Other model components														
Intercept			1.36 ± 0.24	< 0.001	1.79 ± 0.25	< 0.001	1.97 ± 0.05	< 0.001	-0.03 ± 0.08	0.686	0.36 ± 0.06	< 0.001	0.45 ± 0.11	< 0.001
Residual variance			0.37 ± 0.07	< 0.001	0.41 ± 0.08	< 0.001	0.19 ± 0.03	< 0.001	0.15 ± 0.03	< 0.001	0.07 ± 0.01	< 0.001	0.20 ± 0.04	< 0.001

Note: all data of the continuous predictors were standardized into z-scores to reduce the problem of multicollinearity. One unit of z-score equals one standard deviation.

Table 2.5. Back-transformed total nitrogen, and live and ineffective nodule density in the top 12 cm of soil in the wet season

Z – Z-score or number of standard deviations from the mean value of PV; PV – predictor variable; LIDND_A – Live and ineffective nodule density

$Z_{total\ nitrogen}$	$PV_{total\ nitrogen}$ (mg N g^{-1} soil)	Log_{10}(LIDND_A*100+0.5) (10^{-3} nodules cm^{-3} soil)	LIDND_A (10^{-3} nodules cm^{-3} soil)
-2	0.29	0.64	38.65
-1	0.52	0.50	26.62
0	0.75	0.36	17.91
1	0.98	0.22	11.60
2	1.21	0.08	7.02

In the top 12 cm of soil and during the wet season, when the total nitrogen decreased by 0.23 mg N g^{-1} soil, i.e. one standard deviation from the mean value of 0.75 mg N g^{-1} soil, the density of live and ineffective nodules increased by 8.71 x 10^{-3} nodules cm^{-3} soil. When the total nitrogen dropped by two standard deviations from the mean value, the density increased by 20.74 x 10^{-3} nodules cm^{-3} soil. Apart from total nitrogen, when soil moisture decreased by 0.5 and one standard deviation, the density of live and ineffective nodules dropped by 2.73 x 10^{-3} nodules cm^{-3} soil and 5.13 x 10^{-3} nodules cm^{-3} soil respectively.

Back-transformation of the density values resulted in a non-linear relationship between $PV_{total\ nitrogen}$ and LIDND_A. Since all six response variables were log-transformed to meet the assumptions of linear regression analyses (Table 2.4), non-linearity would be expected in the relationships of all back-transformed response variables with their corresponding predictor variables.

The model indicates that if soil bulk density increases by one and two standard deviation(s) from the mean value, the density of live and effective nodules decreases by 2.73 x 10^{-3} nodules cm^{-3} soil and 4.66 x 10^{-3} nodules cm^{-3} soil respectrively. If soil moisture is increased by 0.5 and one standard deviation from the mean value, the density decreases by 1.20 x 10^{-3} nodules cm^{-3} soil and 2.25 x 10^{-3} nodules cm^{-3} soil respectively. As to the change in the dry weight of live and effective nodules, soil moisture plays an important role. For instance, if it is reduced by 0.5 and one standard deviation from the mean value, the model indicates dry weight will rise by 19.26 and 54.72 µg cm^{-3} soil respectively. On the other hand, if it is raised by 0.5 and one standard deviation, the dry weight is predicted to decrease by 10.46 and 16.15 µg cm^{-3} soil respectively.

The models indicate that both soil moisture and crown width play an important role in the change in the dry weight of dead nodules. If soil moisture is reduced by 0.5 and one standard deviation from the mean value, the dry weight is predicted to increases by 60 and 158 µg cm^{-3} soil respectively. If the crown width is reduced by one and two standard deviations from the mean value, i.e. by 2.84 and 5.68 m, the dry weight will decrease by 56 and 79 µg cm^{-3} soil respectively. It therefore appears that crown width had a lesser influence than soil moisture over the dry weight of dead nodules. Also, the models indicate that, in between 1.095 ds m^{-2} and 0.018 ds m^{-2}, soils with slightly higher electrical conductivity (EC) were found with greater nodule dry weights, regardless of the type of nodules. The models indicate a negative relationship between the standing crop of dead nodules and soil organic matter (carbon).

From the above results, it could be demonstrated that increased soil moisture affected ineffective nodules positively but dead and effective nodules negatively. While higher total nitrogen resulted in a lower density of ineffective nodules, it had no significant effects on either dead or effective nodules. Total phosphorus had no significant effect on the densities of all three types of nodules.

The regression for dead nodule dry weight largely resembled that for dead nodule density. The factors affecting the dry weight of effective nodules were almost entirely different from those affecting the density of effective nodules. Overall, deeper soil, the change from the dry to the rainy season, larger crown size, adequate soil drainage, slight increase in electrical conductivity , and less compact soil can encourage effective nodulation.

The regression on dead nodule densities or dry weights should be interpreted with caution because dead nodule density is a cumulative number. When nodules died due to senescence and/or unsuitable environmental conditions, it took some time for their full decomposition. Theoretically, the environmental conditions at the time of sampling might not directly correlate with nodule density. The predictive power of variables in the model appears strong but needs testing. An example of such a test would be repeating the field study for another one to two years, to see if a similar predictive power can be obtained. Nodule production rate might have exceeded that of the decomposition rate such that as the host plant got larger or produced more roots, more nodules were produced. But nodules might have quickly died and accumulated in the soil when soil properties varied over time or at a micro-site spatial scale. This may explain why dead nodules increased with tree size and *Acacia* root biomass.

About 40 to 78% of the variance of nodule densities or dry weights can be explained by the mixed models (Table 2.6). Given the strong spatial heterogeneity of soil properties, the sampling plan was designed to reduce the confounding effects of spatial variation. Thus such a range of explanatory power is reasonable. Only the dry weight of ineffective nodules reached an unsatisfactory low level of about 20%. The variances of dead nodule densities or dry weights explained were particularly high, reaching almost 66 to 78%.

Table 2.6. The proportion of the variance of each of full models specified in Table 2.4 which can be explained by the model components (R_I^2)

See Table 2.5 for a description of the abbreviations used in this table.

	LEDND_DW	LIDND_DW	DDND_DW	LEDND_A	LIDND_A	DDND_A	Average
R_I^2	54.9 %	21.2 %	78.4 %	40.9 %	46.8 %	66.0 %	51.4%

It is clear that higher nodule density and dry weight came with greater crown width of the four sampled *Acacia* species (Fig. 2.7). Surprisingly, high soil moisture was associated with low nodule density and dry weight (Fig. 2.8).

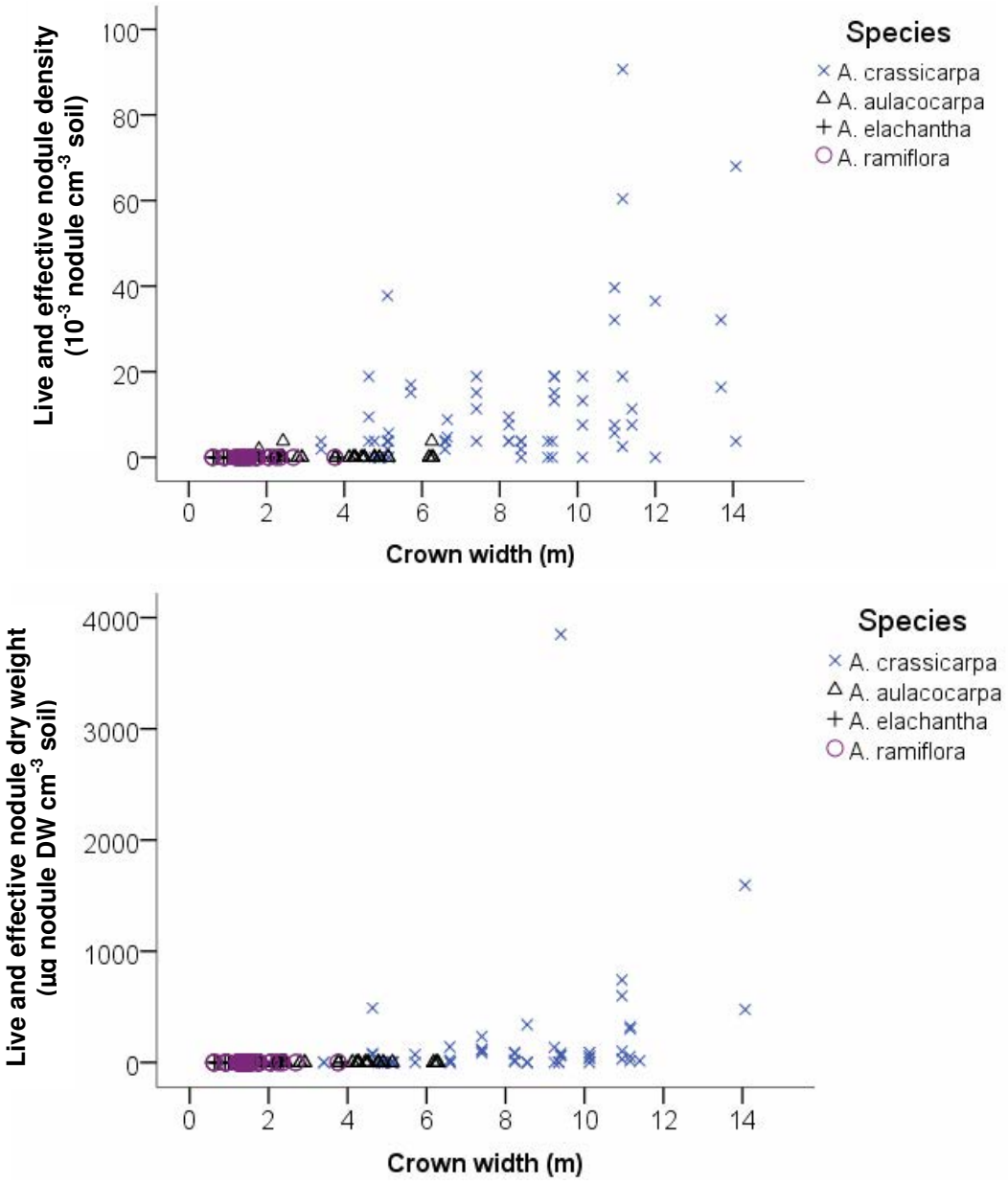


Figure 2.7. The relationship between crown width and effective nodule density (upper), and between crown width and effective nodule dry weight (lower) in *A. crassicarpa, A. aulacocarpa, A. elachantha* and *A. ramiflora.* Each mark is a core average of a specific distance (20 vs 40-80 cm) and depth (0-12 vs 12-24 cm) of each sampled individual.

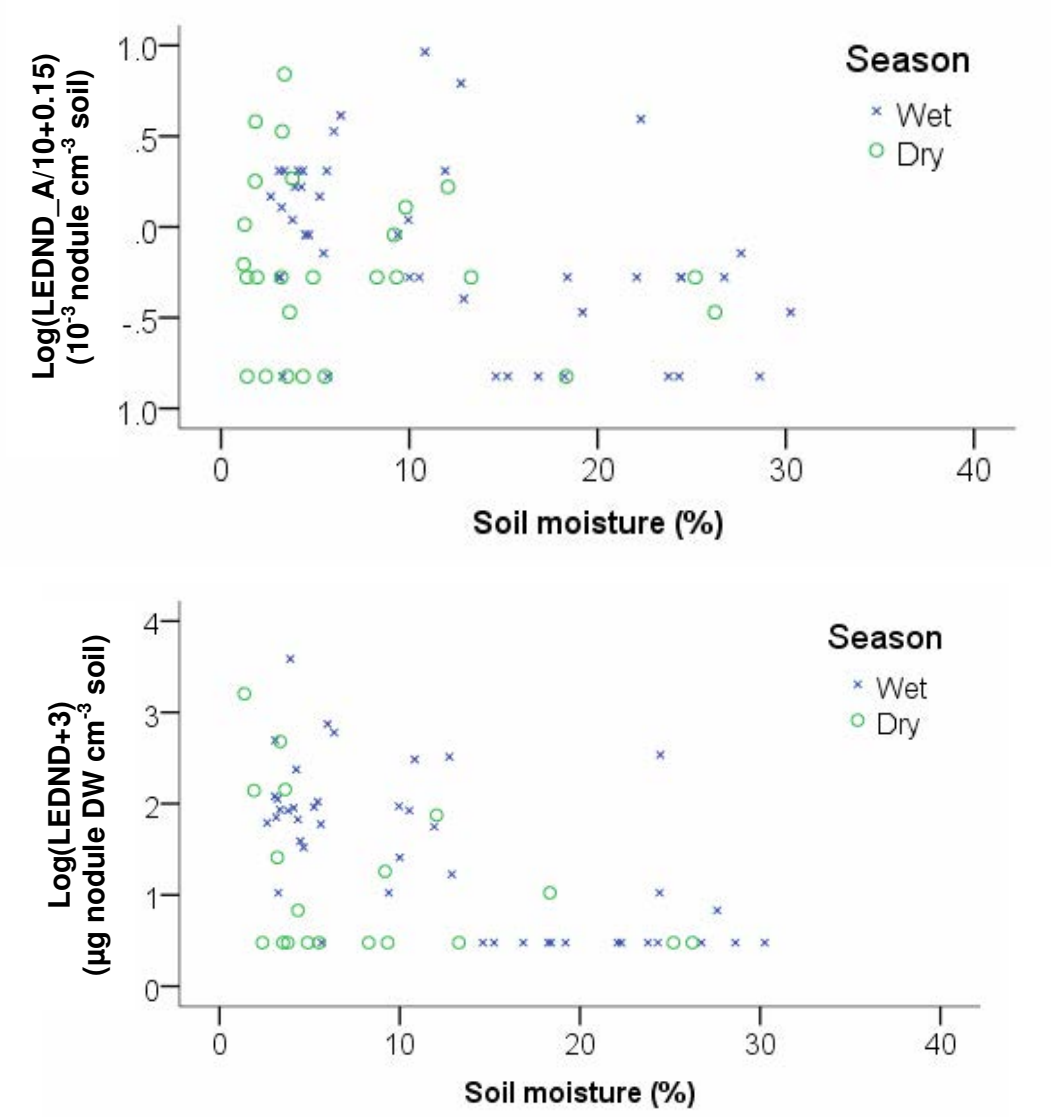


Figure 2.8. The relationship between soil moisture and the logarithmic functions of effective nodule density (upper) and dry weight (lower) of *A. crassicarpa* on the Townsville Town Common. Each mark is a core average of a specific distance (20 vs 40-80 cm) and depth (0-12 vs 12-24 cm) of each sampled individual.

Higher organic carbon at the top 24 cm soil suggested more organic debris which could originate from aboveground litter and surface fine roots. They were known to modulate soil structure , creating an environment that favour higher soil moisture (Brady and Weil 2010).

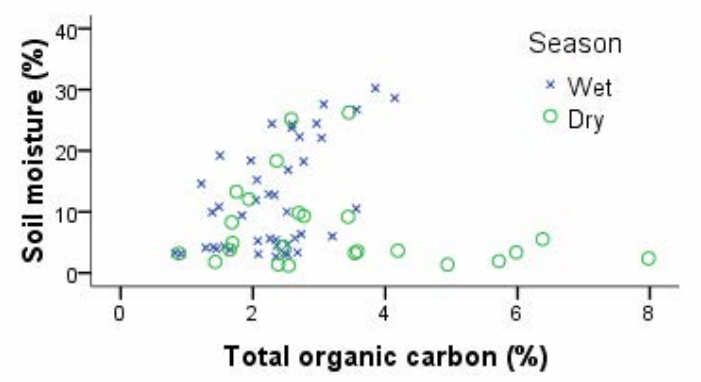


Figure 2.9. The relationship between soil moisture and soil organic carbon under the canopy of *A. crassicarpa* in the wet and dry seasons, on the Townsville Town Common. Note the highest level of soil organic carbon in the dry season was greater than that in the wet season. Each mark is a core average of a specific distance (20 vs 40-80 cm) and depth (0-12 cm vs 12-24 cm) of each sampled individual.

While the modelling indicated soil total nitrogen was regressed negatively with ineffective nodule density ($p < 0.002$) (Table 2.4), seasonality was also a significant factor (Fig. 2.10). The average ineffective nodule density was clearly higher in the dry season than in the wet season. Also, many core samples in the dry season had higher soil total nitrogen while many core samples in the wet season had lower soil total nitrogen.

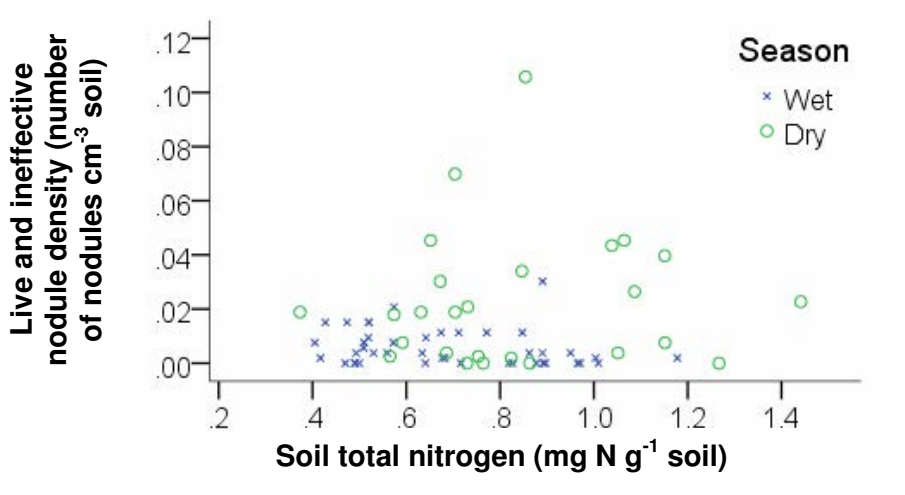


Figure 2.10. The relationship between the density of ineffective nodules of *A. crassicarpa* and soil total nitrogen at Townsville Town Common Conservation Park in the wet and dry seasons. Each mark is a core average of a specific distance (20 vs 40-80 cm) and depth (0-12 cm vs 12-24 cm) of each sampled individual.

2.3.3 *Variation of $\delta^{15}N_{foliage}$, foliage nitrogen concentration and soil nitrogen concentration among species, sites and seasons*

$\delta^{15}N_{foliage}$ values of all *Acacias* were negative, suggesting high ^{15}N discrimination which can occur in symbiotic nitrogen fixation and/or mycorrhizal symbiosis (Fig. 2.11 i). Interestingly, *A. crassicarpa* trees having abundant nodules were found to be less negative in $\delta^{15}N_{foliage}$ than three other *Acacia* shrub species with few or no surface nodules. The mean $\delta^{15}N_{foliage}$ value of *A. crassicarpa* was -1.71‰ in the wet season and -1.68‰ in the dry season. The mean $\delta^{15}N_{foliage}$ values of the other three species ranged from -2.76 to -3.47‰.

No significant differences in $\delta^{15}N_{foliage}$ between seasons occurred for coastal *A. crassicarpa* and *A. aulacocarpa* but for inland *A. elachantha* and *A. ramiflora,* $\delta^{15}N_{foliage}$ was significantly more negative in the dry season. The seasonal difference in mean $\delta^{15}N_{foliage}$ value for *A. ramiflora* was 0.72‰ and 0.03 to 0.32‰ for the other three species. From the results, one can conclude that inland species showed increased reliance on symbiotic nitrogen fixation and/or mycorrhizae in the dry season, so as to obtain sufficient nitrogen to meet their nitrogen requirement.

The phyllodes of *A. crassicarpa, A. elachantha* and *A. ramiflora* were high in nitrogen t (1.71 to 2.17%), while *A. aulacocarpa* phyllodes contained much less nitrogen (1.25 to 1.41%) (Fig. 2.11 ii). However, both *A. crassicarpa* and *A. aulacocarpa* phyllodes were more enriched in nitrogen in the dry season while *A. elachantha* and *A. ramiflora* phyllodes had significantly less nitrogen in the dry season.

Soil nitrogen concentrations below trees/shrubs decreased in the order TTCCP (0.76 mg N g^{-1}) > Horseshoe Bend (0.57 mg N g^{-1}) > Burra Range (0.34 to 0.36 mg N g^{-1}) (Fig. 2.11 iii). Total soil phosphorus was highest under *A. crassicarpa* (123µg P g^{-1}), followed by *A. ramiflora* (78 µg P g^{-1}), *A elachantha* (67 µg P g^{-1}) and *A. aulacocarpa* (64 µg P g^{-1}) (Fig. 2.11 iv).

Phyllode nitrogen content was not directly related to soil nitrogen concentrations. Also, soil nitrogen in the dry season was higher than in the wet season except below *A. ramiflora,* presumably because the limited moisture and lower average temperature inhibits microbial decomposition of organic matter, leading to accumulation of total nitrogen in the soil.

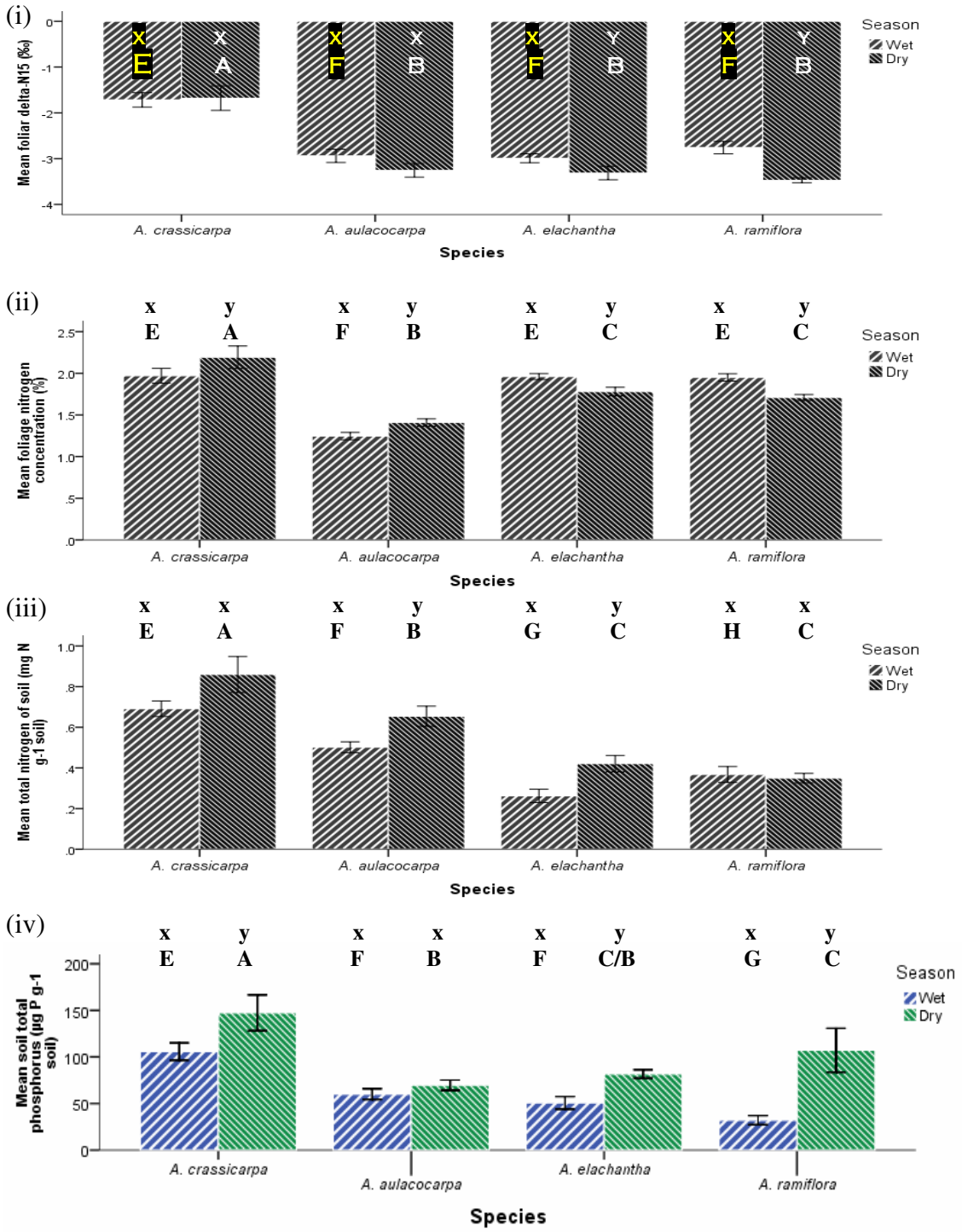


Figure 2.11. The seasonal and inter-specific differences in (i) $\delta^{15}N_{foliage}$, (ii) foliage nitrogen concentration, (iii) soil total nitrogen concentration and (iv) soil total phosphorus concentration, from the field study. Different lowercase indicate significant differences between seasons for each species. Different uppercase letters indicate significant differences between species in dry season (ABC) and wet season (EFGH) only "C/B" in the last bar chart indicates an ambiguous relationship of *A. elachantha* to *A. aulacocarpa* and *A. ramiflora* due to insufficient power of pairwise comparison.

In order to calculate the percentage of nitrogen derived from the atmosphere (%Ndfa), it is necessary to determine the natural abundance of ^{15}N isotope of nitrogen fixing *Acacia* species forced to rely on atmospheric nitrogen as their sole source of nitrogen ($\delta^{15}N_F$), of those taking up both soil nitrogen and atmospheric nitrogen ($\delta^{15}N_A$) and of non-nitrogen fixing reference plants ($\delta^{15}N_R$). Field-collected phyllodes clearly differed from shadehouse samples in terms of $\delta^{15}N$ (Table 2.7). The phyllodes of non-N-fixing seedlings taking up nitrogen only from soil had positive $\delta^{15}N_R$, while field-collected ones had negative $\delta^{15}N_F$. The phyllodes of seedlings relying largely on symbiotic nitrogen fixation for nitrogen had negative $\delta^{15}N_A$.

Table 2.7. Mean $\delta^{15}N_{foliage}$ (±1 S.E.M.) of N-fixers in the field and in the shadehouse, and of non-N-fixers in the shadehouse

$\delta^{15}N_F - \delta^{15}N_{foliage}$ of N-fixers growing in the field; $\delta^{15}N_A - \delta^{15}N_{foliage}$ of N-fixers in shadehouse; $\delta^{15}N_R - \delta^{15}N_{foliage}$ of non-N-fixers in shadehouse; %Ndfa – the proportion of nitrogen obtained from symbiotic nitrogen fixation. Values in parentheses are numbers of samples.

Species	Seasons	$\delta^{15}N_F$ (‰)	$\delta^{15}N_A$ [A] (‰)	$\delta^{15}N_R$ (‰)	%Ndfa
A. crassicarpa	Wet	-1.71 ± 0.16 (11)	-2.14 ± 0.34 (3)	2.22 ± 1.18 (2)	90% [C]
	Dry	-1.68 ± 0.27 (8)			89% [C]
A. aulacocarpa	Wet	-2.94 ± 0.14 (13)	-0.05 (1)	3.34 (1)	185% [D]
	Dry	-3.26 ± 0.15 (11)			195% [D]
A. elachantha	Wet	-2.99 ± 0.10 (11)	-2.92 ± 0.08 (3)	Not available [B]	---
	Dry	-3.32 ± 0.15 (10)			---
A. ramiflora	Wet	-2.76 ± 0.14 (10)	-2.75 ± 0.71 (2)	1.74 (1)	100% [C]
	Dry	-3.47 ± 0.05 (12)			116% [D]

[A] $\delta^{15}N_A$ was less negative than expected because, while ^{15}N taken up by N-fixers in the shadehouse from soil in the first month could be diluted by atmospheric ^{14}N fixed in the remaining four months of growth period, a minute amount of ^{15}N signal might still remain in phyllodes.

[B] Not enough samples for analysis and hence %Ndfa could not be calculated.

[C] %Ndfa were inflated because $\delta^{15}N_A$ was less negative than expected (see footnote A above)

[D] %Ndfa figures were invalid (see text for explanation).

In *A. crassicarpa,* $\delta^{15}N_A$ was more negative than $\delta^{15}N_F$ but in *A. aulacocarpa, A. elachantha* and *A. ramiflora,* $\delta^{15}N_A$ was less negative than $\delta^{15}N_F$. For instance, the $\delta^{15}N_A$ value of *A. aulacocarpa* was only -0.05‰ but its corresponding $\delta^{15}N_F$ value was as negative as -3.26‰. The $\delta^{15}N_A$ value of *A. ramiflora* was -2.75‰ while its $\delta^{15}N_F$ value ranged from -2.76 to -3.47‰. Theoretically $\delta^{15}N_A$ should always be more negative than $\delta^{15}N_F$. This is because air is the only nitrogen source of the former while both air and soil are the sources of the latter. It should be noted that the ^{15}N : ^{14}N proportion of air is much lower than that of soil. The fact that $\delta^{15}N_F$ was less negative than $\delta^{15}N_A$ reflect two possibilities:

- In the field, discrimination against ^{15}N was due to mycorrhizae and/or symbiotic nitrogen fixation; and
- N-fixers growing in the glasshouse obtained some nitrogen from soil such that ^{15}N : ^{14}N in phyllodes was higher than expected.

Mycorrhizae, in particular vesicular-arbuscular mycorrhizae that are known to occur in *Acacia* roots, may be responsible for the first possibility (Schmidt and Stewart 2003). A %Ndfa value of more than 100, caused by extra ^{15}N discrimination, is clearly invalid (Table 2.7). According to Hobbie *et al.* (2000)'s hypothesis, one of the symptoms of mycorrhizal association is the positive correlation between foliage NPUDW and $\delta^{15}N_{foliage}$, which was found significant in the field-collect phyllodes of *A. ramiflora* ($r_{0.05(2),\ 20} = 0.445$; $P = 0.038$).

$\delta^{15}N_A$ is less negative or more positive than expected because, while ^{15}N taken up by N-fixers in the shadehouse from soil in the first month could be diluted by atmospheric ^{14}N fixed in the remaining four months, a minute amount of ^{15}N signal might still remain.

Internal fractionation of ^{15}N can vary $\delta^{15}N$ by 2‰ (Section 2.1.7). Fractionation occurred in nitrogen mobilization and conversion, the extent of which may differ seedlings and mature trees/shrubs. Involving both age groups in calculating %Ndfa may cause invalidity. Also, employing the wrong reference plants could be other reasons. Reference plants of other species having similar growth form, root system and phenology are often selected in the field (Kreibich 2002) (also Section 2.1.7). This is in contrast to the current study using shadehouse-grown seedlings of the same species, in part due to difficulty in identifying suitable reference species in the field. As only the top 24 cm of field soil was collected for growing seedlings, the variation in $\delta^{15}N$ in form of nitrate or ammonium with different soil depths were not reflected in the $\delta^{15}N_R$.

2.4 Discussion

2.4.1 Changes in nodule distribution with depths and seasons

There are reasons to believe that most nodules of *A. aulacocarpa*, *A. elachantha* and *A. ramiflora* in the study area might develop in deeper soil while few were observed in the top 24 cm of soil under the canopies of these three species (Table 2.3). Negative foliage $\delta^{15}N$ of these three species suggested that ^{15}N discrimination occurred in the process of nitrogen acquisition which could be either by symbiotic nitrogen fixation or mycorrhizal symbiosis (Fig. 2.11).

The biomass of effective nodules of *A. crassicarpa* increased significantly in deeper soil which would be a more stable environment without significant daily temperature fluctuation and temperature extremes caused by fire events (Beadle 1940; Williams 2002). There was also a slight but non-significant increase in the density of effective nodules in deeper soil but by holding other factors constant, soil depth was a significant factor (Tables 2.3 and 2.4).

There were, however, no significant seasonal changes of the densities and biomass of effective nodules of *A. crassicarpa*. Coincidentally, there were no significant seasonal changes in the foliage $\delta^{15}N$ of the same species.

2.4.2 Factors affecting the nodulation of A. crassicarpa

Two principles should be borne in mind when interpreting how abiotic factors affect the nodulation of *A. crassicarpa*. Higher nodule numbers can result in higher total nodule weight, assuming that the average weight per nodule is the same. Thus if a higher density of nodules does not result in proportionally higher nodule dry weight, something else is affecting the nodule dry weight. Also since nodule density is nested within nodule biomass, an observed effect of a factor on the biomass could be indirect and hence should be interpreted with caution.

Soil depth was marginally non-significant in influencing both the density and dry weight of effective nodules. This may be attributable to the two measured depths being too close, one right below the other one. There was a significant decrease in effective nodule dry weight during the dry season, compared to the wet season . This decrease in dry weight likely reflected smaller effective nodules (Table 2.4).

Plants with wider crowns, and hence a greater number of phyllodes and branches, would require a higher absolute amount of nitrogen. However, crown width was not significantly correlated with an increase in density of effective nodules alone. Density did rise with crown width but the mixed model suggests that the crown width factor was correlated with other factors too. Nodule density was best explained by other factors, leading to the low P-value of the estimated regression coefficient of the crown width factor. Nevertheless crown width was still a moderate predictor that should be included in the mixed model according to AIC. In contrast, the dry weight of effective nodules was significantly correlated with crown width.

The lower importance of crown width in influencing density of effective nodules but greater importance of crown width in influencing dry weight reflected the fact that individual effective nodules got heavier and larger as crown width increased. This suggests a strategy in nodule development to cope with rising nitrogen demand. Large nodules were usually branched or elongated. The close relationship between nodule size and shape was observed because nodules in either of the two shapes have distinct meristematic tissues that can support an increase in number of bacteroids and the need for greater surface area to volume ratio for air exchange (Dixon and Wheeler 1986). If increasing a company's profit is employed as an analogy to tree growth, then enlarging existing nodules is similar to improving services to stimulate bigger spending by returning customers while producing more nodules is similar to opening more branches to attract more customers.

There was a crown width threshold at about 4 m below which no nodules were recorded (Fig. 2.9). Most individuals with crowns smaller than 4 m were *A. aulacocarpa, A. elachantha* and *A. ramiflora.* The threshold reflected inadequate sampling effort, leading to under-estimation of nodule density. A seedling however small could develop nodules, especially when the soil nitrogen level is insufficient to meet its demand (Section 4.3). Besides, smaller plants may develop nodules much closer to the trunk. Their nodule densities or dry weights would logically be lower as smaller plants have smaller absolute nitrogen demand. The negative $\delta^{15}N_{foliage}$ values of *A. aulacocarpa, A. elachantha* and *A. ramiflora* also fell within the range of nitrogen-fixing plants (*Acacias* in particular) (Shearer and Kohl 1986; Schmidt and Stewart 2003) (Fig. 2.11 i and Table 2.1). Nodules of *A. ramiflora* and *A. elachantha* may have developed in deeper soil where the fluctuations in soil moisture and temperature would have been less than near the surface. It should be noted that the surface soil in Australian savannas can be subject to greater fire disturbance than deeper soil in terms of fire frequency and intensity (Sections 1.1.2 and 1.1.3). It is not known if nodules would have developed in deeper soil so as to avoid fire disturbance. This hypothesis should be tested in the future. Nevertheless, one can conclude that

nodules may have developed on these three *Acacias*. With greater sampling effort (e.g. sampling five locations at each distance instead of three and obtaining soil cores from deeper soil), the whole curve in Fig. 2.7 may be shifted upwards.

The density and dry weight of effective nodules of *A. crassicarpa* were higher under low soil moisture (Table 2.4 and Fig. 2.8), in contrast to the results reported in other literature (Habish 1970; Danso *et al.* 1992). For example, soil moisture of 15% was found to be optimal for *A. mellifera* and some other *Acacia* species in Sudan (Habish 1970). Given that *A. crassicarpa* soil and root nodules were sampled only once in the wet season and once in the dry season, this correlation could be an artefact of temporal variability. Otherwise, adequate moisture was still important but low moisture probably reflected *A. crassicarpa*'s extra requirement for good drainage. Given the general low water-holding capacity of siliceous sand (Murtha and Reid 1992), and the low moisture index or low mean rainfall to evaporation ratio in Australian tropical savannas (Table 1.1), water can quickly evaporate or percolate through soil to great depths. The results indicated that most soil patches sampled were low in moisture content. Those patches with high moisture content probably reflected their texture heterogeneity, containing more organic matter that has good water-holding capacity (Fig. 2.9).

The densities and dry weights of effective and dead nodules of *A. crassicarpa* were not correlated with total phosphorus and total nitrogen in the soil . Ineffective nodules, on the other hand, were negatively correlated with total soil nitrogen, mainly due to an increase in the dry season (Fig. 2.10). In the dry season, soil organic matter increased, apparently due to an observed increase in phyllode abscission and potentially reduced rate of decomposition at low soil moisture. Finally, the mixed linear regression model confirmed that a significantly higher density of ineffective nodules resulted in the dry season (Table 2.4). By correcting for the multicollinearity between total soil nitrogen and seasonal changes, the higher density of ineffective nodules at lower nitrogen levels could be explained by the seasonal effect.

2.4.3 Variations of $\delta^{15}N_{foliage}$, foliage nitrogen concentration and soil nitrogen concentration among species, sites and seasons

Why were the $\delta^{15}N_{foliage}$ values of *A. aulacocarpa* and the two inland *Acacias* more negative than *A. crassicarpa*? Where soil nitrogen is low, symbiotic nitrogen fixation often becomes more important to the nitrogen nutrition of *Acacias* (Brockwell *et al.* 2005). Thus the significantly higher soil NPUDW, recorded under the *A. crassicarpa* canopy (Fig. 2.11 iii), might be responsible for the less negative $\delta^{15}N_{foliage}$ compared with other species.

The negative relationship between soil nitrogen and symbiotic nitrogen fixation may be weakened when the effect of water stress is considered. Firstly, the rainfall to evaporation ratio clearly decreased from the coastal areas to the inland sites (Table 1.1). While soil moisture does not necessarily decrease in the same order, possibly because of different soil textures, the water stress imposed on plants is likely to increase. The soil texture at inland sites contained higher proportions of clay (Table 1.3). The soil moisture at which plants can no longer extract water from soil by transpiration pull, and hence begin to wilt (known as the permanent wilting point), rises with clay content (Rodríguez-Iturbe and Porporato 2006). The lower rainfall to evaporation ratio, coupled with higher permanent wilting point, is likely to lead to higher water stress for inland *Acacias* than coastal *Acacias*.

Secondly, it should be noted that enough water has to be available to maintain turgor pressure and efficient transport of substrates and products in and out of N-fixing bacterioids in nodules (Dixon and Wheeler 1986; Madigan *et al.* 2003). Therefore, when water stress becomes more severe and a deep tap root system capable of accessing soil water at greater depths is not available, plants can neither absorb nutrients directly from the soil nor rely on symbiotic nitrogen fixation.

It is therefore deduced that *Acacias* sampled in this study, when subject to water stress, may increasingly rely on mycorrhizal symbioses to obtain nitrogen. Hyphae networks are known to survive drought reasonably well (Brundrett *et al.* 1996). Hyphae may adapt to drought even better than nodules, but such potential differences in drought tolerance are yet to be tested. Mycorrhizal uptake of nitrogen can cause consistently or even more negative $\delta^{15}N_{foliage}$ values, as shown in another study (Schmidt and Stewart 2003). Insufficient soil nitrogen could result in a significantly lower phyllode nitrogen content of *A. elachantha* and *A. ramiflora* in the dry season, partly because of low direct root uptake and, even with mycorrhizae, much of the nitrogen taken up by hyphae is not transported to roots but retained inside hyphae to meet nutritional demands (Fig. 2.2 and see Section 2.1.7 for detailed explanation of the interaction between hyphae and roots).

For trees of *A. crassicarpa* with a mean height of 9.4 m, water stress might not be as severe as for *A. aulacocarpa*, *A. elachantha* and *A. ramiflora* with mean heights of 4.3 m, 4 m and 6.1 m respectively, if *A. crassicarpa* possesses a deep tap root system and water is available in deeper soil. Deep tap root systems allow plants to tap water from deeper soil for transfer to upper roots and nodules through hydraulic lift (Bacon 2004). Adequate water is important to effective functioning of nodules. With such a structural advantage that the other three studied *Acacias*

might not have, *A. crassicarpa* would likely compensate for the reduced direct root uptake in the dry season by fixing more nitrogen from the air. Comparing the water potential of root xylem in the four studied *Acacias* would help to verify the role of hydraulic lift in nodule functioning in wet and dry seasons.

These factors could explain why the $\delta^{15}N_{foliage}$ values of *A. elachantha*, *A. ramiflora* and *A. aulacocarpa* were more negative than the $\delta^{15}N_{foliage}$ value of *A. crassicarpa* (Fig. 2.11 i).

Whether symbiotic nitrogen fixation plays a significant role in *A. aulacocarpa*, *A. elachantha* and *A. ramiflora* in nature would have to be examined through *in situ* experiments. Apart from the inadequacy of the sampling design in the current study, low nodule densities of the three *Acacias* mentioned above in surface soil might also be due to a compensatory increase at deeper depths up to 50 cm to 1 m, where most nodules may be found (Hogberg and Kvarnstrom 1982; Hogberg and Wester 1998; Peter and Lehmann 2000). Soil moisture is expected to increase with depth and high soil temperatures avoided. Clay has a high water-holding capacity and its proportion within soil tends to increase with depth (Brady and Weil 2010). Inland areas have a higher maximum daily temperature, and the smaller sized *Acacias* provide less shading than a large *A. crassicarpa* tree. Soil immediately around *A. elachantha* and *A. ramiflora* can absorb more heat, and root nodules near the surface are vulnerable to overheating. A resource allocation strategy to develop nodules near the surface appears detrimental to their functioning.

Do we have evidence of increased nodulation as soil gets deeper and as the weather becomes drier? Some evidence is available from *A. crassicarpa* (Table 2.3). In contrast, *A. aulacocarpa* appeared to have more nodules (dead) in the dry season in surface soil than in the wet season but deeper soil had fewer nodules in both seasons. There were too few nodules of *A. elachantha* and *A. ramiflora* to reveal any pattern. It is acknowledged that the 0 to 12 and 12 to 24 cm soil depths can only be deemed as "surface soil" with reference to the general depth that is found with most fine roots and/or nodules. For example, *Acacia mangium* in Malaysian tropical forest that is 3 to 4 m in height has 67% of the total fine root biomass in the top 15 cm of soil and almost all of the total fine root biomass within the top 45 cm (Hogberg and Wester 1998). Most root nodules of *A. mangium*, if any, would logically occur in the same depth range. *Acacia saligna* in dry tropical savanna in North Kenya has the maximum root length density in the top 15 cm of soil, within 25 cm of the tree trunk (Peter and Lehmann 2000). Unfortunately, Peter and Lehmann (2000) did not describe the age of the small trees. In parts of Tanzania with annual rainfall of 870 mm and mean annual temperature of 24.4^{o}C, most fine roots and nodules of *Leucaena leucocephala* can be found in the top 50 cm of soil (Hogberg and Kvarnstrom 1982). It is also recommended that when collecting roots with a soil auger, at least 30 cm of soil

depth should be sampled "*because most of the roots will probably be concentrated in this layer"* (Oliveira *et al.* 2000). Finally, the depth and horizontal distribution of root systems can differ between species, climatic zone and soil properties (Bengough *et al.* 2000). They can also be affected by intraspecific or interspecific competition (Hutchings and John 2003). A full excavation of a few individuals of the four studied *Acacias* would help to determine the "optimal" soil depth to be sampled.

Furthermore, the total soil nitrogen under *A. aulacocarpa* and *A. elachantha* increased significantly in the dry season. Higher concentrations of soil total phosphorus were also recorded under *A. crassicarpa, A. elachantha* and *A. ramiflora* in the dry season, but in each of the four species, soil organic carbon did not differ significantly between seasons. The mean organic carbon of the surface soil under *A. crassicarpa* was apparently higher in the dry season than the wet season but a large standard error makes the statistical comparison not significant. It is possible that while the total amount of debris on the soil's surface was similar between seasons, the rate of microbial decomposition potentially decreased in the dry season due to soil moisture depletion. Lower temperatures can potentially slow down the root uptake kinetics by plants. These two processes may have resulted in a significant amount of nitrogen and phosphorus locked up in the dead organic matter. Unfortunately, plant-available inorganic nitrogen in soil was not measured as it was believed that measuring total soil nitrogen would be sufficient to demonstrate its correlation with symbiotic nitrogen fixation, as shown in other studies (Brockwell *et al.* 2005). One would expect the plant-available inorganic nitrogen level in soil to decrease if most nitrogen in litter got locked up under reduced mineralization. Both mineralization and nitrification are known to be faster in the wet season or during the transition from dry to wet season (Pate *et al.* 1998; Schmidt and Lamble 2002), and at sites with high nitrogen availability (Viani *et al.* 2011). Reduced plant-available inorganic nitrogen in soil, if any, could be one reason for increased reliance on symbiotic nitrogen fixation and/or mycorrhizae symbiosis.

2.5 Conclusion

In short, *A. aulacocarpa*, *A. elachantha* and *A. ramiflora* had fewer nodules than *A. crassicarpa* species in the top 24 cm of soil. Nodules of inland species were rare in the field but in view of the negative foliage $\delta^{15}N$ values, they might have developed deep in the soil or very close to the trunk and were missed by the sampling protocol adopted in this study. The two coastal and two inland species appeared to acquire nitrogen partly through symbiotic nitrogen fixation, but mycorrhizae might also play a role in the nitrogen nutrition of these four species. Calculating

the percentage of nitrogen derived from symbiotic nitrogen fixation compared to soil is problematic and appears to have suffered from a methodological flaw. Soil depth, crown width, season, soil moisture, soil bulk density and *Acacia* root biomass were key factors in the development of effective nodules.

3 Germination of tropical inland and coastal *Acacia* species under different treatments

3.1 Introduction

Seedlings were required for the provenance and drought experiments, so the opportunity was taken to extend the studies of germination of the study species. Many *Acacia* species germinate in response to fire in nature. While some studies investigate the effect of smoke on their germination, some examine the effect of heat. Heat is known to break the seed coat of *Acacias* (thus breaking the physical dormancy) but species vary in their dormancy breaking temperature and duration. The effects of heat treatments on the seed germination of tropical *Acacia* species were previously studied by Congdon *et al.* (in prep.). The current seed germination experiment aims to complement the study by testing the effect of more heat treatments. It investigated whether two inland *Acacia* species are more adapted to the inland semi-arid environment through developing seeds with weaker physical dormancy (hence weaker seed coat) than coastal species, that receive rain more frequently along the eastern coast. It also examines whether the heat sensitivity and tolerance of inland *Aacia* seeds differ from those of coastal *Acacia* seeds. The literature review below introduces the concept of physical dormancy and its relationship with seed germination. Methods known to break physical dormancy, in particular heat treatments, and their effects on the seeds of various *Acacia* species are discussed.

3.2 Seed production, dispersal and germination

Seeds develop into seedlings which as adults replace their parent plants in the short-term or long-term. Before germination, they are usually dispersed by various agents such as birds, wind or mammals. In this way, a species can establish a new population, or expand its current population into new areas with favorable environmental conditions. Seed dispersal is thus essential to sustain or even increase populations through extending gene flow, thus maintaining or increasing the species' long-term survival in natural ecosystems and reducing the extinction risk caused by a single environmental perturbation. The number of seed plants worldwide has been estimated to be from 223 300 to more than 400 000 (Scotland and Wortley 2003; Ungricht 2004), while the total number of flowering plant and gymnosperm species in Australia was estimated at about 21,120 (Chapman 2009). Large trees and shrubs that dominate prominent habitats such as forests, woodlands and mangroves are seed plants, and hence understanding seed biology and ecology will help to explain the role that seeds play in the long-term sustainability of these habitats in nature.

3.3 Seeds of Acacia species

Acacia spp. undergo sexual reproduction and produce seeds which are usually enclosed inside dry dehiscent pods that split soon after maturity. Different *Acacia* spp. have their own flowering and fruiting cycle (Gunn 2001). For example, *A. elachantha* flowers from May to August and fruits from September to November (Gunn 2001; McDonald 2001). On the other hand, *A. aulacocarpa* flowers from April to June and fruits in the second half of the year (Simmons 1981) or September to November (Gunn 2001).

In general, a seed comprises embryos with seed leaves (cotyledons) and endosperms which are protected by the testa (Langkamp 1987). However, the endosperms of *Acacia* seeds very often become vestigial or are entirely absorbed by the cotyledons, so the cotyledons perform both food storage and photosynthetic functions (Guinet and Vassal 1978; Ashcroft and Murray 1979). *Acacia* seed coats are hard and impermeable to water, and such a water-repelling property is due to the presence of a cuticle and one or more layers of impermeable palisade cells underneath. The cuticle is composed of hydrophobic lipids and pectins, while the palisade layer comprises Malpighian or macrosclereid cells which possess lignified secondary walls and water-repellent substances such as cutin, suberin and wax (Baskin and Baskin 1998).

On the seed coat of *Acacia* species can be found the hilum (a scar left by the detachment of the funicle from the developing seed), the micropyle (the pollen tube entry point into an unfertilized ovule) and a small rounded spot known as the lens on the opposite side of the micropyle (Langkamp 1987; Gunn 1989). The different thickness and density of Malpighian cells among these three structures result in different tolerances to rupture, i.e. given the same level of heating treatment, the micropyle and hilum remain relatively impermeable to water while cells comprising the lens rupture to allow imbibition (Burrows *et al.* 2009). The Malpighian cells are relatively thin and short in the lens area compared to near the hilum. Application of a suitable heating treatment in the laboratory, or alternating heating and cooling in the wild, may cause the lens to partially or completely separate from the seed coat (Langkamp 1987; Burrows *et al.* 2009). For this reason, soaking in boiling water for 1 min is recommended for breaking seed dormancy of several *Acacia* spp., such as *A. cincinnata, A. crassicarpa, A. melanoxylon* and *A. mangium* (Gunn 1989).

3.3.1 Defining seed dormancy

There have been several definitions of dormancy and how applicable they are depends on the type of dormancy one is referring to. Seeds which germinate and do not germinate were considered as non-dormant and dormant, respectively (Harper 1959). In a similar sense, dormancy was also defined as *"temporary failure of a viable seed to germinate, after a specified length of time, in a particular set of environmental conditions that later evoke germination when the restrictive state has been terminated by either natural or artificial means"* (Simpson 1990). The third definition stated that seed dormancy was an inability of viable seeds to germinate under favourable environmental conditions (Langkamp 1987; Bewley 1997), which can be apparent optimal soil temperature and moisture. These traditional definitions imply that dormancy is always a binary character. In order to proceed from a dormant to a non-dormant state, dormancy has to be 'broken'. In the first two traditional definitions, when seed germinates, it becomes non-dormant. Seeds do not germinate when they are dormant and viable. However, the third definition describes the seeds' responses to their surrounding environment more accurately. To explain this further, when a seed germinates, it becomes non-dormant. If a seed does not germinate, this can be a result of (i) unfavorable temperature and/or moisture, or (ii) that the seed has an inherent property that prevents it from germination even if the environmental conditions are favorable. This inherent property is called seed dormancy.

The three traditional definitions, however, cannot be applied specifically to seeds with physiological dormancy because their environmental requirements for germination fall within a range (e.g. range of temperature suitable for germination). When this range of requirement is large, seeds have a low degree of dormancy, and vice versa. Thus the dormancy of each individual seed varies along a continuous scale but does not operate like a binary on/off switch. A seed which has a low degree of dormancy does not necessarily germinate once it has been dispersed because the current environment in the field may not match its germination requirement. Seed germination thus requires matching of current environment and the seeds' internal requirement. Based on such logic, seed germination is independent of seed dormancy. Thus the more accurate definition of seed dormancy is that *"seed dormancy is a characteristic, the degree of which defines what conditions should be met to make the seed germinate"* (Vleeshouwers *et al.* 1995).

Germination requirements of physiologically dormant seeds can vary in response to environmental cues. The variation is known as sensitivity changes. Environmental cues may cause sensitivity changes by inducing physiological changes of, and interactions between,

embryo, endosperm and/or seed coat. For example, cold stratification can widen the temperature range at which germination occurs, thus increasing the seeds' sensitivity to environmental conditions and lowering the degree of seed dormancy. For example, seeds of *Polygonum persicaria,* which have a high degree of dormancy, display a very narrow germination temperature range throughout summer and autumn (Vleeshouwers *et al.* 1995). Decreasing temperature in early winter acts as a cue to initiate dormancy relief, lowering the minimum temperature required for germination. The condition of relieved dormancy continues throughout the winter and early spring. In early spring, the rising temperature of the environment matches and even falls within the now widened germination range of the seeds, thus resulting in seed germination. However as the environmental temperature continues to increase from early spring to late spring, dormancy is gradually induced again leading to a narrowing of germination temperature range (Vleeshouwers *et al.* 1995). This example falsifies the binary division of dormancy into dormant and non-dormant, the tradition of defining germination as a reciprocal of dormancy, and the use of the terminology "breaking dormancy".

Seeds which exhibit morphological or physical dormancy have a barrier which they must overcome as a pre-requisite before they can be stimulated to germinate. This barrier can be an ontogenetic barrier, i.e. the embryo differentiating and reaching maturity. It can also be a physical barrier that prevents water imbibition. Germination of seeds with either of the two types of dormancy therefore requires two distinct stages. Stage one requires breaking dormancy. Thus we can apply the third traditional definition "the inability of viable seeds to completely germinate under favourable environmental conditions" (Langkamp 1987; Bewley 1997), which can be apparent under optimal soil temperature and moisture. For a species whose seeds are predominantly physically dormant, we often find exceptional ones which are non-dormant. This was found in the current study for seeds of *Acacia ramiflora, A. aulacocarpa, A. elachantha,* and *A. crassicarpa*. Non-dormant seeds of these species were usually brown instead of black in appearance and revealed a soft seed coat upon squeezing. When dissected, their embryo colors were usually slightly greenish instead of white or yellow, perhaps indicating immaturity. Non-dormant seeds resulted probably because of poor seed development such that seeds remained immature at the time of fruit dehiscence. Thus at the population scale, the proportion of seeds which are dormant or non-dormant can result in a "degree of dormancy" for a particular species.

Stage two of germination requires matching seed germination requirements with environmental conditions. Seeds of species from the families Fabaceae and Convovulaceae, which show physical dormancy, have recently been confirmed as exhibiting sensitivity cycling in response to field temperature and moisture (Jayasuriya *et al.* 2009). It was therefore theorized that changing field temperature and/or substrate moisture can affect a seed's sensitivity to different

potential physical dormancy-breaking methods. However, the difficulty lies in proving whether a treatment breaks dormancy or changes the seed's sensitivity to the environment (if sensitivity cycling does exist in the species concerned). While the current study does not attempt to look for sensitivity-changing behaviour of inland and coastal *Acacia* seeds, the above discussion

Table 3.1. Classification of seed dormancy

Classification by origin	Classification by nature	Brief description	Evidence
Seed coat-related (exogenous dormancy)	Physical	• Preventing imbibition by being a water-impermeable barrier in hard seeds of the plant families Mimosaceae, Fabaceae and Caesalpinaceae.	• The breaking of the 'lens' of *Acacia* seeds after boiling water treatment (or scarification), allowing water entry and stimulating seed germination.
	Mechanical	• Restricting embryo development with mechanical resistance.	• The restraining force of the testa of *Xanthium* seeds and the thrust exerted by the embryo were measured – the former was greater than the latter.
	Physiological	• The possibility of inhibition of embryo development through plant hormones, such as Abscisic acid inside the seed coat, or through reduced oxygen diffusion into the embryo.	• Difficult to exclude confounding factors in manipulation experiments and hence difficult to prove. For example, prolonged washing of seeds may cause leaching of inhibitors but can also soften the seed coat. Isolation of inhibitors from seed coat also is inadequate to prove their role in seed dormancy. • Oxygen enrichment around intact seeds provokes germination, thus indicating that oxygen is a limiting factor to germination.
2. Embryo-related (endogenous dormancy)	Physiological	• Plant hormones, such as Abscisic acid, residing in cotyledons inhibit seed germination.	• Need to establish the state of dormancy and level of inhibitors in embryos. • Increasing germination after half and then three-quarters of the cotyledons are removed from the excised embryos of Apple (*Malus x domestica).*

Table 3.1 Cont'd.

Morphological	• Embryo is differentiated but is not fully grown or the embryo is only a group of undifferentiated cells. ➢ Differentiated but underdeveloped embryos gain their maturity after seeds have been dispersed under suitable moisture, temperature and light or dark environments. These are usually embryos with linear or rudimentary shape out of the 12 embryo shapes identified in the current literature. ➢ Undifferentiated embryos are found only in micro- and dwarf seed shapes.	• Families such as Araceae, Araliaceae, Oleaceae, Podocarpaceae, Smilacaceae have one or more species with rudimentary or linear embryos. • However, not all rudimentary or linear embryos are underdeveloped but nevertheless those families with rudimentary or linear embryos would be a starting point for anatomical examination. • An example of a family with micro- and dwarf seeds is the Orchidaceae.

Source: Langkamp (1987) Baskin and Baskin (1998) Sweedman et al (2006)

provides a framework for future research to better understand the interaction between seed dormancy, germination and the surrounding environment.

3.3.2 Breaking seed dormancy of Acacia species

Seed dormancy can be classified by its nature, origin or the sequence of the causes (Table 3.1). Seed dormancy of *Acacia* species is primarily physical dormancy, i.e. prevention of imbibition by an impermeable seed coat. The embryo of *Acacia* species is believed to be non-dormant. Consequently, much research has focused on methods to soften the seed coat or to break the seed coat to allow imbibition. In general, the following methods have been tested on many plant species with physically dormant seeds including those of *Acacia* species (Langkamp 1987):

- Mechanical scarification
- Acid scarification
- The use of enzymes such as hemicellulase and organic solvents such as ethyl alcohol
- Percussion/impaction
- Immersion in hot water
- Subjecting seeds to dry heat
- Dry storage
- Infrared, radio and microwave radiation, and ultrasound
- Freezing at an extremely low temperature

Mechanical scarification, acid scarification, immersion in hot water and dry heat are of particular interest to breaking the seed dormancy of *Acacia* species. It was argued that subjecting *Acacia* seeds to boiling water could produce erratic germination response, and hence alternative methods such as mechanical or acid scarification should be considered (Langkamp 1987). However, soaking seeds in boiling water for 1 minute or less can achieve more than 80% final germination rate in both arid zone and wet tropics species (Gunn 1989; Burrows ***et al.*** 2009). In fact, a closer look at the four examples quoted by Langkamp (1987) revealed that the boiling water treatments employed in those examples differ significantly. Highest germination percentages (GPs), 33% and 76%, were recorded for *A. crassicarpa, A. oncinocarpa, A. latescens, A. rothii* and *A. torulosa* in two studies that employed the 'pour and soak' method, i.e. by pouring boiling water over seeds and letting the seeds soak in the cooling water until it reaches room temperature (Morton 1982; Barrett and Fox 1983). This method is now known to give variable germination performance since the rate of cooling down can be fast or slow

depending on the room temperature of the laboratory concerned. The heat transfer to the seed coat thus can sometimes be quite inadequate to cause the lens to rupture. In another study, the seeds of *A. torulosa, A. oncinocarpa, A. browniana, A. cyclops* and *A. saligna* were boiled for 10 minutes (Grahame and Nihill 1979). The highest germination recorded for these species ranged from 3% to 41%. This is not unexpected since 10 minutes at high temperature has been confirmed to be too severe for *Acacia* seeds (Gunn 1989; Burrows *et al.* 2009). Similarly, this probably explains the low germination percentages of *A. urophylla* and *A. crassicarpa* in another study that also boiled the seeds for up to 10 minutes (Glossop 1982).

Soil moisture interacts with fire to facilitate seed germination of *A. aneura* from semi-arid or arid regions. It has been suggested that soil moisture reduces the level of heat reaching seeds *per se,* because some of the heat is dissipated during water vaporization (Beadle 1940). This is important in both fine-fuel fires when leaves and stems of grasses and forbs are burnt, and litter fires which burn mainly leaves and stems of woody plants. Fire types differ in terms of fire-line intensity which is the product of heat of combustion of the fuel (unit: kJ kg^{-1}), dry weight of the fuel (kg m^{-2}) and fire velocity (m s^{-1}). The former type of fire can result in a much higher fire-line intensity of shorter duration, and tends to result in much higher temperatures on the surface compared to 1 cm or 2 cm soil depth, while the latter has a lower fire-line intensity but of much longer duration and results in a more uniform temperature increase from the surface to 2 cm below the soil surface (Hodgkinson and Oxley 1990). It was demonstrated in an experimental fine-fuel fire that the surface fire temperature is much lower on wet soil (mean is about $70^{o}C$ min^{-1}) than dry soil (mean is about $105^{o}C$ min^{-1}). Consequently, the germination percentage of *A. aneura* seeds is higher on a wet than dry soil surface under a high fire-line intensity fire (Hodgkinson and Oxley 1990). In addition, the dissipation of heat by soil water is also important in protecting seeds from being killed by a slow-burning fire. In the same experiment, when a slow-burning fire was applied, all seeds of *A. aneura* on the surface of the dry soil failed to germinate but the germination in wet soil was much more uniform across the surface and sub-surface.

Some inland *Acacia* species, which are adapted to drier areas, have been observed to show low levels of seed dormancy and can germinate without additional heat treatment under suitable environmental conditions (Langkamp 1987; Congdon *et al.* in prep.). Examples include *A. cambagei* and *A. harpophylla* (Langkamp 1987). No hypothesis was proposed as to why these two species have low levels of seed dormancy. 20% of *A. aneura* seeds, collected from Vaughan Springs, Northern Territory, germinated when no seed-dormancy breaking treatments were applied (Doran and Gunn 1987), but the authors suspected the seed coat was broken accidentally during extraction rather than being an inherent characteristic of the seeds. A recent

unpublished study revealed that about 80% of *A. ramiflora,* and more than 90% of *A. hyaloneura* seeds, respectively, germinated without treatment (Congdon *et al.* in prep.). Seeds of *A. flavescens* and *A. elachantha,* also collected from the semi-arid Burra Range in Queensland, yielded about 30% and more than 40% germination without treatment. Their germination percentages were all higher than for coastal *A. cincinnata, A. crassicarpa* and *A. mangium* seeds without treatment (Congdon *et al.* in prep.).

On the other hand, some inland species show a relatively high level of seed dormancy. Inland *A. ampliceps,* from Wave Hill in the Northern Territory, had 0% germination without treatment, and *A. stenophylla* from Cow Creek in Western Australia had 10% (Doran and Gunn 1987). Another inland species, *A. platycarpa* from the Burra Range in semi-arid Queensland, had a high level of seed dormancy (Congdon *et al*. in prep.).

In a fire-prone environment, seedlings of many *Acacias* are usually recruited from germinated seeds in the wet season after fire. The mortality rates of seeders in other genera, even if the fire is relatively mild or moderately intense, are often high (Gill 1981; Ojeda *et al.* 2005). In a very broad sense, as with the other genera of the same family Mimosaceae, several *Acacia* species have seeds with a high level of physical dormancy. Thus it seems that *Acacia* seeders in a fire-prone environment are linked to high dormancy. On the other hand, *Acacia* resprouters can recover from fire through storage organs or buds. For instance, *A. ramiflora* adult shrubs are known to resprout from stem bases after fire (Williams *et al.* 2004). Resprouters of other genera often can also survive wildfires, except fires of extreme intensity (Gill 1981; Ojeda *et al.* 2005). It has been suggested that species that divert a substantial amount of resources to fire survival (e.g. thick bark) and fire recovery, rely more on resprouting than seeding to maintain fitness. When a substantial amount of resources were diverted elsewhere to fire survival (e.g. thick bark) and fire recovery, it has been suggested that a plants resprout more instead of seeding to maintain fitness. Since *"soils of many Australia tropical savannas are low in nutrients"* (Williams 2002), it seems possible that fewer resources would be invested into developing seed coat hardness and/or thickness. A weaker or thinner seed coat may result in two possible consequences: poor fire resistance and higher mortality, and seedlings emerging as long as soil moisture and temperature are optimal, thus compensating for dead seedlings in between years of wildfire.

This chapter examines the degree of physical dormancy of a number of *Acacia* species. The effect of climate change may differ between inland and coastal species, and to determine this it is important to distinguish potentially different germination responses between inland and coastal *Acacia* species.

Also, landscape-level revegetation projects require direct seeding to save costs and maximise cost-effectiveness (Thrall *et al.* 2005). Small-scale revegetation projects have even more limited budgets and their plans usually include transplanting seedlings into field sites. Project managers thus look for ways to maximise seed germination rate. This study tested a number of physical dormancy breaking techniques, which together with another similar study (Congdon *et al.* in prep.), should provide valuable information to revegetation managers when their species mix include *Acacias.*

This chapter examines whether semi-arid species have different germination requirements from species from higher rainfall areas, that relate to the environmental conditions where these species occur naturally. The following hypotheses are proposed:

- The two inland species will germinate faster and at a higher percentage without any particular heat treatment than the two coastal species, because rainfall is sporadic in the arid zone. Inland species would have adapted to this to more rapidly take advantage of favorable conditions for growth.

- Physical dormancy exists in *Acacia* seeds, regardless of whether they occur in wetter coastal or drier inland environments. Therefore greater germination and faster germination rates will occur under manual scarification or heating treatments than without treatment.

- Water in association with heat helps to break down the seed dormancy of *A. ramiflora* significantly better than using heat only

3.4 Materials, methods and data analysis

3.4.1 Species and treatments

Germination of two coastal and two inland species was examined in relation to several treatments to test for dormancy (Table 3.2). It is acknowledged that the four species were not necessarily representative of other species in the two climatic regions. The seeds were collected in 2011 from their natural habitats, from at least ten individuals which were at a distance twice

the average height of trees or at least 100 m apart (Gunn 2001). As many seeds as possible from each site were collected to represent a population.

All seeds received experimental treatments within three months after collection. Some of the seeds from each species were manually scarified. These seeds were used for another experiment and manual scarification was shown to result in high germination percentage. The other treatments included a combination of 60°C, 80°C, 100°C for one minute or five minutes. They were chosen to supplement a recent study that examined the germination of similar *Acacia* species (Congdon *et al*. in prep.). Several treatment combinations of temperature and time period were used in the current study to extend the information from the previous study. For example, where 80°C water was applied for five minutes in the previous study, this study examined the effect of applying 80°C water for one minute. This study also compared the effect of dry heat and wet heat. No treatment was the control for every species.

3.4.2 Experimental procedure

As some seedlings would be grown in a shadehouse without nodulation for another experiment, seeds and petri dishes were dipped into 4.7% (v/v) bleach for three minutes and then rinsed three times with UV-sterilised or autoclaved water. Manual scarification of the seed coat was undertaken with a knife sterilised with 70% ethanol. Care was taken to avoid damaging cotyledons. Another seed would be used if cotyledons were damaged significantly during excision. Those receiving wet heat were put in a mesh bag. The sealed mesh bag was then immersed into hot water of a certain temperature. The required temperature was confirmed by two thermometers in the same water bath. The mesh bag was immersed for a specified period of time, removed from hot water and the seeds were carefully transferred from the mesh bag to a petri dish. Treatments were prepared separately for each replicate to prevent pseudoreplication (Morrison and Morris 2000) (Table 3.2). Those receiving dry heat were put in an oven, also with a thermometer for temperature verification. Seeds of each replicate were spread over a folded paper towel in a petri dish and sterilised water was added until the paper became completely wet. The petri dishes were loosely covered with lids. Seeds were incubated at 28°C in a constant temperature room with a 12 h light / 12 h dark cycle (Congdon *et al.* in prep.). The paper towel was kept moist every day or every two days with sterilised water, or a 2 g L^{-1} fungicide solution (Thiram™) if mould was present (Congdon *et al.* in prep.).

Table 3.2. Germination treatments applied to seeds collected from two inland and two coastal *Acacia* species in 2011

'dry' refers to oven heat while 'wet' refers to heated water

Species	Site of collection	Treatment			No of seeds and replicates
Acacia crassicarpa	Town Common Conservation Park, QLD	1) Without treatment 2) Manual scarification	3) 80°C wet, 1 min 4) 80°C dry, 1 min	5) 100°C wet, 1 min 6) 100°C dry, 1 min	Total 270 seeds - For each treatment, 3 replicates and 15 seeds per replicate
Acacia aulacocarpa	Horseshoe Bend near Paluma, QLD	1) Without treatment 2) Manual scarification	3) 80°C wet, 1 min 4) 80°C dry, 1 min	5) 100°C wet, 1 min 6) 100°C dry, 1 min	Total 270 seeds - For each treatment, 3 replicates and 15 seeds per replicate
Acacia elachantha	Burra Range, White Mountain National Park, QLD	1) Without treatment 2) Manual scarification	3) 80°C wet, 1 min 4) 80°C dry, 1 min	5) 100°C wet, 1 min 6) 100°C dry, 1 min	Total 270 seeds - For each treatment, 3 replicates and 15 seeds per replicate
Acacia ramiflora	Burra Range, White Mountain National Park, QLD	1) Without treatment 2) Manual scarification	3) 60°C wet, 5 mins 4) 60°C dry, 5 mins	5) 100°C wet, 1 min 6) 100°C dry, 1 min	Total 270 seeds - For each treatment, 3 replicates and 15 seeds per replicate

A separate study was conducted to examine the effect of "bleach and rinse". Three replicates of *A. aulacocarpa, A. elachantha* and *A. ramiflora* were subject to the "bleach and rinse" treatment as described in the previous paragraph. Another three replicates of the three species received no treatment and served as controls.

Germination was recorded daily or once every two days, as emergence of the radicle, for 109 days. At the end of the experiment, the ungerminated seeds in the control group were tested for viability using the tetrazolium method. This method requires seeds to be cut in longitudinal section so that the cut surface touches a filter paper saturated with 2,3,5-triphenyltetrazolium chloride (1%) at 20°C for six hours (Burrows *et al.* 2009). A pink or red colouration in the embryo indicates a seed was viable (Burrows *et al.* 2009).

3.4.3 Data analysis

Data was first pooled to calculate the germination percentage, and time to 50% germination. The time to 50% germination (T_{50}) was calculated using the formula:

$$T_{50} = t_i + \left[\frac{(N+1)/2 - n_i}{n_j - n_i}\right].(t_j - t_i)$$

(where N is the final number of seeds germinating and n_1, and n_2 are the total number of seeds germinated by adjacent counts at times t_i and t_j, where $n_i < (N+1)/2 < n_j$) (Beadle 1940)

3.4.3.1 Effect of "bleach and rinse" on germination percentages and speed

A two-way ANOVA was undertaken on log-transformed germination percentage to examine the potential interactions between species (*A. aulacocarpa, A. elachantha* and *A. ramiflora*) and bleach and rinse treatment. For the time to 50% germination, because the homogeneity of variance assumption could not be met for a two-way ANOVA after data transformation, the data was split into two groups – one with *A. aulacocarpa* and one with *A. elachantha* and *A. ramiflora*. The bleach and rinse treatment was compared to its control for *A. aulacocarpa* using a non-parametric Mann-Whitney test. For the other group, a two-way ANOVA was undertaken on log-transformed time to 50% germination.

3.4.3.2 Relevance to revegetation

The cumulative number of seeds germinated was plotted against the experimental period for inland and coastal species so that the germination rates in the early, mid- and later experimental period could be visually compared. These three pieces of information would allow a project manager to time the germination events with the rest of a revegetation programme.

3.4.3.3 Species responses to heat treatments

Two-way ANOVAs were conducted on time to 50% germination and germination percentage to look for a significant interaction between species factor and treatment groups. Note that treatments of the all the four species were divided into control group, manual scarification, 100°C wet and 100°C dry for one minute treatments. In addition to that, however, only *A. crassicarpa, A. aulacocarpa* and *A. elachantha* received 80°C wet and 80°C dry heat treatments for one minute. Similarly, only *A. ramiflora* received 60°C wet and 60°C dry for five minutes treatments. Therefore, two separate two-way ANOVAs were conducted, one with six treatments and three species and one with four treatments and four species (excluding both 60°C treatments from the analysis). Data had to be log-transformed to meet the normality and homogeneity of variance assumptions. Because the interaction factor species X treatments was significant, the simple main effects were compared between species at each treatment level or between treatments at each species level using the least significant difference test ($P < 0.05$).

3.4.3.4 Germination of two coastal species versus two inland species without treatment

Two 2-sample t-tests were conducted on germination percentage and time to 50% germination (after data transformation) between inland species and coastal species when no treatment was applied.

3.4.3.5 Comparison between control, manual scarification and heat treatments

A Welch's one-way ANOVA, which assumed unequal variance, was conducted on the time to 50% germination between the control group, manual scarification and heat treatments. For germination percentage, a Kruskal-Wallis test was conducted because data were non-normal, even after data transformation. After significant differences were detected for the time to 50% germination, two planned contrasts between control and the heat treatment, and between control and manual scarification, were undertaken. Dunn's test of multiple comparison was used where significant differences in germination percentages were detected.

3.4.3.6 Wet heat versus dry heat for A. ramiflora and across all species

A 2-sample t-test and a Mann-Whitney test were conducted respectively, on the time to 50% germination and germination percentages of *A. ramiflora* so that the effect of wet heat and dry heat could be compared. A separate set of analyses were conducted when all species were pooled together.

3.5 Results

3.5.1 Effect of "bleach and rinse"

Compared to their controls, the "bleach and rinse" procedures did not significantly affect the mean germination percentages of *A. elachantha, A. ramiflora* and *A. aulacocarpa* ($F_{1, 12} = 2.17$; $P = 0.166$). The T_{50} of *A. elachantha* and *A. ramiflora* also were not significantly changed by "bleach and rinse" ($F_{1, 8} = 1.001$; $P = 0.346$). For *A. aulacocarpa,* "bleach and rinse" shortened the time to 50% germination by 19 days on average but this was not significant ($U_{3, 3} = 9$; $P = 0.1$). The effect of "bleach and rinse" followed by heat treatments was not tested because there were not enough seeds. Since all seeds were washed thoroughly after they were treated with bleach, any residual bleach on the seed surface was extremely minute and diluted. Interaction between residual bleach and heat treatments was unlikely.

3.5.2 Relevance to revegetation

Nursery managers are interested in which treatments should be applied to the seeds of particular species. Manual scarification always resulted in the shortest time to 50% germination, from the mean of two days to five days. It also resulted in the highest germination percentages for *A. aulacocarpa* (96%) and *A. elachantha* (96%). For *A. ramiflora,* highest mean germination percentage of 93% was achieved by treating seeds in 60°C wet heat for five minutes. While manual scarification produced the highest germination (except for *A. crassicarpa*), it was time-consuming. Generally, the use of 60°C wet heat for five minutes (for *A. ramiflora)* and 80°C wet heat for one minute (for the other three species) was the most efficient: they achieved high and fast germination (Table 3.3 and Fig. 3.1).

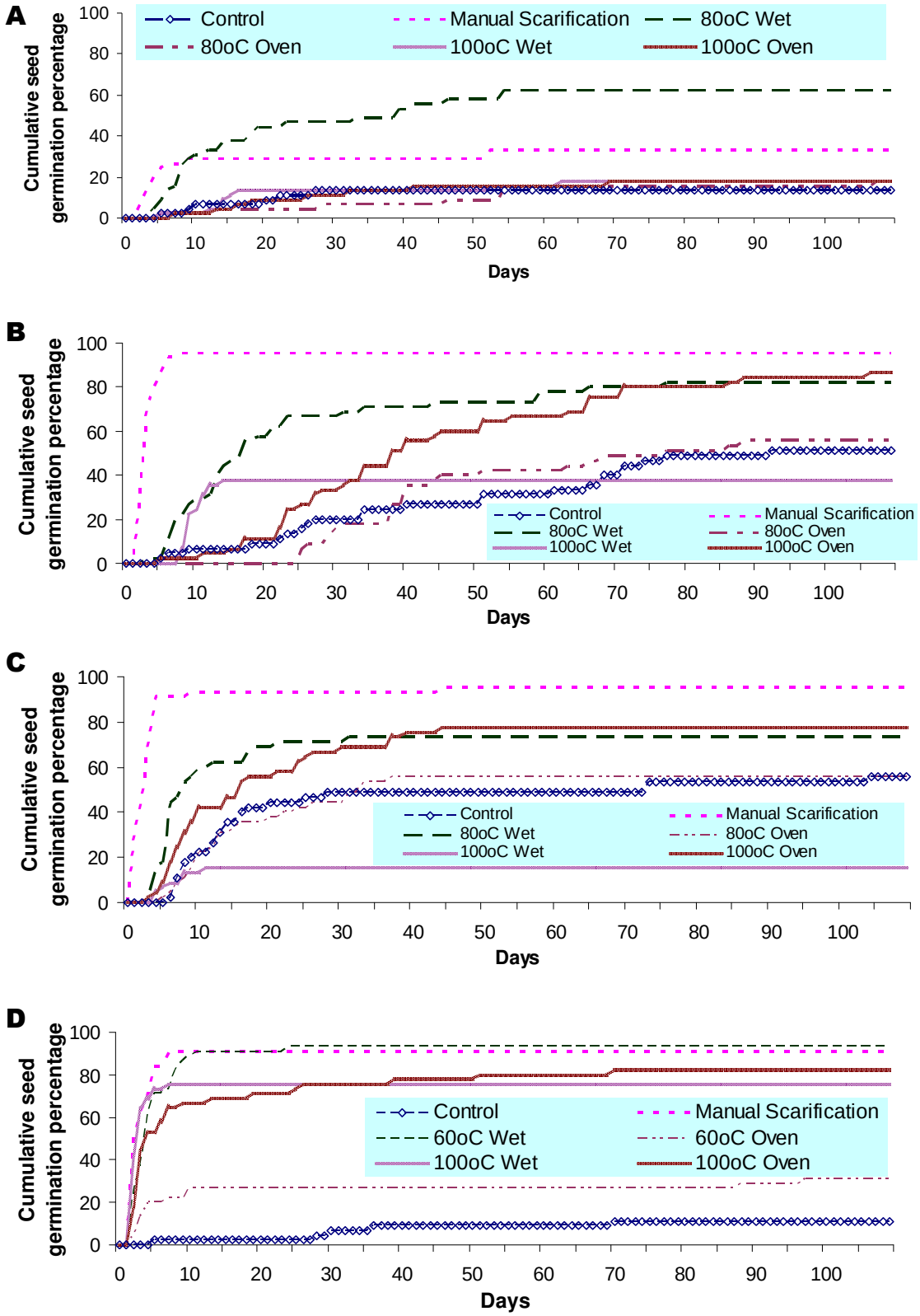


Figure 3.1. The cumulative seed germination percentages over 109 days of the experimental period. Six different treatments were tested and four species (A - *A. crassicarpa*; B – *A. aulacocarpa*; C – *A. elachantha*; D – *A. ramiflora*) were investigated. Each point is an average of three replicates of 15 seeds.

3.5.3 Species responses to heat treatments

In the current study, the effect of four treatments on germination percentage and the time to 50% germination differed between the four species, hence resulting in significant interaction (T_{50}: $F_{9,29} = 5.752$; $P < 0.001$ and GP: $F_{9,32} = 7.811$; $P < 0.001$). The ANOVA modelling of the effect of six treatments and three species on germination percentages and the time to 50% germination also gave significant interaction (T_{50}: $F_{10,33} = 5.567$; $P < 0.001$ and GP: $F_{10,36} = 2.537$; $P = 0.02$).

Seeds of *A. crassicarpa* yielded low germination percentages of 18% at both 80°C dry-1 min and 100°C dry-1 min groups compared with other species. This suggests that the seed coats of *A. crassicarpa* were less easily broken by heat. When more heat was applied, by extending the duration from one minute to five minutes, higher germination percentages of 36% and 84% were recorded respectively. The germination percentages of *A. crassicarpa* increased from 62% in the 80°C wet-1 min treatment to 92% in the 80°C wet-5 mins treatment. This result suggests that the seeds of *A. crassicarpa* have relatively high heat tolerance.

The germination of *A. elachantha* seeds was more readily stimulated by heat as its germination percentages under three of the four 1-minute heat treatments were all higher than those of *A. crassicarpa* (80°C wet-1 min: 73% vs 62%, 80°C dry-1 min: 56% vs 18%, 100°C dry-1 min: 78% vs 20%). When the duration of heat was extended to five minutes, germination percentages always decreased. The decreases were as low as 11% for 80°C wet or as high as 70% for 100°C dry. This suggests that five minutes of heat duration delivered too much heat, causing increased mortality. This indicates that the heat tolerance of its seeds is lower than that of *A. crassicarpa.*

A. aulacocarpa exhibited similar responses to heat to *A. elachantha*. The germination percentages of the two species were similar in the 80°C wet-1 min and 80°C dry-1 min treatments. More seeds of *A. aulacocarpa* germinated than *A. elachantha* under the 100°C wet-1 min (87% vs 78%) and 100°C dry-1 min treatments (38% vs 16%), but the power of the least significance test was too low to confirm the statistical significance.

The germination of *A. ramiflora* was stimulated the most by heat since it had the highest germination percentages under each of the 100°C wet-1 min treatment. The higher germination percentages of *A. ramiflora* in response to 60°C wet- and 60°C dry-5 mins treatment than *A. elachantha* suggested that its heat tolerance was higher. The differences in germination percentages of the control and 60°C dry-5 mins groups between the current and Congdon *et al.*

(in prep.) experiments might be attributed to inter-annual variation in seed quality (seed coat in particular).

By comparing the simple main effects, the sensitivity of seed coats to heat is in the ascending order of *A. crassicarpa, A. elachantha* and *A. aulacocarpa*, and *A. ramiflora* (Table 3.3 and Fig. 3.1).

Table 3.3. Mean (± 1 S.E.M.) germination percentages and time to 50% germination of four *Acacia* species under 11 treatments including one control

Within each treatment, species with different lower case letters are significantly different ($P < 0.05$). Within each species, treatments with different upper case letters are significantly different ($P < 0.05$). Numbers not labelled have an unclear relationship with other species or treatments due to the low statistical power of multiple comparison tests.

Treatment	Species	Mean germination of current experiment (%)	Mean germination of Congdon *et al.* (in prep.) experiment (%)	Mean time to 50% germination (days)
Control	*A. crassicarpa*	13 ± 7 $^{x A}$	4	12 ± 2 ^{x}
	A. aulacocarpa	51 ± 2 ^{y}	NA	41 ± 13 ^{x}
	A. elachantha	56 ± 10 ^{y}	43	12 ± 1 ^{x}
	A. ramiflora	11 ± 2 $^{x A}$	78	35 ± 14 ^{x}
Manual Scarification	*A. crassicarpa*	33 ± 14 ^{x}	NA	5 ± 1 ^{x}
	A. aulacocarpa	96 ± 2 $^{y B}$	NA	3 ± 0.16 ^{x}
	A. elachantha	96 ± 4 $^{y B}$	NA	2 ± 0.04 ^{x}
	A. ramiflora	91 ± 4 $^{y B}$	NA	2 ± 1 ^{x}
80°C wet-5 mins	*A. crassicarpa*	NA	92	NA
	A. aulacocarpa	NA	NA	NA
	A. elachantha	NA	62	NA
	A. ramiflora	NA	92	NA
80°C wet-1 min	*A. crassicarpa*	62 ± 2 $^{x B}$	NA	12 ± 1 ^{x}
	A. aulacocarpa	82 ± 15 ^{y}	NA	14 ± 3 ^{x}
	A. elachantha	73 ± 15 ^{y}	NA	7 ± 1 ^{x}
	A. ramiflora	NA	NA	NA
80°C dry- 5 mins	*A. crassicarpa*	NA	36	NA
	A. aulacocarpa	NA	NA	NA
	A. elachantha	NA	24	NA
	A. ramiflora	NA	78	NA
80°C dry-1 min	*A. crassicarpa*	18 ± 4 ^{x}	NA	33 ± 12 ^{x}
	A. aulacocarpa	56 ± 4 ^{y}	NA	40 ± 3 ^{x}
	A. elachantha	56 ± 11 ^{y}	NA	13 ± 2 ^{y}
	A. ramiflora	NA	NA	NA

Table 3.4 (Cont'd)

100°C wet-1 min	*A. crassicarpa*	18 ± 2 ^{x}	NA	28 ± 9 ^{y}
	A. aulacocarpa	38 ± 8 $^{x A}$	NA	10 ± 1 ^{y}
	A. elachantha	16 ± 8 $^{x A}$	NA	7 ± 1
	A. ramiflora	76 ± 4 $^{y B}$	NA	2 ± 0.45 ^{x}
100°C dry- 5 mins	*A. crassicarpa*	NA	82	NA
	A. aulacocarpa	NA	NA	NA
	A. elachantha	NA	8	NA
	A. ramiflora	NA	32	NA
100°C dry-1 min	*A. crassicarpa*	20 ± 4 ^{x}	NA	38 ± 11 ^{y}
	A. aulacocarpa	87 ± 7 ^{y}	NA	35 ± 3 ^{y}
	A. elachantha	78 ± 10 ^{y}	NA	13 ± 5
	A. ramiflora	82 ± 11 $^{y B}$	NA	5 ± 2 ^{x}
60°C wet-5 mins	*A. crassicarpa*	NA	53	NA
	A. aulacocarpa	NA	NA	NA
	A. elachantha	NA	73	NA
	A. ramiflora	93 ± 4	92	4 ± 0.18
60°C dry-5 mins	*A. crassicarpa*	NA	31	NA
	A. aulacocarpa	NA	NA	NA
	A. elachantha	NA	25	NA
	A. ramiflora	31 ± 2	60	17 ± 14

3.5.4 Germination of two coastal species versus two inland species without treatment

Without treatment, the paired coastal and inland species required a similar amount of time to achieve 50% germination ($t_{0.05(2),8}$ = 0.641; P = 0.540). The mean germination percentages were also similar ($t_{0.05(2),10}$ = 0.067; P = 0.948). Although based on only two species from each environment, such results do not support the hypothesis that inland species germinate faster and more frequently than coastal species in the absence of any treatment.

3.5.5 Comparison between control, manual scarification and heat treatments

By pooling species data together, significant differences between heat treatment, manual scarification and control groups were found in terms of time to 50% germination ($F_{2,66}$ = 17.568; P < 0.001), and germination percentage (χ^2 = 13.825; P = 0.001). Heat treatment or manual scarification significantly shortened the time to 50% germination compared to the control ($t_{19.711}$ = 2.37; P = 0.028 and $t_{16.196}$ = 8.499; P < 0.001 respectively). The mean time to 50% germination of heat treatment, control and manual scarification were 18 days, 25 days and 3

days. Manual scarification achieved 79% germination which was higher than the 33% of the control group. Heat treatments resulted in germination percentages intermediate between the two (55%), but the differences from either of the two groups were not significant. This ambiguous relationship was caused by low power of the test and inadequate number of replicates (Zar 1999). The results confirmed that substantial physical dormancy exists in *Acacia* seeds but this dormancy can be broken by heat or physical erosion.

3.5.6 Wet heat versus dry heat for A. ramiflora and across all species

The time to 50% germination and germination percentage under wet heat and dry heat (combining 60 and 100°C) for *Acacia ramiflora* were not significantly different ($t_9 = -1.314$; $P = 0.221$ and $U_{6,6} = 9$; $P = 0.145$ respectively), though wet heat appeared to shorten the time to 50% germination and achieved a higher germination percentage than dry heat at 60°C. The lack of significant difference was caused by the great variance in results for the dry heat treatment, which could be reduced with more replicates.

When the data for all the species were combined, wet heat significantly decreased the time to 50% germination from 23 days to 10.5 days ($t_{44} = -2.858$; $P = 0.006$). The mean germination percentage under the wet heat treatment was 57% which was not significantly different from the 53% under the dry heat treatment ($U_{24,24} = 266.5$; $P = 0.656$).

3.6 Discussion

3.6.1 Heat tolerance and sensitivity of the seeds of the four Acacia species

The amount of heat applied onto seeds varied with treatments. The results of the current experiment and the 2003 experiment by Congdon *et al*. (in prep.) were analysed together. In both studies, the heat treatments were divided into 60, 80 and 100°C. To facilitate interpretation of results, treatments of the same temperature were further separated into three groups according to the the duration and type of heat strong heat (wet heat-5 mins), moderate heat (wet heat-1 min and dry heat-5 mins) and low heat (dry heat-1 min). The specific heat capacity of water is four times that of air (Myers 2006), and thus seeds receive a greater amount of heat energy from each unit mass of heated water than heated air from their surroundings, given that they are of the same temperature. Also, raising the duration of exposure to heat logically leads

to an increase in the amount of heat applied to seeds. Wet heat-5 mins (very strong heat) thus delivers more heat to seeds than dry heat-1 min (low heat). Wet heat-1 min and dry heat-5 mins are thus in between the two groups.

The seeds of the four *Acacia* species differ in the optimal amount of heat that stimulates the greatest germination (Fig. 3.2). Three responses to heat treatment were apparent: low germination due to inadequate heat applied to break seed dormancy (sub-optimal response), maximum germination after applying an optimal amount of heat (optimal response), low germination because excessive heat resulted in significant seed mortality (supra-optimal response).

Whether a heat treatment produces optimal seed germination varies with species. For instance, under wet heat-5 mins, *A. ramiflora* and *A. crassicarpa* could achieve an optimal germination while *A. elachantha* experienced some mortality and hence the germination under wet heat-5 mins could only be considered as supra-optimal. *A. elachantha* had optimal seed germination at 80^{o}C wet-1 min, and *A. aulacocarpa* at 100^{o}C dry-1 min and 80^{o}C wet-1 min.

Acacia seeds achieving optimal germination when subject to strong heat logically depend on and can tolerate a greater amount of heat. Therefore, with 92% seed germination under 80^{o}C wet-5 min (strong heat group), the seeds of *A. ramiflora* and *A. crassicarpa* had high heat tolerance. *A. aulacocarpa* and *A. elachantha* had optimal germination at 80^{o}C wet-1 min and thus the heat tolerance of their seeds is moderate.

By comparing the germination in the lower heat treatments (80 ^{o}C / 100^{o}C dry-1 min), the sensitivity of the seed coat to heat was shown to vary with species. It should be noted that any low germination percentage is unlikely to be a result of seed mortality from excessive heat because, if seed mortality is the reason, the germination percentage is unlikely to rise when stronger heat, such as the 80^{o}C wet-1 min or 80^{o}C wet-5 mins treatments, is applied. Yet we observed a significant increase in germination percentages in all four studied species in these higher heat treatments. The seed coats of *A. aulacocarpa* and *A. ramiflora* had higher heat sensitivity as their seed germination rates were 87% and 82% respectively (Fig. 3.2). The seed coat of *A. elachantha* was intermediate in heat sensitivity while that of *A. crassicarpa* had the lowest heat sensitivity.

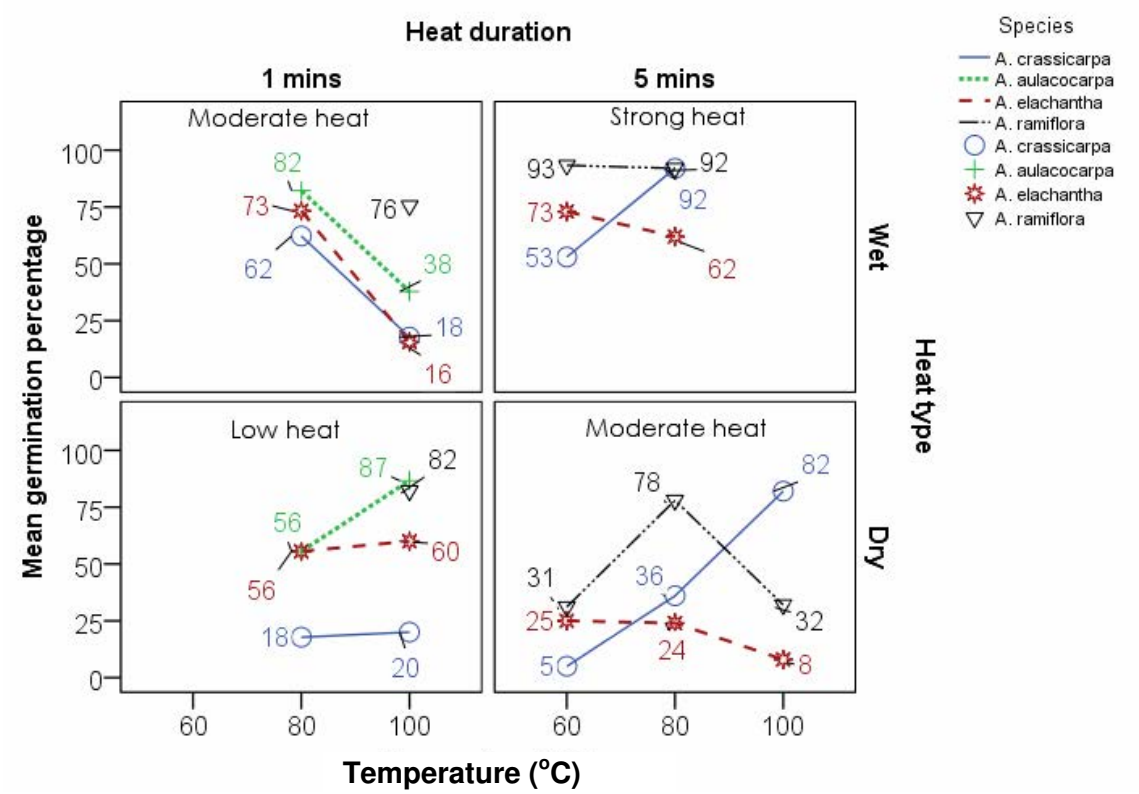


Figure 3.2. The mean germination percentages of the seeds of four *Acacia* species subject to heat of two durations, three temperatures and two types (wet v.s. dry). All the results of the 1-min heat treatments on all four species and 60°C wet-5 mins on *A. ramiflora* came from the current experiment while the results of other heat treatments came from the experiment by Congdon *et al.* (in prep.). Heat treatments are grouped into three general categories: strong heat, moderate heat and low heat.

3.6.2 Responses of the seeds of inland and coastal Acacia species to rainfall variability and wild fire in savanna woodland

In the absence of treatment, inland and coastal *Acacia* species did not differ in terms of germination speed or percentages. It was hypothesised that seeds of inland *Acacia* species adapted to high inter-annual rainfall variability and seasonality would respond to favourable moisture more rapidly in the absence of treatment. The inter-annual rainfall variability and mean percentage of MAR falling between December and March for the Burra Range where seeds of inland *Acacia* species were collected were respectively 2.9% higher and 7.4% lower than the coastal Town Common Conservation Park, where seeds of *A. crassicarpa* were obtained. Such small geographical differences in climate variability are probably the reason why the chosen inland and coastal species had similar germination performance in the absence of treatment.

Many researchers suggest that physical dormancy is an adaptive trait to fire regime in a fire-prone environment (Gill 1993; Baskin and Baskin 1998; Keeley *et al.* 2011). Adaptive traits are defined as traits which enhance the fitness of an individual in their current habitats (Keeley *et al.* 2011). Since the original hypothesis was falsified, a new hypothesis is proposed: compared to coastal species, the seeds of inland *Acacia* species were expected to germinate more prolifically and faster when non-lethal heat is applied. Their lethal fire temperature should be lower since they appear less adapted to frequent fire than the coastal species. In order to test this hypothesis, the following aspects will be evaluated:

- Frequent fires occur in tropical savannas and the fire frequency of coastal savannas is higher than that of inland ones
- *Acacia* seeds have physical dormancy and germinate following fire.
- Seeds of inland *Acacia* species germinate faster and more abundantly than seeds of coastal *Acacias* when subject to low heat. When subject to higher heat, the reverse is true.

Many coastal tropical savanna woodlands experience higher fire frequency and higher fuel load (hence burning a greater proportion of area and potentially leading to more intense fire) than the inland ones (Fensham 1994; Williams 2002; Russell-Smith and Yates 2007; Turner *et al.* 2008), Fire can also stimulate seed germination of many legume species (Williams 2002). Therefore, coastal and inland *Acacias* seeds may develop adaptations to the differences in fire regimes of their habitats.

Firstly, post-fire seedling recruitment is usually high compared to recruitment without fire for a number of *Acacia* species in semi-arid Australia. Post-fire seedling recruitment of *A. ramiflora* was recorded in the wet season following a prescribed burn in the late dry season in the Burra Range (Williams *et al.* 2004). Also in the Girraween/Bald Rock region of New South Wales, a wild fire occurred in October 1994. In August 1995, a large number of new seedlings of the rare *A. latisepala* were observed on the burnt granitic outcrops but not on the nearby unburnt areas (Hunter 1995).

Secondly, in the current experiment, the seed coat of inland species appeared to be more sensitive to low heat than coastal species. *A. elachantha* or *A. ramiflora* reached 50% germination in a significantly shorter period of time than coastal *A. crassicarpa* and *A. aulacocarpa* when subjected to 80^{o}C-1 min and 100^{o}C dry-1 min (Table 3.3). However, in terms of germination percentage, no such clear difference between inland and coastal species

was observed. For instance, the seed germination rates *A. aulacocarpa* and *A. ramiflora* were 87% and 82% respectively under 100°C dry-1 min (low heat) (Fig. 3.2). The seed coat of *A. elachantha* was intermediate in heat sensitivity (moderate germination percentage) while that of *A. crassicarpa* had the lowest heat sensitivity (lowest germination percentage).

The treatments in the current experiment were selected not only for ensuring enough germinated seeds for the provenance experiment (Chapter 4), but also to supplement an experiment by Congdon and his colleagues. In their experiment, the seeds of coastal *A. cincinnata, A. crassicarpa, A. flavescens* and *A. mangium,* as well as the seeds of inland *A. elachantha, A. hyaloneura, A. platycarpa* and *A. ramiflora*, were collected and germinated in 2003. The seed sources of *A. crassicarpa, A. elachantha* and *A. ramiflora* were the same as those in the current experiment. Some seeds of each species were not treated, to provide a control group, and others were subject to heating in an oven at 40, 60, 80, 100 or 120°C for five minutes. In a third group of treatments, the seeds of each species were immersed in a water bath at 60 or 80°C for five minutes.

It appeared that the differences in heat tolerance of the seeds between inland and coastal *Acacias* varied from dry to wet heat. For instance, in the earlier experiment, in the 80°C wet-5 mins treatment which delivered more heat than 80°C dry-5 mins, the germination percentages of coastal *A. mangium* and inland *A. ramiflora* both achieved about 95% germination while inland *A. hyaloneura* and coastal *A. flavescens*, *A. crassicarpa* and *A. cincinnata* achieved about 90% germination. Inland *A. elachantha* and *A. platycarpa* achieved only about 60% and 40% germination respectively. In the current study, one coastal species and one inland species were found to have higher heat tolerances than another coastal and inland species. Therefore, by comparing the germination responses to wet heat-5 mins in the earlier experiment and by comparing the heat treatments that caused optimal germination in the current experiment, there were no significant differences in heat tolerance between the seeds of inland and coastal *Acacia* species.

On the other hand, in the 2003 experiment, the seed germination percentages of all the coastal *Acacias* increased with temperatures from 60°C to 80°C and then to 100°C dry-5 mins, but the seed germination percentages of all the inland *Acacias* either increased or remained similar for dry heat-5 mins from 60°C to 80°C and then decreased significantly when temperature was increased to 100°C (Table 3.3 and Congdon *et al.* in prep.). Since inland *Acacias* have an optimal germination percentage at a lower temperature than coastal *Acacias* in that study, their heat tolerance can be considered to be lower than that of coastal *Acacias.*

The 80°C dry-5 mins optimal temperature in the 2003 experiment could probably imply that the selected inland *Acacia* species have adapted to fire in years of average rainfall. It should be noted that extremely intense fire in savanna woodland can occur in years of above-average rainfall or following several hot and drought years (Williams 2002). An experiment that deliberately burned *Eucalyptus* savannas at Cape Cleveland examined the surface and sub-surface temperatures during early and late dry season fires (Williams 2002). On the soil surface, the modal temperature was always 182°C regardless of early or late dry season fires. At 3 mm below the surface during a fire, the modal temperature either remained 182°C (late dry season) fell between 54 and 77°C (early dry season). At 10 mm below the surface, the temperatures were only 71°C (late dry season) and 27.5°C (early dry season).

It should be noted that *Acacia* seeds drop to the soil surface and/or get carried away to great depths by ants after their fruit pods become mature and split-open. Ants are known to carry seeds of *Acacia* in both inland and coastal areas from the soil to their nests since elaiosomes are part of their diet (Berg 1975). Furthermore, sub-surface sandy soil might get exposed (and vice versa) in windy conditions. Small seeds might have a greater chance of moving into deeper soil (Bekker *et al.* 1998). However, it is not known if seed size is a significant factor in influencing the vertical distribution of *Acacia* seeds when the effect of seed dispersal by ants is incorporated into seed bank dynamics. Recently, the dispersal of *Acacia* seeds by ants was discovered to interact with fire in Australian tropical savannas (Parr *et al.* 2007).

A positive correlation was found between seed size and maximum temperature recorded with germination. The seeds of five plant species belonging to the Fabaceae and Rhamnaceae in Ethiopian savanna and grassland habitats can germinate even after a dry heat treatment of 150 or 200°C for one or five minute(s) (Gashaw and Michelsen 2002). Four of the five selected species have big seeds with a length ranging from 12 to 25.5 mm while the fifth is only 5.5 mm. If the seeds of coastal *Acacia* species are found on the surface soil or at a shallower depth range than inland *Acacia* seeds, this may be the reason why the seeds of coastal *Acacia* species are found to have a higher dry heat tolerance. Surveying the depths of inland and coastal *Acacia* seed banks in the wild would fill in the missing piece of this hypothesis.

The finding that the 80°C dry-5 mins treatment was the optimal germination temperature for some inland *Acacia* species probably suggests that their seeds would be favoured by being buried below the soil surface. At this depth, the surrounding soil would protect seeds from excessive heat from wild fires at the soil's surface. It appears that inland *Acacia* seeds have not adapted to extremely intense fires that could occur following years of above average rainfall. If they have, they would have developed extreme heat tolerance. Correlation between lethal

temperature to seeds and germination depth was found in tropical savanna species *Galactia tenuiflora* (a twiner) and *Indigofera hirsuta* (an ephemeral) (Williams 2002).

3.6.3 Seed coat sensitivity, not seed metabolism, causes differences in germination rate

The differences in time to 50% germination between inland and coastal *Acacias* were not due to different rates of radicle production. As there was no difference in germination rate of inland and coastal species following manual scarification and in control groups, but there was for the heat treatments, this indicates that it was the seed coat sensitivity to heat that caused the differences in seed germination rate, not the rate of metabolic activities.

3.6.4 Relevance to revegetation

From a revegetation programme perspective, to produce the greatest germination percentage at the fastest rate with minimal preparation time, *A. crassicarpa, A. aulacocarpa and A. elachantha* species should be exposed to 80^{o}C in water for one minute. This treatment can achieve 62% to 82% final germination and half of the seeds germinated in only 7 to 14 days. For *A. ramiflora,* the best treatment was exposure to 60^{o}C in water for five minutes. It achieved 93% final germination and only took 4 days for 50% germination.

3.7 Conclusion

In terms of the time to 50% germination, inland *A. elachantha* and *A. ramiflora* were more sensitive (germinate faster) to heat than coastal *A. crassicarpa* and *A. aulacocarpa*. The two inland *Acacia* species were less tolerant to dry heat than the two coastal species since their optimal germination percentages occurred at lower dry heat level than those of the coastal species. However, in response to wet heat, there were no significant differences between the two coastal and two inland *Acacia* species. Interestingly, the more flammable nature of many coastal savannas than inland savannas was documented in remote sensing studies and explorers' records. It is possible that historic fire regimes facilitate the current heat tolerance and sensitivity patterns of the four *Acacia* species studied.

4 Growth and nodulation of inland and coastal *Acacia* species using different sources of soil

4.1 Introduction

This study investigated the growth and nodulation of inland and coastal *Acacia* species using different soil sources. Different soil sources are assumed to have different rhizobial bacterial assemblages. Rhizobial bacteria are known to infect the roots of many *Acacia* species and induce the development of root nodules in which they inhabit and help to fix atmospheric nitrogen into ammonium that is further converted into amino acids for the host plant. In return the host plant will provide carbohydrates to the bacteria for their growth and reproduction. Some legume species only form the most effective symbiotic relationship with a sympatric bacteria population while some are more promiscuous in both nodulation and/or symbiotic effectiveness. The former relationship is known as local co-adaptation. Several previous studies using temperate *Acacias* in Australia suggested that *Acacias* lack strong local co-adaptation in their symbiosis. This study investigates the possibility of local co-adaptation using Australian tropical *Acacias* and compares the performance of inland and coastal *Acacias* by growing species with their own soil and with soil from other species.

Host-symbiont interactions can be divided into host-parasite or host-mutualist interactions. A mutualist can provide missing nutrients to the host or even protect the host (Thrall *et al.* 2006). Parasites rely on the host's nutrients without providing benefits to the hosts in return. Those symbionts that might be expected to provide benefits but do not are known as cheaters in a host-symbiont symbiosis (Douglas 2007). Both parasites and cheaters can drain the host's resources and decrease its fitness. Host-symbiont interactions are therefore influential to the survival and growth of an organism and even the population. Two important factors affecting successful nodulation and the effectiveness of the interaction are the origins of the host and the mutualists / parasites.

Examining the occurrence of nodules and effectiveness of nitrogen fixation in *Acacia* requires knowledge of host-rhizobial strain specificity and promiscuity. In a comprehensive review regarding nitrogen fixation in *Acacia*, it was concluded that this genus is generally promiscuous in nodulation (Brockwell *et al.* 2005). For effective nitrogen fixation, however, many *Acacia* species require specific rhizobial strains. The nodulation effectiveness of nursery grown seedlings may depend on seed provenance and rhizobial provenance.

Seed provenance refers to *"place of origin"* and is defined as *"the geographical area and environment in which parent trees grow and within which their genetic constitution has been developed through natural selection"* (Gunn 2001). This means that in the course of evolution, genetic variation within a population of a species is shaped by the ecological conditions in that particular location (Gunn 2001). Thus two populations of the same species that come from two separate locations with very different climatic, edaphic or other environmental patterns, may exhibit markedly different growth or physiological responses when grown under the same environmental conditions.

Rhizobial provenance is defined here as the rhizobial communities coming from soil with different host species. Rhizobia and bradyrhizobia are the main types of nodulating bacteria of *Acacia* (Sprent 1995; Madigan *et al.* 2003), and can generally be found in the rhizosphere of nodulating leguminous plants (Brockwell *et al.* 2005). *Acacia* species grown in soil from their source of origin (hereafter referred to as "own soil") rather than soils from other locations (foreign soil) may sometimes grow faster, possibly due to different rhizobial populations. For example, in a glasshouse study where *A. stenophylla* and *A. salicina* were grown with their own soils or in each other's soil, *A. stenophylla* grew better on its own soil which had a higher rhizobial population than did *A. salicina* soil (Thrall *et al.* 2007). In fact, rhizobial population density estimated with *A. salicina* or *A. stenophylla* was strongly correlated with nodulation effectiveness and number as well as dry plant weight. By inoculating specific rhizobial strains, it was found out that strains from own soil helped form effective nodules, while those from other soil fixed atmospheric nitrogen poorly (Thrall *et al.* 2000; Thrall *et al.* 2007).

A narrower definition of rhizobial provenance refers to rhizobial communities coming from soil of different populations of the same species. It has been shown that rhizobial isolates from different populations of the same species can elicit different responses in those populations (Burdon *et al.* 1999). According to a study of 22 *Acacia* species within the temperate regions of eastern Australia, i.e. New South Wales, Victoria, South Australia and Tasmania, the variation in responses between *Acacia* individuals within a population and between populations of the same species, as well as variation between different rhizobial isolates within each site and among sites, resulted in variation in the root nodulation effectiveness (Burdon *et al.* 1999). Furthermore, rhizobial and seed provenances can interact significantly. Among the three species tested for rhizobial-seed interaction, *A. implexa* responded positively but *A. dealbata* and *A. mearnsii* did not. This means that the significant variation between rhizobial isolates in effecting symbiotic association differed between populations of *A. implexa* (Burdon *et al.* 1999). Also, it was demonstrated that the effectiveness of rhizobial strains of some *Acacias* is generally

consistent. For instance, for *A. implexa, A. mearnsii, A. irrorata, A. melanoxylon,* rhizobial strains that were highly effective in one population of a species could exhibit the same effectiveness in other populations of that species. This level of consistency was observed with moderately effective and the least effective strains.

Many councils and land care groups use *Acacia* in revegetation programmes. However they may not have adequate funding to purchase inoculum nor sufficient expertise and time to screen for the most effective inoculum. Growing seedlings or sowing seeds on a species' own soil could be an easy and convenient alternative to sourcing pure inoculum, assuming the most effective rhizobial strain occurs in its own soil. There is always the possibility that the most effective rhizobial strain may not be present in a species' own soil. But the extent of this is largely unknown because literature on this, specifically for legumes, is still scant.

In revegetation projects, cultivating *Acacia* seedlings in nurseries with indigenous soils to ensure root nodulation instead of inoculating with a selected mix of rhizobia may be necessary because managers may have limited expertise about which rhizobium is most effectively associated with a particular *Acacia* species of interest. Many studies have examined the inoculation with a mix of rhizobia strains onto *Acacia* seeds prior to sowing in the field, and onto seedling pots in the nursery, for better growth performance. Many of these studies have practical applications to revegetation and agroforestry/plantations in Australia (Brockwell ***et al.*** 2005). However, due to the strict legislative regulations regarding rhizobium inocula (only a single rhizobial strain inoculum but not mixed-rhizobial strains inoculum can be produced), and the small market demand in Australia, it is difficult to find local manufacturers able to produce different strains of inoculum in small amounts at a relatively low cost (Brockwell ***et al.*** 2005). This causes problems if a revegetation project has a limited budget.

An experiment was conducted to test the effect of soil and seed provenances on the growth and nodulation of two inland and two coastal *Acacia* species. The coastal species are recommended in the draft revegetation plan of the Townsville City Council. One of the inland species, *A. ramiflora,* has been listed since 16 July 2000 as "Vulnerable" under the Environment Protection and Biodiversity Conservation Act 1999. Seedlings of this species were grown in a glasshouse and survived transplantation to man-made rocky substrates along the Burdekin Highway at Burra Range, as part of a revegetation plan (Congdon ***et al.*** in prep. and pers. observ. 2010). Thus the relationship between nodulation effectiveness and growth rate may aid revegetation programmes using this species. *A. elachantha*, a shrub found at the Burra Range in woodland and open woodland, was also compared with *A. ramiflora.*

The experiment aimed to find out to what extent individuals of *Acacia* species, potentially suitable for revegetation, adapt to their own soil versus foreign soil in forming effective nodules. It is hypothesised that for each species tested, the soil sourced from their natural population would result in faster growth and higher plant dry weight, compared with the results obtained with soil sourced from other provenances.

4.2 Materials, methods and data analysis

4.2.1 Species chosen

The four species chosen for the field study were used, so that some of the field work for both investigations could be conducted more efficiently. Thus the study species were *A. crassicarpa, A. aulacocarpa A. elachantha* and *A. ramiflora.*

4.2.2 Sources of seeds and soil

For each species, seeds and soil were collected from the sites visited in the field study (Table 1.2). Seeds were collected from at least ten individuals which were at a distance twice the average height of trees or at least 100 m apart (Gunn 2001). Surface soil (to about 20 cm depth) in the immediate vicinity of the trunks of 10 to 15 individuals was collected from each species.

4.2.3 Shadehouse experiment

A generalized randomised block design was adopted so that a factorial combination of four species, four soil/rhizobia sources and two nodulaton conditions were arranged in each of the five blocks (Fig. 4.1 and 4.2). Nodulation was either facilitated by provision of unsterilised soil or prevented by soil sterilisation, as detailed below. In the original plan, three replicates from every treatment were planned in each block so that 480 pots could be arranged in five blocks and each block consisted of 96 pots. However, the number was modified because of unexpected conditions (see the next paragraph). In general, 15 to 20 replicates were suggested for genetically heterogeneous species (Bergersen 1980), while another author suggested 10 to 15 replicates for similar provenance experiments (Thrall *et al.* 2007).

Due to cold weather and a limited number of germinated seeds, the number of established seedlings at the beginning of the experiment was only 391. In the first month after the first batch of seeds was sowed, another 47 germinated seeds were available for sowing in two separate batches. Eventually, 438 seedlings established and were ready for the experiment.

Seeds were surface sterilised in 70% ethanol for three minutes to kill any attached rhizobia. They were then manually scarified or boiled for two minutes to break the seed coat. Seeds were then put on petri-dishes lined with moist sterilized paper towels to germinate. Two seedlings were placed in a pot of 100 g of the collected soil together with 1:1 vermiculite : perlite mixture (Bergersen 1980; Thrall *et al.* 2007). For treatments without soil rhizobia the collected soil and vermiculite : perlite mixture were autoclaved at 124^{o}C for two hours beforehand to kill all bacteria. The autoclaving procedure was repeated two to three times to ensure that all soil rhizobia were killed; soil was mixed using a rod sterilised with 70% ethanol before each repetition. The autoclaving period was higher than the 50 minutes or one hour as previously suggested (Schmidt *et al.* 1997; Shaw *et al.* 1999; Pérez-Fernández and Lamont 2003), to ensure full sterilization of the large volume of soil involved in this experiment. The top of each pot was covered in orange polyethylene beads that had been autoclaved in 124^{o}C for 2 hours.

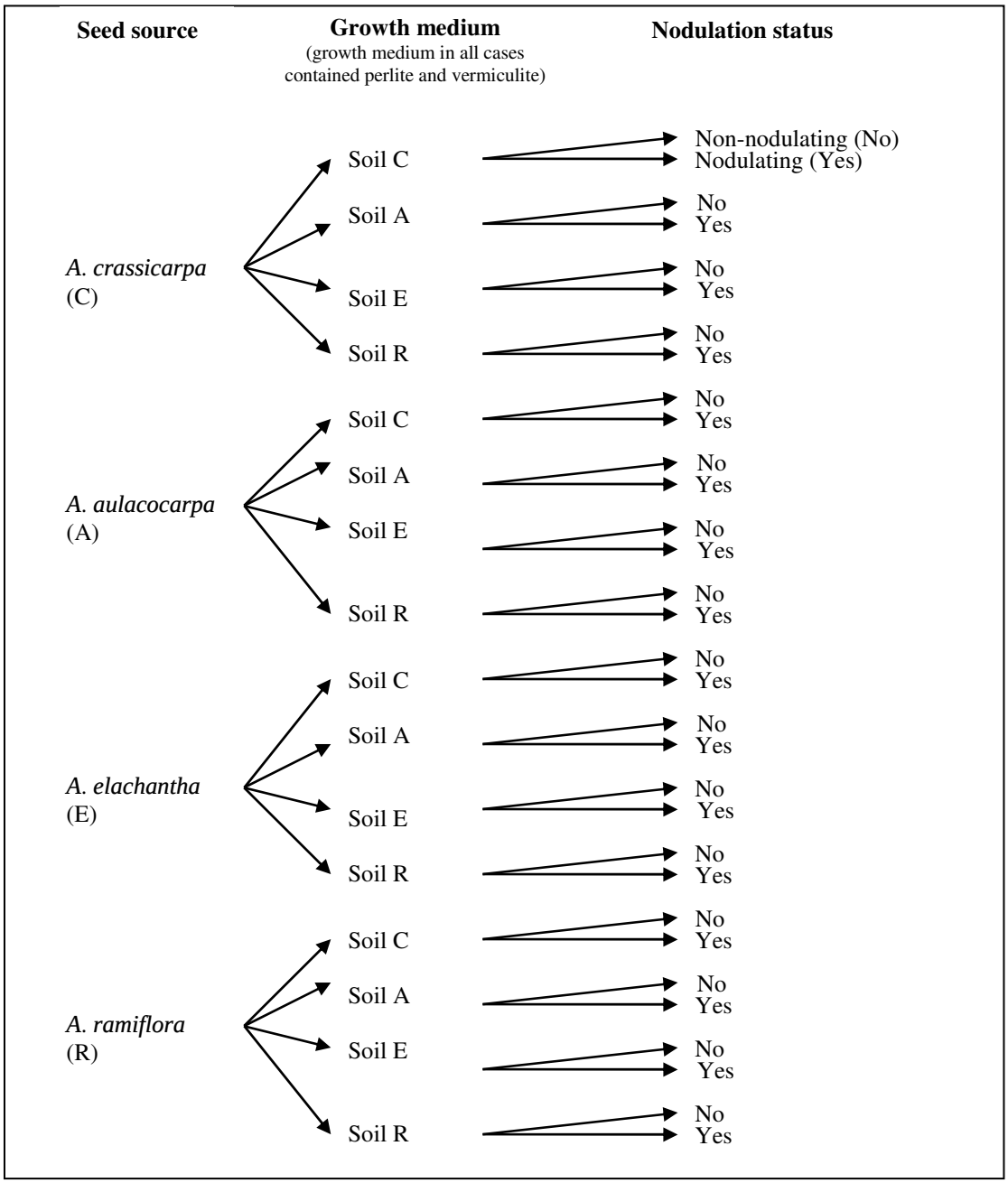


Figure 4.1. The fully factorial combinations of 32 treatments of the provenance experiment.

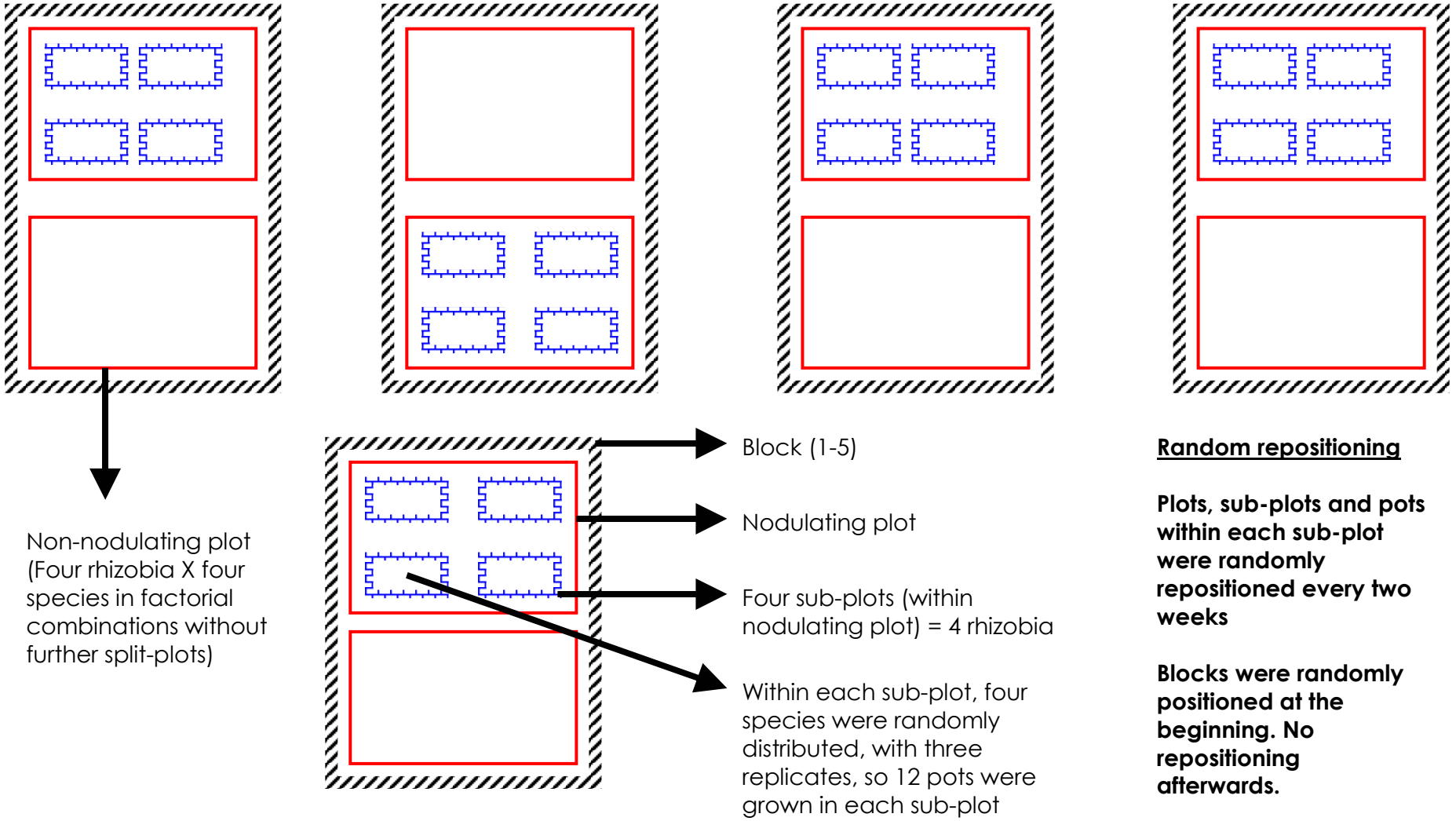


Figure 4.2. The spatial arrangement of the 32 treatment combinations of the provenance experiment in a shadehouse.

If non-nodulating controls were grown too close to nodulating pots, there would be a risk of contamination even after practising all precautionary measures (Section 4.2.4). Therefore, the non-nodulating and nodulating pots formed two spatially separate plots within each block. Within each plot, the same source of soil rhizobia was grouped to form four separate sub-plots. The positions of each block, each plot (nodulating vs non-nodulating), each sub-plot (soil/rhizobia source) and each pot within sub-plot were randomly repositioned every two weeks (Fig. 4.2), to reduce the confounding effect of variation of light intensity, temperature and air movement on the growth and nodulation of seedlings.

Germinated seeds were sown into prepared pots in batches A, B and C, which were grown from 4 June to 7 November 2011, from 27 June to 30 November 2011 and from 7 July to 10 December 2011 respectively. The number of seeds sown in batches A, B and C were 391, 41 and 7 respectively. They were grown in the shadehouse at the Townsville campus of James Cook University for a total of 157 days.

Batches B and C were necessary because the number of germinated seeds was inadequate and some seedlings in batch A failed to establish. Since batches B and C were delayed for three to four weeks, there were concerns about the validity of using datasets from both batches. However it should be noted that the temperature regimes of batches "B" and "C" were fairly similar (Fig. 4.3 and Table 4.1). For instance, the average daily maximum and minimum temperatures for batch A were 33.4 and 14.8^{o}C respectively while those of batch B were 33.8 and 15.5^{o}C, and those of batch C were 34.6 and 16.4^{o}C. Also, the mean and the range of growth parameters of batch B and C overlapped with batch "A". Thus data from all three batches was used for the analysis.

Table 4.1. Summary of the growing conditions and the number of three sets of seedlings designated as batches A, B and C

Batch	Number of seeds / seedlings			Growth period	Average daily temperature (^{o}C)	
	Seeds sown	Survival analysis	Biomass / nutrient analyses		Max.	Min.
A	391	374	371	4 June to 7 Nov 2011	33.4	14.8
B	41	39	37	27 June to 30 Nov 2011	33.8	15.5
C	7	7	7	7 July to 10 Dec 2011	34.6	16.4

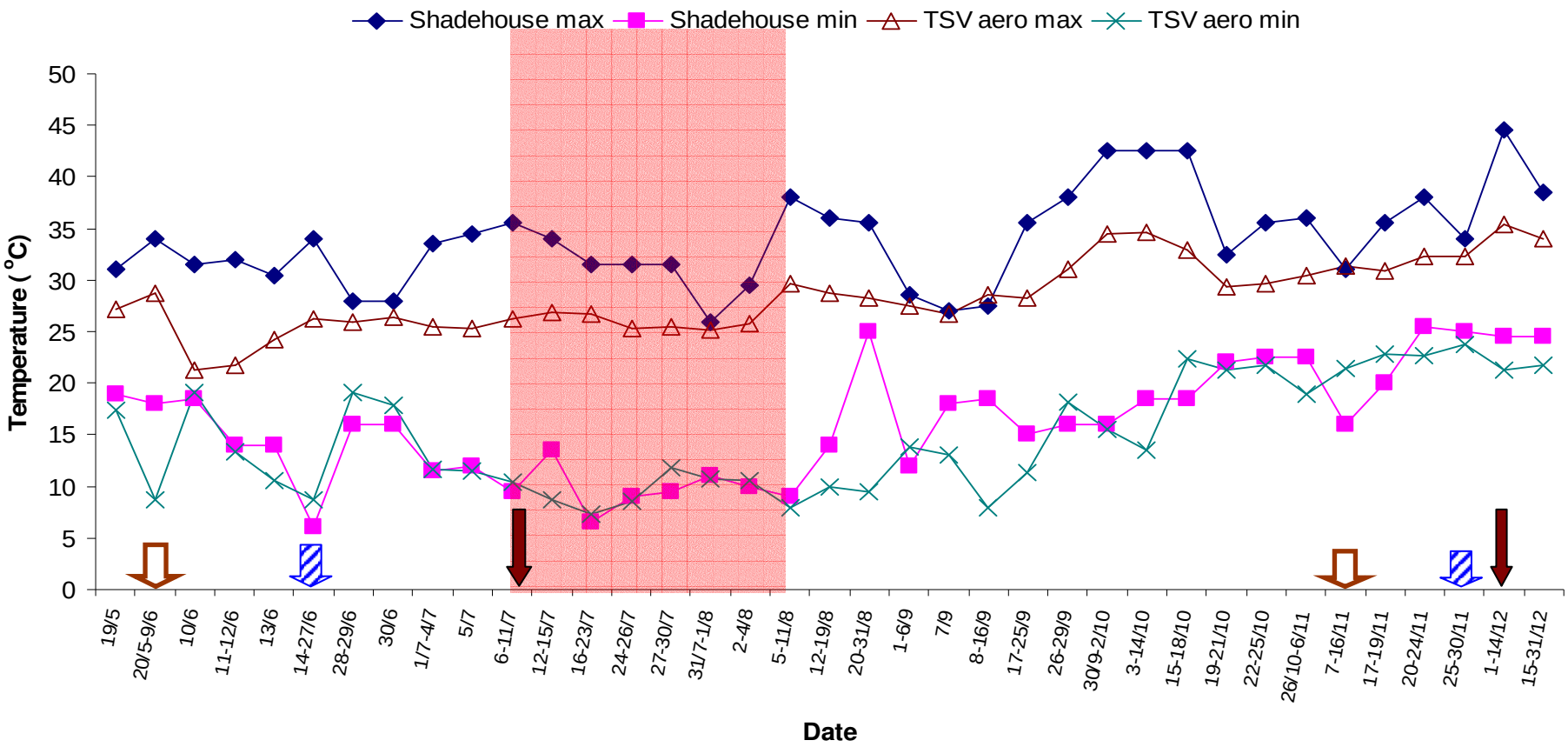


Figure 4.3. The temperature regimes of batches A and B seedlings inside the shadehouse for the provenance experiment. The temperature recorded by the Townsville airport weather station is also provided for comparison (BOM 2012b) Arrows indicate seed sowing and seedling harvest dates. Open arrows represent batch A and hatched arrows represent batch B. Filled arrows represent batch C. Hatched rectangle indicates the period when seedlings' growth was halted (see text).

Most seedlings were observed to have stopped growing between 6 July and 11 August 2011, i.e. their heights stopped increasing, old foliage stopped enlarging and new foliage failed to develop. This was probably because of the cold weather. From 6 July to 11 August 2011, the average minimum shadehouse temperature was only 9.8^{o}C. The minimum temperature between 16 and 23 July were as low as 6.5^{o}C.

On the harvest days, pots were moved to the laboratory, which was cool enough to forestall further growth. A maximum period of two weeks was allowed for processing to reduce unexpected changes. Among the 388 seedlings from batch A, only 374 and 371 seedlings were included for survival and biomass/nutrient analyses respectively after excluding the contaminated and late processed ones. Among the 48 seedlings from batches B and C, only 46 and 44 seedlings were included for survival and biomass/nutrient analyses respectively for the same reasons.

4.2.4 Shadehouse experiment – precautions

Many precautionary measures were practised to prevent contamination of non-nodulating seedlings. Pots were washed with tap water and brushed clean, then thoroughly sterilised with 2 to 4% (v/v) bleach. Pots were in contact with bleach for at least 30 minutes to kill all soil rhizobia. Sterilised pots were submerged in tap water three times to remove all residual bleach. The storage containers used for washing, the plastic bags enclosing the bottom of every pot, and metal benches on which the experiment was carried out were sterilised with 70% ethanol beforehand. They were then allowed to air dry before usage. Drainage saucers were not prepared under pots as they could not be rinsed out regularly without great difficulty and without the risk of contaminating the pots (Bergersen 1980). The bottom of each pot was enclosed with a plastic bag and the top was covered with plastic beads to reduce cross-contamination (Thrall *et al.* 2000; Thrall *et al.* 2007).

N-free modified McKnight solution was added once to twice a week to each pot (Claussen 2000; Thrall *et al.* 2007) (Table 4.2). The volume of nutrient solution added each time was about 100 to 300 mL. To prevent excessive salinity, distilled water was added where necessary.

In preparing nutrient solution, 1 M stock solutions were prepared first. Then depending on the nutrients, 1 to 4 mL per litre was pipetted out from each stock solution and diluted with 200 to 300 mL of distilled water. Diluted working nutrient solutions were autoclaved. The diluted working solutions of potassium chloride, magnesium sulphate and micronutrients were

autoclaved together. Those of calcium sulphate and potassium dihydrogen phosphate were autoclaved separately to avoid reaction with other chemicals (A. Richardson, pers. comm. 2011). As a large volume of nutrient solution was prepared regularly, tap water was used instead of distilled water to save cost and time. Tap water and Fe-EDTA were sterilised with ultra-violet radiation.

Table 4.2. The composition of modified N-free McKnight nutrient solution

Nutrient salt	Molar mass (g)	Nutrient salt concentration [A] (mg L^{-1})
KH_2PO_4	136.0857	200
$CaSO_4 * 2 H_2O$	172.1723	1500
$MgSO_4 * 7 H_2O$	246.4755	200
KCl	74.5515	300
FeNa-EDTA	348.089	313

Note: Trace solution was made by mixing the following amounts of trace elements in 1L solution. This solution was applied at a rate of 1mL per litre of the modified N-free McKnight solution: H_3BO_3 - 2.86 g; MnCl2 * $4H_2O$ - 1.81 g; $ZnSO_4 * 7 H_2O$ - 0.22 g; $CuSO_4 * 5 H_2O$ - 0.08 g; $MoO_4 * 4 H_2O$ - 0.025g

[A] Bergersen (1980)

Watering frequency was about once a week in the cooler dry season and twice a week as the weather became warmer. Occasionally, pots were watered twice a week in the dry season to ensure that the growth medium had an optimum nutrient level, especially when some pots displayed signs of nutrient deficiency or excessive salinity. Some wilting of phyllodes indicated water stress. Yellowing of phyllodes could be due to deficiency in certain macronutrients such as sulphur, nitrogen and magnesium, and micronutrients such as iron and manganese (Guha 1988).

Prolonged yellowing of foliage reflected nitrogen deficiency. This symptom was observed in almost all the pots during the cold and dry season when nodule formation was unlikely to occur. However, as the weather got warmer in early August, the foliage of most of the nodulating seedlings enlarged and developed moderately green color while seedlings of the non-nodulating treatment remained pale green or yellow.

Insect attack was never a problem in the shadehouse. The foliage of a few seedlings showed some signs of fungal infection but the infection appeared not to affect the growth in terms of height and foliage area.

4.2.5 Measurements

All plants were harvested after 157 days of growth and the dry weights of phyllodes, leaflets, stems, roots and root nodules were determined. The total nitrogen concentration of leaves, stems, roots and nodules were determined with the modified Kjeldahl digestion method (Rayment and Higginson 1992). The number of seedlings remaining for each treatment was recorded to calculate the survival rate.

4.2.6 Measuring symbiotic effectiveness

The absolute increase in the growth of a nodulating treatment was divided by the corresponding non-nodulating baseline. This proportion was then multiplied by 100 to obtain the symbiotic effectiveness (equation no. 4.1). The plant dry weight or nitrogen content of the non-nodulating treatment were treated as the background level that came from the seeds' reserves and sterilized soil in the prepared pots. Subtraction from the nodulating treatment permits a calculation of the net total amount of nitrogen fixed by nodules and the corresponding stoichiometric rise in carbon as nitrogen became no longer limiting. Percentage standardization facilitates fair comparison across species of different growth habits (tall tree – *A. crassicarpa* vs shrub/small tree – *A. aulacocarpa*, *A. elachantha* and *A. ramiflora*).

$$\text{Symbiotic effectiveness (\%)} = [(Y_{\text{Sp-Rh-Nodulating}} - Y_{\text{Sp-Rh-Non-nodulating}}) / (Y_{\text{Sp-Rh-Non-nodulating}})] * 100 \quad \text{..... (4.1)}$$

Adapted from Bergerson (1980)

In relation to biomass, "Y" can be the following growth parameters (abbreviations and units in parentheses):

- plant biomass (PB) (mg)
- stem biomass (mg)
- root biomass (mg)
- phyllode biomass (mg)
- phyllode area (cm^2)
- relative growth rate in terms of plant biomass (RGR) (mg DW gain g^{-1} PB d^{-1})

In relation to the nitrogen contents, "Y" can be the following growth parameters (units in parentheses):

- plant nitrogen (PN) (mg N)
- foliage nitrogen (mg N)
- nodule dry weight (NDW) (mg)
- relative growth rate in terms of plant nitrogen content (RGR-N) (mg N gain mg^{-1} PN d^{-1})

Net specific nitrogenase activity (SNA) (unit: µg N gain mg^{-1} NDW d^{-1}) is also included (Schortemeyer *et al.* 2002). It refers to the amount of nitrogen fixed per unit nodule biomass per day. The $PN_{Non\text{-}nodulating}$ is subtracted from $PN_{Nodulating}$ to calculate the net amount of nitrogen fixed by nodules. Net SNA can be calculated by either of the following two formulae:

$$\text{Net SNA} = [(PN_{Nodulating} - PN_{Non\text{-}nodulating}) / NDW] / 157 \text{ days} \quad \ldots (4.2)$$

$$\text{Net SNA} = [(RGR\text{-}N_{Nodulating} - RGR\text{-}N_{Non\text{-}nodulating}) / NMR] * PNC \quad \ldots (4.3)$$

$$RGR\text{-}N_{Nodulating} = [\text{Ln} (\text{PN of seedling}_{Sp\text{-}Rh\text{-}Nodulating}) - \text{Ln} (\text{PN of seed}_{Sp})] / 157 \text{ days} \quad \ldots (4.3a)$$

$$RGR\text{-}N_{Non\text{-}nodulating} = [\text{Ln} (\text{PN of seedling}_{Sp\text{-}Rh\text{-}Non\text{-}nodulating}) - \text{Ln} (\text{PN of seed}_{Sp})] / 157 \text{ days} \quad \ldots (4.3b)$$

(Note that in the case of $RGR_{nodulating}$ or $RGR_{non\text{-}nodulating}$, PN would be replaced by PB in equations 4.3a and 4.3b)

$$\text{Nodule mass ratio (NMR)} = NDW / PB \quad \ldots (4.3c)$$

$$\text{Plant nitrogen concentration (PNC)} = (PN_{Nodulating} - PN_{Non\text{-}nodulating}) / (PB_{Nodulating} - PB_{Non\text{-}nodulating}) \quad \ldots (4.3d)$$

Plant nitrogen concentration is calculated by dividing the net change in total nitrogen by net change in total biomass. Nodule mass ratio refers to the proportion of nodule biomass in relation to the whole plant biomass. The equations for calculating relative growth rates reflect the logarithmic nature of plant growth.

4.2.7 Data analysis

4.2.7.1 Survival rates of different treatments

The survival rates of different treatment combinations were calculated and compared.

4.2.7.2 Model construction and validation

The data structure of this experiment is a two-level hierarchy. Since pots were arranged in five blocks, block is the group variable (level two variable). Each block consists of 32 treatment combinations which belong to level one variable. Blocks one to five were randomly allocated so block is also a random variable. All treatments are fixed factors since species, rhizobia and nodulation status have been selected deliberately to test the hypotheses specified in Section 4.1.1.

With random and fixed factors, linear mixed models (LMMs) can be constructed with S-PLUS 8.0 (Pinheiro and Bates 1998). The LMM function in S-PLUS allows for differences of within-group variance among treatments. For example, if nodulation status contributes to most of the residuals heteroclasdecity, S-PLUS can allow for this by specifying "nodulation status" as the variable of within-group variance. This powerful function reduces the need to transform the response variables to meet the homoclasdecity assumption of linear models (LMs). In the current provenance experiment, the absolute differences in PB between species, rhizobia, nodulation status and all their interactions were modelled by S-PLUS. Differences in variance between every treatment combination were allowed for by specifying "species x rhizobia x nodulation status" as the group variable.

It should be noted that all the non-nodulating pots of one treatment were dead, so it was not possible to calculate the percentage increase of various growth parameters of the corresponding nodulating treatment. It also happened that one non-nodulating treatment had no phyllodes but its corresponding nodulating treatment had, so it was not possible to calculate the percentage change in phyllode area or phyllode dry weight for the nodulating treatment. The absence of all observations in a particular treatment resulted in a missing cell. Unknown errors were sometimes encountered in S-PLUS when constructing LMMs on response variables with missing cells. When this occurred LMMs were constructed with SPSS v. 20. Thus some models in Tables 4.1 to 4.5 were constructed with SPSS and some with S-PLUS.

In the LMM function of SPSS, variances were allowed to vary across different treatment combinations. For example, sometimes various block x species combinations had heterogeneous variances and so specifying this in the "subject combination" option in SPSS might help to meet the assumption of homocledasticity. However, it was found that the distribution of residuals sometimes remained significantly skewed or their variances continued to be heterogeneous. Logarithmic transformation of data was thus necessary.

Parameters in a mixed model were estimated using the REML method. Whether an LM or an LMM fit best was determined by CAIC. Models with smaller information criterion values fit better.

It should be noted that no matter whether the analysis used S-PLUS or SPSS, block was always included as a random factor first. It was then removed to see if the model fit better. Most models were found to fit better without including block as a random factor. This reflects that block was not a significant random factor in most cases. In addition, allowing for differences in variance between treatments in terms of varying intercepts could sometimes result in better fit but sometimes it could not. Parameters estimated by REML in models without random factor and without varying intercepts resembled those estimated by OLS in a LM.

Though the experimental layout resembled a split-split plot design (Fig. 4.2), the effects of plots and sub-plots were removed by random repositioning with a two-week interval. There was thus no need to allow for this added variance in ANOVA (T. Glasby, pers. comm. 2012 and R. Jones, pers. comm. 2012).

Residual normality assumption was checked by histogram, Q-Q plot, Shapiro-Wilk test, and descriptives (especially for the severity of skewness and kurtosis). Homogeneity of variance of residuals was examined through inspecting the scatter plot of residuals against fitted values which should have no observable pattern to meet the relevant assumptions.

4.2.7.3 Statistical analyses

Potential differences in PB of each species due to variation of soil rhizobia (provenance) were examined first with a three-way ANOVA. The fixed factors included species, rhizobia, nodulation condition (NodC) and their interactions (Table 4.1).

The second part of the analysis focused on the effect of species, rhizobia and their interactions on growth parameters related to biomass (Table 4.3). These parameters were all standardized as mentioned in Section 4.2.6 and included percentage changes in PB, root biomass, stem biomass, phyllode biomass, phyllode area and RGR. Models were built and the effects of the aforementioned factors were examined with ANOVA.

The effect of species, rhizobia and their interactions on the nitrogen-related variables was also examined by ANOVA (Table 4.4). Response variables related to nitrogen-related variables were again standardized and included percentage changes in PN, foliage nitrogen and RGR-N, and NDW.

Since NDW was believed to have a close relationship with the biomass and nitrogen contents, the effect of species, rhizobia and their interactions on such a relationship was also examined. This was by means of comparing the regression slopes between different rhizobia for each species. Models were thus constructed by fitting species, rhizobia, NDW, all of the two-way and three-way interactions onto an LMM for each of the response variables (Table 4.5 and 4.6).

Table 4.3. Models used to investigate the effects of four species, four sources of rhizobia and their interactions on biomass-related variables

LMM – linear mixed model; LM – linear model. NodC – nodulation conditions (nodulating vs non-nodulating).

Response variable	Unit	Fixed factor	Software	Model type	Random factor	Variance function
Plant biomass [A]	mg	Species Rhizobia NodC Species x Rhizobia Species x NodC Rhizobia x NodC	S-PLUS	LMM	Block, with varying intercept	Within-group variance : Identity Group variable: Block x Species x NodC
Log_{10}(% increase in plant biomass +100)	%	Species Rhizobia Species x Rhizobia	SPSS	LM	Nil	Nil
Log_{10}(% increase in root biomass + 80)	%	Same as above	SPSS	LM	Nil	Nil
Log_{10}(% increase in stem biomass + 90)	%	Same as above	SPSS	LMM	Varying intercept	Random effect covariance type: Variance component Subject combination: Block x Species x Rhizobia
Log_{10}(% increase in phyllode biomass + 95)	%	Same as above	SPSS	LMM	Varying intercept	Random effect covariance type: Variance component Subject combination: Block x Species x Rhizobia
Log_{10}(% increase in phyllode area + 100)	%	Same as above	SPSS	LMM	Varying intercept	Random effect covariance type: Variance component Subject combination: Block x Species x Rhizobia
Log_{10}(% increase in relative growth rate in terms of plant biomass + 150)	%	Same as above	SPSS	LMM	Varying intercept	Random effect covariance type: Variance component Subject combination: Block x Species x Rhizobia

[A] When the species X rhizobia X nodC factor was included as a fixed factor, the model failed to converge. Potential three-way interaction was thus verified by profile plot.

Table 4.4. Models used to investigate the effects of four species, four sources of rhizobia and their interactions on nitrogen-related variables

LMM – linear mixed model; LM – linear model.

Response variable	Unit	Fixed factor	Software	Model type	Random factor	Variance function
Log_{10}(% increase in plant nitrogen +60)	%	Species Rhizobia Species x Rhizobia	SPSS	LMM	Varying intercept	Random effect covariance type: Variance component Subject combination: Block x Species x Rhizobia
Log_{10}(nodule dry weight + 15)	mg	Same as above	SPSS	LM	Nil	Nil
Log_{10}(% increase in foliage nitrogen + 140)	%	Same as above	SPSS	LM	Nil	Nil
Net specific nitrogenase activity	µg N gain mg^{-1} NDW d^{-1}	Same as above	S-PLUS	LMM	Block with varying intercept	Within-group variance : Identity Group variable: Block x Species
% increase in relative growth rate in terms of plant nitrogen content	%	Same as above	S-PLUS	LMM	Block with varying intercept	Within-group variance : Identity Group variable: Block x Species

Table 4.5. Models used to investigate the effects of four species and four sources of rhizobia on the regression between nodule dry weight (NDW) and biomass-related variables

LMM – linear mixed model; LM – linear model.

Response variable	Unit	Fixed factor	Software	Model type	Random factor	Variance function
Log_{10}(% increase in plant biomass +100)	%	Species Rhizobia NDW Species x Rhizobia Species x NDW Rhizobia x NDW Species x Rhizobia x NDW	SPSS	LMM	Varying intercept	Random effect covariance type: Variance component Subject combination: Block x Species x Rhizobia
Log_{10}(% increase in root biomass + 80)	%	Same as above	SPSS	LMM	Varying intercept	Random effect covariance type: Variance component Subject combination: Block x Species x Rhizobia
Log_{10}(% increase in stem biomass + 90)	%	Same as above	SPSS	LMM	Varying intercept	Random effect covariance type: Variance component Subject combination: Block x Species x Rhizobia
Log_{10}(% increase in phyllode biomass + 95)	%	Same as above	SPSS	LMM	Varying intercept	Random effect covariance type: Variance component Subject combination: Block x Species x Rhizobia
Log_{10}(% increase in phyllode area + 100)	%	Same as above	SPSS	LMM	Varying intercept	Random effect covariance type: Variance component Subject combination: Block x Species x Rhizobia
Log_{10}(% increase in relative growth rate in terms of plant biomass + 150)	%	Same as above	SPSS	LMM	Varying intercept	Random effect covariance type: Variance component Subject combination: Block x Species x Rhizobia

Table 4.6. Models used to investigate the effects of four species and four sources of rhizobia on the regression between nodule dry weight (NDW) and nitrogen-related variables

LMM – linear mixed model; LM – linear model.

Response variable	Unit	Fixed factor	Software	Model type	Random factor	Variance function
Log_{10}(% increase in plant nitrogen + 60)	%	Species Rhizobia NDW Species x Rhizobia Species x NDW Rhizobia x NDW Species x Rhizobia x NDW	SPSS	LMM	Varying intercept	Random effect covariance type: Variance component Subject combination: Block x Species x Rhizobia
Log_{10}(% increase in foliage nitrogen + 140)	%	Same as above	SPSS	LMM	Varying intercept	Random effect covariance type: Variance component Subject combination: Block x Species x Rhizobia
Net specific nitrogenase activity	µg N gain mg^{-1} NDW d^{-1}	Same as above	S-PLUS	LMM	Block with varying intercept	Within-group variance : Identity Group variable: Block x Species
% increase in relative growth rate in terms of plant nitrogen content	%	Same as above	S-PLUS	LMM	Block with varying intercept	Within-group variance : Identity Group variable: Block x Species

4.3 Results

4.3.1 Survival rates of different treatments

The average survival of *A. aulacocarpa*, *A. crassicarpa*, *A. elachantha* and *A. ramiflora* were respectively 48%, 87%, 43% and 67% (Fig. 4.4). After pooling the data from all rhizobia groups, nodulation was found to always result in higher survival of all species. For example, only 37% of non-nodulating *A. aulacocarpa* survived while 58% of nodulating *A. aulacocarpa* survived. An 8 to 11% increase in survival was observed when *A. crassicarpa* and *A. ramiflora* nodulated. For *A. elachantha*, nodulation increased survival by about 33 to 66%.

The effect of nodulation on survival varied with rhizobial sources. For example, in the presence of *A. elachantha* rhizobia, the survival rates of nodulating and non-nodulating *A. crassicarpa* were similar. *A. aulacocarpa* rhizobia, however, increased the survival of *A. crassicarpa* from 64% to 90% (Fig. 4.4).

Nodulation sometimes appeared to reduce survival. Nodulating *A. crassicarpa* in the presence of *A. ramiflora* rhizobia had a survival of 80% compared to 89% in the non-nodulating treatment. Nodulating *A. ramiflora* grown in *A. elachantha* soil had a survival of about 67% which was 20% lower than its non-nodulating counterpart.

Nodulation resulted in a higher survival of 12 treatment combinations (Fig. 4.4). For *A. aulacocarpa*, the increase in survival due to inland rhizobia was higher than that due to coastal rhizobia, however, for *A. crassicarpa,* inland rhizobia had a poor or even negative effect on the survival. The survival increase of *A. elachantha* was at least 36% in the presence of *A. crassicarpa* rhizobia or reached a maximum of 66% with *A. ramiflora* rhizobia. Regarding the survival of *A. ramiflora*, *A. elachantha* rhizobia resulted in a 20% decrease while other rhizobia resulted in an increase (13% with *A. ramiflora* rhizobia compared to the 19% and 20% with coastal rhizobia).

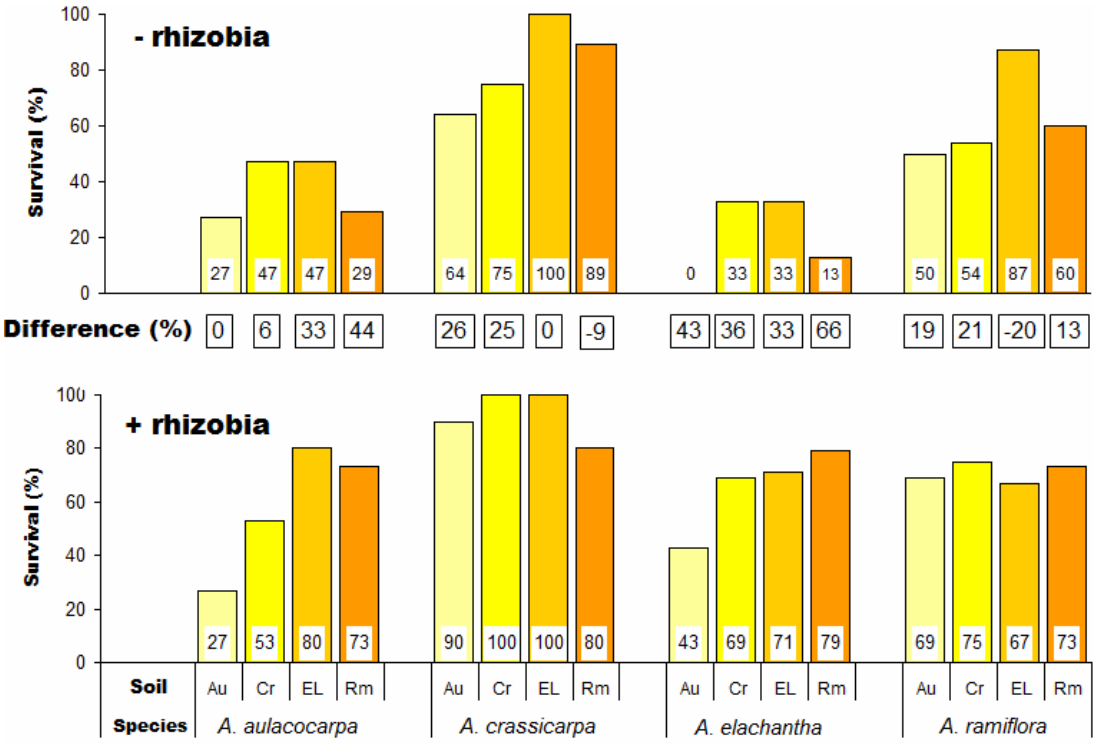


Figure 4.4. The percentage survival of 32 treatment combinations in the provenance experiment. Numbers in the top and bottom panels represent the survival rates of non-nodulating and nodulating treatments respectively. Each number in the intermediate panel represents the difference between each nodulating treatment and its non-nodulating counterpart. Au, *A. aulacocarpa*; Cr, *A. crassicarpa*; EL, *A. elachantha*; Rm, *A. ramiflora.*

4.3.2 Plant biomass of nodulating and non-nodulating treatments

Nodulation resulted in significantly higher PB of most species-rhizobia treatments ($P < 0.001$) (Table 4.7). Nodulation had no significant effect on the PB of *A. ramiflora* grown in *A. crassicarpa* soil. The mean PB of nodulating *A. crassicarpa*, *A. aulacocarpa*, *A. elachantha* and *A. ramiflora* across all rhizobial sources were 3481, 365, 959 and 141 mg respectively. These values were 16, 1.4, 27 and 3.5 times higher than their respective non-nodulating counterparts.

Table 4.7. The measured mean (± 1 S.E.M.) plant biomass of non-nodulating and nodulating treatments

Values are the measured means while S.E.M. is estimated by the Restricted Maximum Likelihood (REML) method, after fitting a linear mixed model. An asterisk indicates significant difference between a specific nodulating treatment combination and its corresponding non-nodulating combination. PB – plant biomass. Au, *A. aulacocarpa*; Cr, *A. crassicarpa*; EL, *A. elachantha*; Rm, *A. ramiflora*. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; NS – $P > 0.05$.

Species	Source of rhizobia / soil	Non-nodulating PB (mg)	Nodulating PB (mg)	P-value
Au	Au	56 ± 24	199 ± 74	***
Au	Cr	86 ± 17	250 ± 68	***
Au	EL	78 ± 18	510 ± 68	***
Au	Rm	37 ± 26	502 ± 69	***
Cr	Au	147 ± 27	3236 ± 129	***
Cr	Cr	488 ± 35	3 661 ± 126	***
Cr	EL	150 ± 26	3 989 ± 125	***
Cr	Rm	100 ± 26	3038 ± 127	***
EL	Au	All died	102 ± 282	***
EL	Cr	52 ± 14	1 297 ± 128	***
EL	EL	33 ± 1	1 214 ± 128	***
EL	Rm	24 ± 1	1 222 ± 127	***
Rm	Au	48 ± 7	266 ± 32	***
Rm	Cr	36 ± 5	137 ± 30	NS
Rm	EL	48 ± 4	79 ± 39	*
Rm	Rm	30 ± 5	84 ± 32	*

4.3.2.1 Non-nodulating treatments

The plant biomass varied depending on the source of sterilised soil (Table 4.8). The PB of non-nodulating *A. aulacocarpa* (37 to 86 mg) and *A. ramiflora* (30 to 48 mg) seedlings did not differ significantly between soil sources (Table 4.8). In contrast, the PB of non-nodulating *A. crassicarpa* seedlings grown in their own soil (488 mg) was significantly higher than those in foreign soil (100 to 150 mg). *A. crassicarpa* soil had higher nitrogen levels than other soils (Table 1.2). When *A. crassicarpa* seedlings could not develop root nodules from the sterilised growth medium, soil within the growth medium became their sole source of nitrogen. Furthermore this nitrogen was the only limiting nutrient when other nutrients were provided to

seedlings regularly in the form of N-free modified McKnight solution (Table 4.1). Changing the nitrogen level by means of varying the source of soil should thus affect the PB of non-nodulating *A. crassicarpa.* The higher nitrogen content in *A. crassicarpa* soil was probably also the reason for the fact that *A. elachantha* seedlings grown in *A. crassicarpa* soil gave a significantly higher PB than those grown in their own soil (52 mg vs 33 mg) (Table 4.8).

Table 4.8. The measured mean (± 1 S.E.M.) plant biomass of 32 treatment combinations

Each value in bold is the measured mean plant biomass (unit: mg) of each species grown with their own soil rhizobia. An asterisk attached to a specific treatment combination indicate significant differences between that combination and its corresponding sympatric combination (Au-Au, Cr-Cr, EL-EL or Rm-Rm). The S.E.M. was estimated by the Restricted Maximum Likelihood (REML) method. Au, *A. aulacocarpa*; Cr, *A. crassicarpa*; EL, *A. elachantha*; Rm, *A. ramiflora*. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

Nodulating condition	Species	Source of soil			
		Au	Cr	EL	Rm
Non-nodulating	Au	**56 ± 24**	86 ± 17	78 ± 18	37 ± 26
	Cr	147 ± 27 ***	**488 ± 35**	150 ± 26 ***	100 ± 26 ***
	EL	All died	52 ± 14 ***	**33 ± 1**	24 ± 1
	Rm	48 ± 7	36 ± 5	48 ± 4	**30 ± 5**
Nodulating	Au	**199 ± 74**	250 ± 68 *	510 ± 68	502 ± 69 ***
	Cr	3236 ± 129	**3 661 ± 126**	3 989 ± 125 ***	3 038 ± 127 ***
	EL	102 ± 282 ***	1 297 ± 128	**1 214 ± 128**	1 222 ± 127
	Rm	266 ± 32 ***	137 ± 30	79 ± 39	**84 ± 32**

4.3.2.2 Nodulating treatments

Nodulating *A. aulacocarpa* yielded the lowest PB in the presence of its own rhizobia. The mean PB was 199 mg (Table 4.8). It was significantly lower than the PB of *A. aulacocarpa* in the presence of *A. crassicarpa* or *A. ramiflora* rhizobia. The failure to detect a significant difference between *A. aulacocarpa* with its own rhizobia and *A. aulacocarpa* with *A. elachantha* rhizobia may indicate the inadequacy of the mixed model, despite that it is the best model derived from S-PLUS.

A. crassicarpa had the highest PB when rhizobia originated from *A. elachantha* soil. The mean PB was 3 989 mg. The PB in the presence of its own and *A. ramiflora* soil were significantly less ($P < 0.001$). Their mean PB were 3 661 and 3 038 mg respectively. The PB of *A.*

crassicarpa in the presence of *A. aulacocarpa* soil was similar to that in the presence of its own soil.

A. elachantha grown with *A. aulacocarpa* soil rhizobia had a significantly lower PB (102 mg) than when grown with other soil rhizobia (1 214 to 1 297 mg). These other PB were not significantly different. In contrast, , *A. ramiflora* with *A. aulacocarpa* rhizobia had a mean PB of 266 mg, which was significantly higher than the PB in the presence of other rhizobia.

It appears that local soil rhizobia do not have advantages over foreign soil rhizobia in yielding the highest PB. However, in order to verify this conclusion, the performance of soil rhizobia of different species should be standardized. The next section will compare standardized results across species and rhizobia.

4.3.3 Effect of nodulation on plant biomass and nitrogen content

Seedlings of nodulating *A. elachantha* yielded a 2 990% greater plant biomass than non-nodulating seedlings, while nodulating *A. crassicarpa* yielded 1 845% more than non-nodulating seedlings (Table 4.9). The effectiveness of nodulation was less for *A. aulacocarpa* and *A. ramiflora* as their nodulating seedlings yielded only 220% and 154% more than non-nodulating ones. The estimated mean increase in the PB of different species in decreasing order was: *A. elachantha* > *A. crassicarpa* > *A. aulacocarpa* = *A. ramiflora*. Thus the effectiveness of nodulation appears to have little relationship to whether a species was inland or coastal.

Other plant organs (except stems) showed similar patterns. The estimated mean increase in stem biomass was 1 566% for *A. crassicarpa* and 1 082% for *A. elachantha* but only 110% and 95% for *A. aulacocarpa* and *A. ramiflora* respectively (Table 4.9).

The increase in biomass varied with plant organs. In *A. aulacocarpa* and *A. crassicarpa,* the greatest changes occurred in phyllodes, followed by stems and then roots. In *A. ramiflora,* the increases in stem and root biomass were similar and that of phyllodes was 4.5 times that of the stem. In *A. elachantha,* the increase in phyllode biomass was almost five times that of stems, which in turn was less than that of roots by 50%.

Thus the inland *A. elachantha* and *A. ramiflora* allocated a greater or an equal amount of biomass in developing roots compared to stems. For coastal *A. aulacocarpa* and *A. crassicarpa,*

the biomass allocation towards stem development was higher than for roots. For both coastal and inland species, phyllode development was always greater when nitrogen was not limiting.

Table 4.9. The estimated mean increases in the biomass of plant organs of the four studied species

The biomass of each plant organ was fitted into a linear mixed model or linear model. Every model incorporated three fixed factors: species, rhizobia and species x rhizobia interaction. 95% confidence intervals are given in parentheses .

Plant organ	Estimated mean increase in biomass (%)			
	A. aulacocarpa	*A. crassicarpa*	*A. elachantha*	*A. ramiflora*
Whole plant	220 (134 to 337)	1 845 (1 366 to 2 488)	2 990 (2 175 to 4 107)	154 (92 to 237)
Root	64 (27 to 113)	789 (585 to 1 055)	1 546 (1 136 to 2 088)	68 (34 to 111)
Stem	110 (49 to 197)	1 566 (1 088 to 2 233)	1 082 (721 to 1 600)	95 (43 to 169)
Phyllode	363 (180 to 667)	2 769 (1 683 to 4 508)	5 202 (2 691 to 9 951)	431 (232 to 757)

Foliage (phyllodes) of nodulating *A. elachantha* seedlings contained 17 725% more nitrogen than non-nodulating seedlings (Table 4.10). Even for *A. crassicarpa,* the estimated mean foliage nitrogen of the nodulating seedlings was only 7 569% greater than that of non-nodulating seedlings. Nodulation of *A. aulacocarpa* and *A. ramiflora* resulted in much smaller increases in foliage nitrogen (491% and 397% respectively).

The plant nitrogen of nodulating *A. elachantha* and *A. ramiflora* were 4 051% and 159% greater than the non-nodulating seedlings respectively (Table 4.10). The PNs of nodulating *A. aulacocarpa* and *A. crassicarpa* were respectively 380% and 445% greater than that of their non-nodulating counterparts,

Each mg of NDW of *A. crassicarpa* and *A. elachantha* fixed 5.55 to 5.59 µg N per day on average, a much faster rate than *A. aulacocarpa* and *A. ramiflora.* The mean NDW showed a similar pattern among species: *A. crassicarpa* > *A. elachantha* > *A. aulacocarpa* > *A. ramiflora*.

Table 4.10. The estimated mean values of nitrogen-related variables of four nodulating species

Each response variable was fitted into a linear mixed model that examined the effect of species, rhizobia and species x rhizobia interaction. The net specific nitrogenase activity was fitted into a linear mixed model which only compared species differences. Parentheses enclose 95 % confidence intervals.

Response variable	Unit	Estimated mean			
		A. aulacocarpa	*A. crassicarpa*	*A. elachantha*	*A. ramiflora*
Increase in foliage nitrogen	%	491 (307 to 753)	7 569 (5 522 to 10 331)	17 725 (12 537 to 25 037)	397 (223 to 653)
Increase in plant nitrogen	%	380 (223 to 624)	4 448 (2 960 to 6 670)	4 051 (2 582 to 6 323)	159 (86 to 269)
Nodule dry weight	mg	15 (10 to 21)	275 (230 to 329)	46 (36 to 57)	6 (3 to 10)
Net specific nitrogenase activity	µg N gain mg^{-1} NDW d^{-1}	2.81 (1.77 to 3.84)	5.59 (3.20 to 7.99)	5.55 (2.80 to 8.29)	1.00 (-1.18 to 3.18)

4.3.4 Effect of rhizobial sources on biomass increases

It is assumed that symbiotic effectiveness was mainly attributed to the presence of rhizobia. Autoclaving was employed to kill soil bacteria but was found to release some nutrients from microbes (Schmidt *et al.* 1997). Increased soil nitrate or ammonium might lead to greater biomass and nitrogen contents. Autoclaving was also known to alter soil chemical properties such as increasing the soluble organic carbon or lowering the pH by possibly making organic acid from humus available (Shaw *et al.* 1999). The effect of lower pH and nutrient release from microbes was assumed to be small to each seedling because each non-nodulating replicate was added with 100 g of autoclaved soil only.

A. aulacocarpa and *A. crassicarpa* showed small increases in the biomass of whole plants and individual organs when grown in soil from *A. crassicarpa* (Table 4.11). For both species grown in *A. crassicarpa* soil, the increases in RGR was the lowest (respectively 42% and 59%) compared to the increases in the presence of other sources of rhizobia (respectively 49% to 108% and 136% to 188%).

In the case of *A. aulacocarpa,* the estimated increases in the biomass of the whole plant and individual organs were higher when the rhizobia were sourced under the canopies of *A. elachantha* or *A. ramiflora*. For *A. elachantha,* the estimated increases in the biomass of whole plant and of individual plant organs were higher with either *A. ramiflora* or *A. elachantha* rhizobia but small with *A. crassicarpa* rhizobia.

In contrast, the estimated increases in the PB and NDW of *A. ramiflora* grown with *A. aulacocarpa* rhizobia were highest (393% and 17% respectively). The effectiveness of *A. crassicarpa* soil rhizobia came second in these two parameters (192% and 4% respectively). The estimated mean increase in phyllode biomass of *A. ramiflora* grown with *A. aulacocarpa* rhizobia was higher than that with *A. crassicarpa* rhizobia but the low power of the pairwise comparison rendered it impossible to verify the statistical significance. *A. elachantha* and *A. ramiflora* rhizobia resulted in low increases in PB, phyllode biomass and root biomass, and NDW of *A. ramiflora* seedlings. The increase in phyllode area largely resembled the pattern observed in the increase phyllode biomass.

There is no evidence that local soil rhizobia stimulated the greatest increase in the biomass of the host plant and its organs.

Table 4.11. The effects of four species, four sources of rhizobia and their interactions on biomass-related variables

Each response variable was fitted into either a linear mixed model (LMM) or linear model (LM). Every model has three fixed factors: species, rhizobia and species x rhizobia interaction. The F-ratios and significances of the fixed factors are shown below; when the P-value of the species x rhizobia interaction was less than 0.05, multiple comparisons of the estimated means between soil rhizobia for each of the species were undertaken.

Au, *A. aulacocarpa*; Cr, *A. crassicarpa*; EL, *A. elachantha*; Rm, *A. ramiflora*. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS – $P > 0.05$.

Response variable	Fixed factor	F-ratio	P-value	Species	Multiple comparison between rhizobia per species [A, B]			
Log_{10}(% increase in RGR + 150)	Species	$F_{3, 52} = 29.43$	***	Au	Rm =	EL =	Au =	Cr
	Rhizobia	$F_{3, 53} = 4.811$	**	Cr	Rm >	(Au) =	(EL) >	Cr
	Species x Rhizobia	$F_{8, 52} = 2.217$	*	EL	Rm =	EL >	Cr	
				Rm	Au >	(Cr) =	(Rm) >	EL
Log_{10}(% increase in plant biomass + 100)	Species	$F_{3, 125} = 71.772$	***	Au	Rm =	EL >	(Au) >	Cr
	Rhizobia	$F_{3, 125} = 4.099$	**	Cr	Rm =	EL =	Au >	Cr
	Species x Rhizobia	$F_{8, 125} = 2.744$	**	EL	Rm >	(EL) >	Cr	
				Rm	Au >	(Cr) >	Rm =	EL
Log_{10}(% increase in root biomass + 80)	Species	$F_{3, 126} = 77.045$	***	Au	Rm =	EL =	Au >	Cr
	Rhizobia	$F_{3, 126} = 4.727$	**	Cr	EL =	Rm =	Au >	Cr
	Species x Rhizobia	$F_{8, 126} = 2.227$	*	EL	Rm >	EL =	Cr	
				Rm	Au =	Cr =	Rm =	EL

Table 4.11. Cont'd.

Log_{10}(% increase in stem biomass + 90)	Species	$F_{3, 62} = 44.709$	***	Au	EL >	(Rm) >	Au =	Cr
	Rhizobia	$F_{3, 62} = 2.449$	NS	Cr	Rm =	EL =	Au >	Cr
	Species x Rhizobia	$F_{8, 61} = 3.489$	**	EL	EL =	Rm =	Cr	
				Rm	Au =	Cr >	(Rm) >	EL
Log_{10}(% increase in phyllode biomass + 95)	Species	$F_{3, 42} = 21.213$	***	Au	EL =	Rm =	Cr =	Au
	Rhizobia	$F_{3, 42} = 2.311$	NS	Cr	Rm =	EL >	(Au) >	Cr
	Species x Rhizobia	$F_{7, 40} = 2.497$	*	EL	EL =	Cr		
				Rm	Au >	(Cr) >	Rm =	EL
Log_{10}(% increase in phyllode area + 100)	Species	$F_{3, 46} = 21.72$	***	Au	EL >	(Rm) >	Cr =	Au
	Rhizobia	$F_{3, 45} = 1.496$	NS	Cr	Rm =	Au =	EL >	Cr
	Species x Rhizobia	$F_{7, 44} = 3.428$	**	EL	EL =	Cr		
				Rm	Au >	(Cr) >	Rm =	EL

[A] Non-nodulating *A. elachantha* grown in *A. ramiflora* soil did not have phyllodes but only leaflets so the increase in phyllode biomass of its nodulating counterpart could not be calculated. Non-nodulating *A. elachantha* seedlings grown in *A. aulacocarpa* soil were all dead and so the increases in the biomass of all plant organs of its nodulating counterpart could not be calculated.

[B] A rhizobial source in parenthsis has a mean value in between the other two sources of rhizobia not in parenthesis but the pairwise comparison lacks sufficient power to distinguish it from either one of them.

4.3.5 Effect of rhizobial sources on increases in nitrogen contents

This experiment was designed in a way such that, by subtracting the nodulating treatments from the non-nodulating ones using equation 4.1, most of the confounding effects of soil physical and chemical properties on the nitrogen contents of all four *Acacia* species were removed (assuming that sterilization did not significantly alter these two properties). By comparing the symbiotic effectiveness between soil sources, the varying nitrogen contents could likely be attributed to different sources of rhizobia instead of some properties of the unsterilized soil.

A. aulacocarpa and *A. crassicarpa* had significantly lower increases in RGR-N (81% and 133% respectively) and foliage nitrogen (15% and 2 083% respectively) when grown in their own soil than with foreign soil (Table 4.12). For each species, the increases in foliage nitrogen in the presence of other sources of rhizobia were similar but significantly higher. *A. aulacocarpa* achieved the greatest increase in RGR-N with *A. elachantha* rhizobia (220%); as for *A. crassicarpa,* that could be achieved with rhizobia from *A. ramiflora* (378%).

A. aulacocarpa also had significantly higher NDWs when it developed a symbiotic relationship with *A. ramiflora* (27 mg) rhizobia or *A. elachantha* rhizobia (24 mg) (Table 4.12). The source of rhizobia did not play a role in altering the NDW of *A. crassicarpa*. The net SNAs of *A. aulacocarpa* and *A. crassicarpa* in the presence of coastal rhizobia (0.3 to 1.9 and 4.1 to 5 µg N gain mg^{-1} NDW d^{-1} respectively) were lower than those in the presence of inland rhizobia (3.2 to 4.9 and 5.1 to 7.5 µg N gain mg^{-1} NDW d^{-1}).

The increases in PN of both coastal species tended to be higher in treatments with inland rhizobia than with coastal rhizobia. In general, it appears that the two coastal species could form more effective symbioses with inland rhizobia than the coastal ones.

For *A. elachantha, A. aulacocarpa* rhizobia resulted in significantly lower NDW (7 mg) than other rhizobia did (64 to 76 mg). However, *A. ramiflora* gained the highest NDW (17 mg) and net SNA (2.3 g N gain mg^{-1} NDW d^{-1}) with *A aulacocarpa* rhizobia. The increase in RGR-N of *A. ramiflora* was significantly higher with either *A. crassicarpa* or *A. aulacocarpa* rhizobia than with other sources of rhizobia; the increase in RGR-N of *A. elachantha* was highest in the presence of *A. ramiflora* rhizobia. The increase in foliage nitrogen of *A. ramiflora* did not vary significantly with the source of soil rhizobia. In contrast, the increase in foliage nitrogen of *A. elachantha* was highest in the presence of either of the two inland rhizobia.

The increases of PN of the two inland species were not significantly affected by the source of rhizobia although its components, RGR-N, NDW, and net SNA, did. It should be noted that the increases in PN of *A. ramiflora* with coastal rhizobia (214% to 280%) had lower values than those with inland rhizobia (74% to 123%). Multiplication of the components probably resulted in the high standard error, making it impossible to detect significant differences.

Table 4.12. The effects of four species, four sources of rhizobia and their interactions on nitrogen-related variables

Each of response variable was fitted into either a linear mixed model or linear model. Every model has three fixed factors: species, rhizobia and species x rhizobia interaction. The F-ratios and significances of the fixed factors are shown below; when the P-value of species x rhizobia factor was less than 0.05, multiple comparisons of the estimated means between rhizobial sources for each of the species were undertaken.

Au, *A. aulacocarpa*; Cr, *A. crassicarpa*; EL, *A. elachantha*; Rm, *A. ramiflora*. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS – $P > 0.05$.

Response variable	Fixed factor	F-ratio	P-value	Species	Mulitple comparison between rhizobia per species [A, B]			
% change in the relative growth rate in terms of plant nitrogen	Species	$F_{3, 127} = 53.428$	***	Au	EL >	Rm =	Cr >	Au
	Rhizobia	$F_{3, 127} = 133.215$	***	Cr	Rm >	Au =	EL >	Cr
	Species x Rhizobia	$F_{9, 127} = 14.662$	***	EL	Rm >	EL =	Cr	
				Rm	Au =	Cr >	Rm =	EL
Log_{10}(% change in plant nitrogen + 60)	Species	$F_{3, 51} = 53.904$	***	Au	EL >	Rm =	Cr =	Au
	Rhizobia	$F_{3, 51} = 1.249$	NS	Cr	Rm =	EL >	(Au) >	Cr
	Species x Rhizobia	$F_{8, 50} = 1.675$	NS [C]	EL	EL =	Rm =	Cr	
				Rm	Cr =	Au =	Rm =	EL
Log_{10}(% change in foliage nitrogen + 140)	Species	$F_{3, 102} = 93.894$	***	Au	Rm =	EL =	Cr >	Au
	Rhizobia	$F_{3, 102} = 12.141$	***	Cr	Rm =	EL =	Au >	Cr
	Species x Rhizobia	$F_{8, 102} = 4.228$	***	EL	Rm =	EL >	Cr	
				Rm	Au =	Cr =	EL =	Rm

Table 4.12. Cont'd.

Log_{10}(nodule dry weight + 15)	Species	$F_{3, 131} = 184.435$	***	Au	Rm =	EL >	(Cr) >	Au
	Rhizobia	$F_{3, 131} = 3.713$	*	Cr	Cr =	Au =	EL =	Rm
	Species x Rhizobia	$F_{9, 131} = 5.691$	***	EL	Cr =	Rm =	EL >	Au
				Rm	Au >	Cr =	Rm =	EL
Net specific nitrogenase activity	Species	$F_{3, 127} = 42.479$	***	Au	EL >	(Rm) >	Cr =	Au
Unit: µg N gain mg^{-1} NDW d^{-1}	Rhizobia	$F_{3, 127} = 1.332$	NS	Cr	EL >	Rm =	Au =	Cr
	Species x Rhizobia	$F_{9, 127} = 5.632$	***	EL	Rm =	EL=	Cr	
				Rm	Au >	(Cr) >	Rm =	EL

[A] Non-nodulating *A. elachantha* grown in *A. ramiflora* soil did not have phyllodes but only leaflets so the increase in phyllode biomass of its nodulating counterpart could not be calculated. Non-nodulating *A. elachantha* grown in *A. aulacocarpa* soil was all dead and so the increases in the biomass of all plant organs of its nodulating counterpart could not be calculated.

[B] A rhizobial source in parentheses has a mean value in between two other rhizobial sources without parentheses but pairwise comparisons lack sufficient power to distinguish it from either one of them.

[C] This species x rhizobia interaction factor was not significant since the variation in plant nitrogen increase between species did not vary significantly with rhizobial sources. But the variation in plant nitrogen increase between rhizobial sources did vary significantly with species. So even though the interaction factor was not significant, multiple comparisons in plant nitrogen increase were still performed between rhizobial sources for every species.

4.3.6 Relationship between nodule biomass and plant biomass / nitrogen content

The relationships between NDW and increases in RGR, PB, root biomass, and stem biomass were positive in some species (see the F-ratios and P-values of the species x NDW factor in Table 4.13). The slopes of those positive relationships did not vary significantly with the sources of rhizobia, as indicated by the lack of significant three-way interactions in Table 4.13.

The relationship between phyllode biomass or area increase and NDW was species-specific (Table 4.13). In the scatter plot of individual species, *A. crassicarpa* did not display any pattern between the two variables but all three other species showed a distinct positive relationship (Fig. 4.5). Pairwise comparisons indicated that rhizobial sources made a significant difference to the regression slope of *A. aulacocarpa* only (Table 4.13). *A. crassicarpa* rhizobia resulted in significantly higher regression slope than *A. ramiflora* rhizobia. The effect of *A. elachantha* soil was in between these two soils but its relationship to them was unclear due to insufficient power of the pairwise comparison. The pattern observed in phyllode area largely resembled that of phyllode biomass with two differences: in *A. aulacocarpa*, both *A. elachantha* and *A. ramiflora* rhizobia were ranked second instead of just *A. ramiflora* rhizobia; in *A. ramiflora, A. aulacocarpa* rhizobia resulted in a significantly weaker relationship than the other three rhizobial sources.

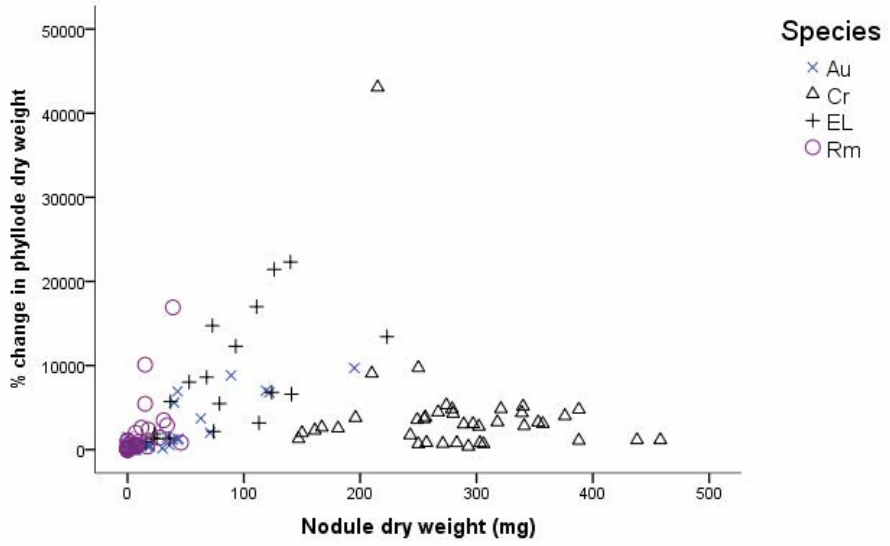


Figure 4.5. The relationship between the percentage change in phyllode biomass and the nodule dry weight of the four studied *Acacia* species.

A positive relationship between RGR-N increase and NDW was observed in *A. aulacocarpa* and *A. ramiflora* only. Also, a positive relationship between PN increase and NDW was observed in *A. aulacocarpa, A. elachantha* and *A. ramiflora* only. The source of soil / rhizobia did not significantly influence both relationships but it did significantly influence the relationship between foliage nitrogen and NDW ($F_{8, 87}$ = 2.713; P < 0. 05) (Table 4.14). ,For instance, soil from *A. aulacocarpa* or *A. crassicarpa* resulted in significantly weaker relationship in *A. crassicarpa*, *A. elachantha* and *A. ramiflora* while soil from *A. elachantha* and/or *A. ramiflora* resulted in a stronger relationship.

Regarding the relationship between net SNA and NDW, no distinctive contrast could be drawn between inland or coastal species (Table 4.14). The relationship between net SNA and NDW of *A. aulacocarpa* with *A. elachantha* or *A. crassicarpa* rhizobia was significantly stronger than that with *A. ramiflora* or *A. aulacocarpa* rhizobia. The relationship between net SNA and NDW of *A. crassicarpa* was not significantly altered by rhizobial sources. The strongest relationship between net SNA and NDW of *A. elachantha* was achieved with its own rhizobia while for *A. ramiflora*, that was achieved with from *A. crassicarpa* rhizobia. The same relationship for *A. ramiflora* caused by its own rhizobia was the second strongest while *A. aulacocarpa* rhizobia resulted in the weakest relationship. The relationship between net SNA and NDW of *A. aulacocarpa* in the presence of its own rhizobia was weaker than that with other rhizobial sources.

Table 4.13. The effects of four species and four sources of rhizobia on the regression between nodule dry weight and biomass-related variables

Each response variable was fitted into a linear mixed model which had seven fixed factors: species, rhizobia, nodule dry weight (NDW) and all the interactions. The F-ratios and significances of the relevant factors are shown below; when the P-value of the species x rhizobia x NDW interaction was less than 0.05, pairwise comparisons of regression coefficients between rhizobial sources for each of the species were undertaken. Au, *A. aulacocarpa*; Cr, *A. crassicarpa*; EL, *A. elachantha*; Rm, *A. ramiflora*. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS – $P > 0.05$..

Response variable	Factor	F-ratio	P-value	Species	Pairwise comparison of regression coefficients between rhizobial sources per species when three-way interaction is significant [B C]
Log_{10}(% increase in relative growth rate in terms of plant biomass + 150)	NDW	$F_{1, 103} = 0.941$	NS		
	Species x NDW	$F_{3, 110} = 11.501$	***		
	Species x Rhizobia x NDW	$F_{8, 110} = 1.15$	NS		
Log_{10}(% increase in plant biomass + 100)	NDW	$F_{1, 91} = 0.638$	NS		
	Species x NDW	$F_{3, 110} = 12.275$	***		
	Species x Rhizobia x NDW	$F_{8, 110} = 1.925$	NS		
Log_{10}(% increase in root biomass + 80)	NDW	$F_{1, 84} = 0.83$	NS		
	Species x NDW	$F_{8, 89} = 3.893$	*		
	Species x Rhizobia x NDW	$F_{8, 111} = 1.529$	NS		
Log_{10}(% increase in stem biomass + 90)	NDW	$F_{1, 95} = 2.301$	NS		
	Species x NDW	$F_{3, 111} = 7.236$	***		
	Species x Rhizobia x NDW	$F_{8, 111} = 0.701$	NS		

Table 4.13 Cont'd.

Log(% increase in phyllode area + 100)	NDW	$F_{1, 96} = 1.665$	NS	Au	Cr >	EL =	Rm	[Au][A]
	Species x NDW	$F_{3, 98} = 19.314$	***	Cr	No numerical differences nor statistical differences			
	Species x Rhizobia x NDW	$F_{7, 98} = 3.163$	**	EL	EL =	Cr		
				Rm	EL =	Rm =	Cr >	Au
Log_{10}(% increase in phyllode biomass + 95)	NDW	$F_{1, 86} = 0.213$	NS	Au	Cr >	(EL) >	Rm	[Au][A]
	Species x NDW	$F_{3, 101} = 12.899$	***	Cr	Au =	EL =	Cr =	Rm
	Species x Rhizobia x NDW	$F_{7, 89} = 2.354$	*	EL	EL =	Cr		
				Rm	EL =	Rm =	Cr =	Au

[A] The regression coefficient under this soil treatment is the highest among other rhizobial sources but its variance is too large to be significantly different from the regression coefficients of other rhizobial sources.

[B] Non-nodulating *A. elachantha* grown in *A. ramiflora* soil did not have phyllodes but only leaflets so the increase in phyllode biomass of its nodulating counterpart could not be calculated. Non-nodulating *A. elachantha* grown in *A. aulacocarpa* soil was all dead and so the increase in the biomass of its nodulating counterpart could not be calculated.

[C] This rhizobial source in parentheses has a regression coefficient in between the coefficients of the other two rhizobial sources not in parentheses but pairwise comparisons lack sufficient power to distinguish it from either one of them.

Table 4.14. The effects of four species and four sources of rhizobia on the regression between nodule dry weight and nitrogen-related variables

Each response variable was fitted into a linear mixed model. Every model had seven fixed factors: species, rhizobia, nodule dry weight (NDW) and all their interactions. The F-ratios and significances of the relevant factors are shown below; when the P-value of the species x rhizobia x NDW interaction was less than 0.05, pairwise comparisons of regression coefficients between rhizobial sources for each species were undertaken. Au, *A. aulacocarpa*; Cr, *A. crassicarpa*; EL, *A. elachantha*; Rm, *A. ramiflora*.* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS – $P > 0.05$.

Response variable	Factor	F-ratio	P-value	Species	Pairwise comparison of regression coefficients between rhizobial sources per species when three-way interaction is significant [A B]			
% increase in relative growth rate in terms of plant nitrogen content	NDW	$F_{1, 110} = 117.548$	***					
	Species x NDW	$F_{3, 110} = 29.573$	***					
	Species x Rhizobia x NDW	$F_{9, 110} = 1.174$	NS					
Log_{10}(% increase in plant nitrogen + 60)	NDW	$F_{1, 81} = 0.823$	NS					
	Species x NDW	$F_{3, 95} = 10.373$	***					
	Species x Rhizobia x NDW	$F_{8, 95} = 1.853$	NS					
Log_{10}(% increase in foliage nitrogen + 140)	NDW	$F_{1, 75} = 5.421$	*	Au	Cr >	(EL) >	Rm =	Au
	Species x NDW	$F_{3, 87} = 9.142$	***	Cr	Rm =	Au =	Cr =	EL
	Species x Rhizobia x NDW	$F_{8, 87} = 2.713$	*	EL	EL >	(Cr) >	Rm	
				Rm	EL >	(Rm) >	Cr =	Au

Table 4.14 Cont'd.

Net specific nitrogenase activity	NDW	$F_{1, 111} = 9.928$	**	Au	EL =	Cr >	Rm >	Au
(Unit: µg N gain mg^{-1} NDW d^{-1})	Species x NDW	$F_{3, 111} = 35.915$	***	Cr	Cr =	Au =	EL =	Rm
	Species x Rhizobia x NDW	$F_{9, 111} = 4.834$	***	EL	EL >	Cr =	Rm	
				Rm	Cr >	Rm >	EL =	Au

[A] Non-nodulating *A. elachantha* grown in *A. aulacocarpa* soil were all dead and so the increase in the biomass of their nodulating counterpart could not be calculated.

[B] This rhizobial source in parentheses has a regression coefficient in between the coefficients of the other two rhizobial sources not in parentheses but pairwise comparisons lack sufficient power to distinguish it from either one of them.

4.4 Discussion

4.4.1 The performance of each Acacia species when grown with its own rhizobia

Though nodulating *A. crassicarpa* had similar nodule biomass under all four sources of rhizobia, the nodules occupied by rhizobia from *A. elachantha* soil fixed more nitrogen per unit mass per day. Most biomass and nitrogen-related variables (e.g. increase in phyllode biomass and PN, and net SNA) of *A. crassicarpa* with either of the two coastal rhizobial sources were lower than with those with *A. elachantha* or *A. ramiflora* rhizobia. There is thus no evidence of local adaptation for *A. crassicarpa*-rhizobia symbiosis.

Nevetheless, nodulation by the coastal rhizobia did increase the survival of *A. crassicarpa* more than nodulation by the inland rhizobia did. The increase in survival as a function of nodulation means that more nodulating replicates experience better nitrogen nutrition and hence their metabolism can achieve more than simple maintenance, and vice versa. Zero change in survival of *A. crassicarpa* due to *A. elachantha* rhizobia implied that the nutritional effect of mutualistic symbionts was constrained by competitive parasitic symbionts. Parasitic symbionts probably had a higher chance of outcompeting the mutualistic symbionts in inland soil when *A. crassicarpa* was used as the host, thus leading to lower survival on inland soils than coastal soils.

Nodulating *A. crassicarpa* with inland rhizobia had greater increase in biomass than with coastal rhizobia. Therefore, among those surviving replicates, the effectiveness of inland mutualistic symbionts was much higher than that of the coastal symbionts. Thus strong rhizobial competitors in nodulation might not be effective partners.

There was also no evidence of local adaptation for *A. ramiflora*-rhizobia symbiosis. *A. ramiflora* grown with *A. aulacocarpa* rhizobia had the highest NDW and net SNA, increases in phyllode biomass, phyllode area, PB and RGR. Nevertheless the increases in PN, foliage nitrogen, and root biomass did not differ significantly between rhizobial sources.

In *A. ramiflora*, increases in survival by 13 to 21% could be observed when rhizobia came from *A. aulacocarpa, A. crassicarpa* and *A. ramiflora* soil. However, *A. ramiflora* grown with *A. elachantha* rhizobia had a 20% decrease in survival. Among the three rhizobial sources with

increased survival, the effectiveness of the mutualistic symbionts of *A. aulacocarpa* soil appeared to be the highest in terms of the NDW, the net SNA and biomass increases.

For *A. aulacocarpa*, there was no significant difference in increases in RGR and phyllode biomass between rhizobial sources. *A. aulacocarpa* with *A. elachantha* and *A. ramiflora* rhizobia had greater increases in PB, stem biomass, phyllode area (*A. elachantha* rhizobia only), RGR-N and PN, and NDW and net SNA (*A. elachantha* rhizobia only) than with *A. aulacocarpa* rhizobia. Nodulation by inland rhizobia also appeared to confer survival advantage in terms of percentage increase in survival.

When *A. elachantha* grew with *A. ramiflora* rhizobia, the increase in PB, root biomass, and RGR-N were significantly higher than the increases with *A. elachantha* and *A. crassicarpa* rhizobia. On the other hand, the increases in foliage nitrogen and RGR of *A. elachantha* with *A. ramiflora* were similar to increases with *A. elachantha* soil rhizobia but higher than the increases with *A. crassicarpa* rhizobia. Rhizobial sources did not significantly change the other variables. Also, nodulation appears very important to increasing the survival of *A. elachantha* compared to other species. For instance, nodulation increased *A. elachantha*'s survival by a mean value of 46% but only 21%, 11% and 8% in *A. aulacocarpa*, *A. crassicarpa* and *A. ramiflora* respectively. *A. ramiflora* rhizobia produced the highest net mutualistic effect on nodulating *A. elachantha* (66% survival). Nodulation by *A. aulacocarpa* rhizobia increased *A. elachantha*'s survival by 43% while increases in survival in the presence of the other two sources of rhizobia were similar (36% and 38%).

In short, *A. aulacocarpa* established and grew best with inland rhizobia. *A. crassicarpa* established best with coastal rhizobia but grew best with inland rhizobia. *A. elachantha* established and grew best with *A. ramiflora* rhizobia. *A. ramiflora* grew best with *A. aulacocarpa* rhizobia. The increase in survival of *A. ramiflora* under *A. aulacocarpa* rhizobia, among other rhizobial sources, was also one of the highest.

In *A. aulacocarpa*, *A. elachantha* and *A. ramiflora*, the symbiotic effectiveness in terms of net SNA or increase in foliage nitrogen was positively correlated with NDW. It is clear that the most effective rhizobial partner of a species had the smallest regression coefficient in the relationship between NDW and net SNA / percentage change in foliage nitrogen (Tables 4.14 and 4.12). It can thus be deduced that a rhizobial partner of a species is most effective not by causing the greatest net SNA or increase in foliage nitrogen per unit NDW. Rather, it triggered development of the highest NDW in its host plant.

As the theory of nutrient co-limitation suggests that each plant species would have a largely constant stoichiometric ratio of carbon to nitrogen (Craine 2009), the contrasting pattern between the sources of rhizobia partners, in terms of the plant nitrogen available for organ synthesis of each species of the host plants, would result in a similar pattern in biomass (Table 4.11).

Reduced survival of *A. crassicarpa* and *A. ramiflora* after nodulation by *A. ramiflora* and *A. elachantha* rhizobia, respectively, suggests that the symbiotic interaction became antagonistic. In other words, the rhizobia in the root nodules of the two species were predominantly parasitic instead of mutualistic. Mutual signals released by both the host and rhizobia still resulted in nodulation but the rhizobia inside root nodules cheated: it failed to provide adequate nitrogen nutrient in return for the carbon provided by the host.

4.4.2 Reasons for failure to reveal any pattern of local adaptation of Acacia symbiosis

In contrast to the hypothesis, no evidence of higher symbiotic effectiveness from local rhizobia was found for the *Acacia* species studied. Theoretically, to achieve effective symbiotic nitrogen fixation when a plant is supplied with its own rhizobial communities, a high degree of symbiosis specificity and the predominant occupancy of root nodules by the appropriate mutualistic symbionts would be required.

4.4.2.1 Source of legume-rhizobia specificity

Specificity is determined by the multistep exchanges of genetic signals between rhizobia and the host (Thompson 2005). In symbiotic nitrogen fixation, it commonly refers to the tight association of a pair of species in a host-mutualist interaction instead of a host-parasite interaction, though rhizobia inside root nodules can be mutualists and sometimes cheaters (one form of parasites). On one hand, the genetic signals lead to the activation of the *nod* gene (for recognition, infection and nodulaton) in rhizobia. On the other hand, the genetic signals require activation of the relevant nodulin genes of the host which were found to be less than ten in several legumes including soybean, with a low number of alleles for each locus (Parker 1999). The combinations of alleles of the different loci determine whether the host is a specialist to a few partners or a generalist to many partners (Parker 1999). The exchange of genetic signals

begins from the host's production of flavonoids in stimulating the growth of rhizobia at the root's rhizosphere, to the release of bacteroids into host cells from infection threads to form the symbiosomes (Thompson 2005; Sprent 2009).

Before an interaction can be established in a symbiotic manner, a host must find its effective bacterial partner(s). The partner is either transferred from the parent host to the offspring (vertical transmission) or it is reacquired from the surrounding soil out of the many rhizobial strains available (horizontal transmission). Rhizobia can only be horizontally transmitted to legumes (Thompson 2005; Douglas 2007). The genotypes of rhizobia partners can become different through sexual reproduction and *"the lateral gene transfer of plasmids among rhizobial taxa"* (Thompson 2005). Reacquisition of rhizobia among the many strains available gives hosts the opportunities to identify and match up with the most effective mutualistic partner.

4.4.2.2 Co-evolutionary responses to environmental heterogeneity and development of geographic mosaic of co-evolved symbiosis

Legumes and rhizobia could have adapted to the spatial differences in abiotic factors (e.g. climate and soil properties) and biotic factors (e.g. intra-specific competition) among populations in terms of varying effectiveness of certain traits with different genotypes. The abiotic and biotic factors could have changed the nutrient requirement of both the host legumes and the relative nodulation abilities of different rhizobial strains, altering the effectiveness of the symbiosis. In other words, the fitness of the genotype of each partner is not just directly affected by changes in soil, climatic and biotic environment but is also indirectly affected by changes in the fitness of the genotype of the other partner. Local adaptation of symbiotic partners to each other in different populations of the same species can logically lead to a distinct geographic structure of co-evolved symbiosis (Thompson 2005). In a multispecies community, local adaptations of many symbioses result in a geographic mosaic. The mosaic is ecologically and evolutionarily meaningful, since several legume species may form nodules using the same rhizobia species and rhizobia species may transfer their plasmids laterally to one another.

4.4.2.3 Shifting of geographic mosaic of co-evolved symbiosis

The geographic structure of co-evolved symbiosis is also shifting because of trait remixing: *"the genetic structure of coevolving species also changes through mutations, gene flow across*

landscapes, random genetic drift, and extinction of local populations" (Thompson 2005). Parker (1999) also argued for a shifting mosaics outcome from a community dynamics perspective. He modelled the population growth and the competition of two plant genotypes (one symbiotic specialist and one generalist) in the presence of one bacteria generalist. It would be difficult for a new specialized bacteria symbiont to invade into the community of the three genotypes because the population density of the existing bacteria generalist was a function of the both host genotypes, while that of the new specialized bacteria could only be a function of the specialized host. However, it should be noted that the nodulation abilities of symbiont specialist and generalist could differ, which compensated for the differences in population densities of the two symbionts at the beginning of the invasion. He predicted a successful invasion by the new bacteria specialist based on the fact that the number of nodules formed by the legume *Amphicarpaea bracteata* with specialized symbionts was two orders of magnitude greater than with a symbiont generalist (Wilkinson 1996). It was deduced that the invasion of a new specialized symbiont could upset the relative symbiotic effectiveness of the previously established symbiont generalist genotype. Thus any current pattern of relatively ineffective nodulation or symbiotc nitrogen fixation with certain bacterial strains may simply reflect such a transitional state instead of absence of local adaptation.

4.4.2.4 Indicators of geographic mosaic of legume-rhizobia interactions

The following observations are indicative of a strongly divergent geographic mosaic of legume-rhizobia interaction (Burdon *et al.* 1999; Thrall *et al.* 2000; Thrall *et al.* 2007):

a) rhizobial communities / specific rhizobial strains sourced from one population of a species, when re-applied onto the same population, can differ in the symbiotic effectiveness (e.g. dry weight) from the rhizobial communities / same strains sourced from another population of the same species. Here local adaptation refers to adaptation of co-evolved symbiosis to individual populations of the same species.

b) rhizobial communities / specific rhizobial strains sourced from a species, when applied onto itself, should achieve higher symbiotic effectiveness than the rhizobial communities / same strains sourced from other co-occuring species. Here local adaptation refers to adaptation of co-evolved symbiosis to certain species.

Point (b) forms the theoretical basis for the original hypothesis of the provenance experiment in this chapter. However, in one study, even among the 14 *Acacia* species (five common and nine

uncommon) inoculated with the most effective local and foreign rhizobial strains, only four species, uncommon *A. filicifolia* and *A. nano-dealbata*, and common *A. cincinnata* and *A. implexa* performed better with their own local rhizobia (Thrall *et al.* 2000). Thus the rarity of a species and local adaptation of symbiosis are not necessarily correlated. Recall that no evidence of higher symbiotic effectiveness from local rhizobia was found for the four *Acacia* species studied in this chapter. This observation leads to an important question: have rhizobial communities failed to adapt to their own species, or was the current experiment unable to reveal such a pattern ?

4.4.2.5 Possible reasons for failure to observe a geographic mosaic of legume-rhizobia interactions in the current study

Different reasons that might limit geographic divergence have been proposed. Some gene flow between populations of the hosts and rhizobia will reduce the level of local adaptations (Thompson 2005). Stochastic loss or reduction in populations of species and/or symbionts will also dilute or reverse any local adaptations (Thompson 2005). Some gene flow between rhizobia from *A. ramiflora* and *A. elachantha* was possible as both species occurred at the same site. No recent signs of fire were recorded or observed at all study sites.

In addition, using peas (*Pisum sativum*) as an example, Lie *et al.* (1987) demonstrated that most of the *Rhizobium leguminosarum* strains found in 230 soil samples in the Middle East but none of the *R. leguminosarum* strains from 58 soil samples of north-west and southern Europe, and North and South America could nodulate the primitive landrace pea cv. Afghanistan. In the same review study, it was also demonstrated out that only *R. leguminosarum* from Israel and Iran (24 soil samples) could form effective nodules with fulvum pea line Fu 3. Fulvum pea was naturally found in Israel, Lebanon, Syria and some parts of Turkey. Likewise, genotypic correlations were found between the different plant phenotypes of *Amphicarpaea bracteata* and certain symbionts. The populations of *Amphicarpaea bracteata* were separated by less than 60 m to as far as 1 000 km apart (Parker 1999). Thus when the hosts or the host genotypes change in plant succession, one can deduce that their favoured symbiont genotypes may also change. Successional dynamics can also potentially shift the geographic structure of a coevolved host-rhizobia symbiosis by altering the relative abundance of parasites and mutualists, the nodulation rate and the specificity of symbiosis (Thrall *et al.* 2006). This temporal shift may obscure any geographic structure of local adaptation of coevolved traits. However, *A. crassicarpa*, *A. elachantha* and *A. ramiflora* have been established in the study sites for decades. The rhizobial communities associated with these three species are expected to form effective nodules with the

three host species should the aforementioned genotypic correlations between rhizobia and legume be applied to them.

Furthermore, recall that the symbiotic effectiveness of each host-rhizobia combination in the provenance experiment was the net competitive result of parasitic and mutualistic symbionts. The shift in the continuum between the two symbiont extremes has recently been hypothesized to be dependent on environmental productivity and biotic complexity of a habitat (Fig. 4.6) (Thompson 2005; Thrall *et al.* 2006).

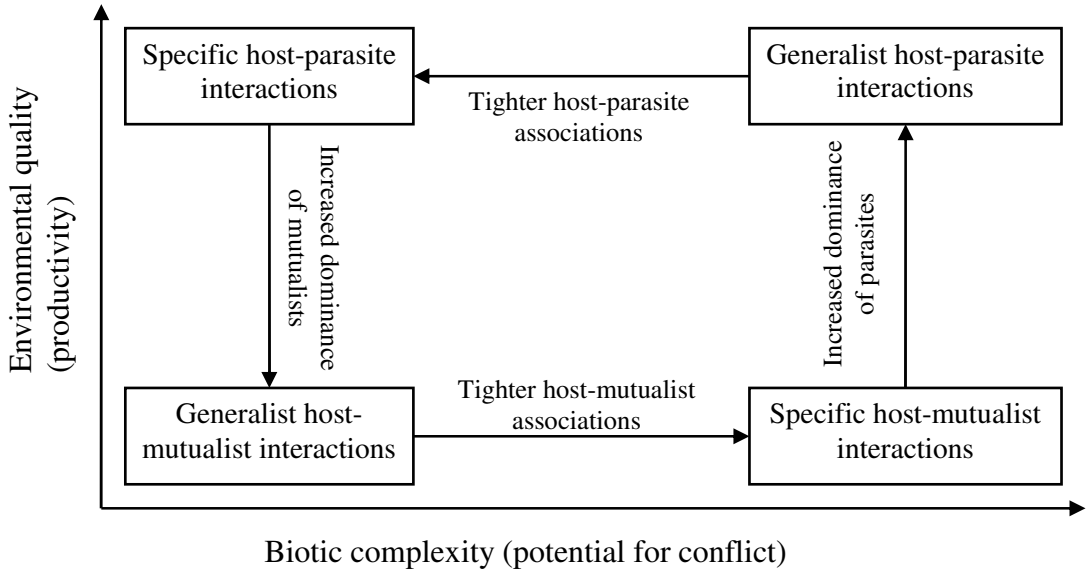


Figure 4.6. The dominance of mutualists and parasites, and the specificity of the host-symbiont interactions under varying levels of environmental quality and biotic complexity. Adapted from Thrall *et al.* (2006).

Environmental quality refers to the resources, physical environment and disturbance regimes that affect the growth, survival and reproduction of an organism, the population density and hence the probability of transmitting diseases within the population (Thrall *et al.* 2006). Increased nitrogen fertilization rate was known to induce a higher density of less beneficial rhizobial and mycorrhizal strains (Thrall *et al.* 2006). Economic models also suggested that mutualism would decline with increasing nutrient availability (Thrall *et al.* 2006). Other mathematical models suggested that in an environment when the host population was highly productive and was the main source of new growth, both virulent parasitic and mutualistic symbionts would be favoured, but no empirical evidence was avialable to support this prediction (Thompson 2005). The total nitrogen and phosphorus levels of the current study sites were in the following descending order: Town Common> Horseshoe Bend> Burra Range. As

such, the mutualistic effects of rhizobia should rise proportionally more than the parasitic effects with distance further inland. The experiment found that the mean dry weights and nitrogen parameters of *A. aulacocarpa, A. crassicarpa* and *A. elachantha* were higher in the presence of inland rhizobia than coastal rhizobia. This pattern appears to fit into the above productivity gradient hypothesis. This may be due to the presence of a greater proportion of fast-growing rhizobial strains in the nodules because the number of fast-growing rhizobial strains, while often fewer than the number of slow-growing ones in soil, were found to increase as climate becomes increasingly arid (Martins *et al.* 1997). The effect of inland rhizobia was only statistically significant in the symbioses with *A. aulacocarpa* and *A. elachantha.*

Increased host or symbiont diversity may lower the specificity of parasites; in return the host will evolve a general all-purpose defence strategy (Fig. 4.6). It has been suggested that mutualistic symbionts might also decrease their host specificity by nodulating more hosts and the hosts might also interact with more bacterial strains (Thrall *et al.* 2006). But in a host-mutualist interaction, increased diversity of the mutualistic symbionts may encourage cheating that can be detrimental to the host's fitness. As such, sanctions are often imposed by the hosts such as legumes, *Yucca* plants and reef fishes on the cheating rhizobia, moths and cleaner fish symbionts respectively (Kiers *et al.* 2003; Douglas 2007). In the presence of sanctions, more specific host-mutualist interactions have been predicted to be the ecological and evolutionary outcomes of higher biotic complexity (Thrall *et al.* 2006). The effect of biotic complexity on the specificity of rhizobial parasitism and mutualism could not be verified in the current experiment as no surveys of plants, in particular Mimosaceae, Caesalpinaceae, Fabaceae, and rhizobial diversity have been undertaken.

It appears that the ability to establish the geographic structure of co-evolved symbiosis might be limited by experimental designs. Employing just one population for each species in the current experiment cannot represent a species since the population can be a potential outlier among the many populations available for three of the *Acacia* species (Fig. 4.7).

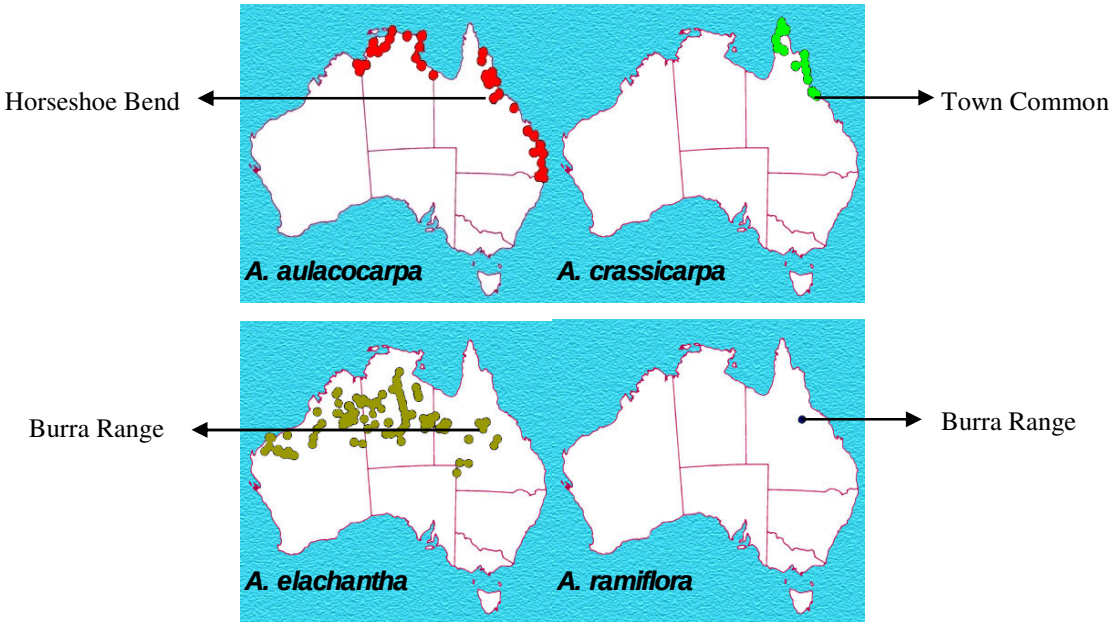


Figure 4.7. The distribution of all the populations of the four *Acacia* species investigated in this study. The four study sites from which the seeds and soil/rhizobia were sourced for the current experiment are indicated. Adapted from ABRS Flora of Australia Online: (http://www.anbg.gov.au/abrs/online-resources/flora/main-query-styles.html)

In another study that compared the nodulation of *A. stenophylla* and *A. salicina* using their own or the other's soil, the soil of each species was sourced from at least 29 sites that were sometimes less than 50 m away from one another within a 700 x 850 km grid (Thrall *et al.* 2007). The 29 sites were fairly representative of the whole ranges of the two species in Australia. In the current experiment, the soil rhizobia from each of the species were collected from 10 to 15 trees in three to four plots that were at least 500 m apart in each season. But the whole ranges of three of the studied species were comparable or greater than the whole ranges of *A. stenophylla* and *A. salicina* (McDonald 2001; Tindale and Kodela 2001a, 2001b). Nevertheless, each site represented one bioclimatic site and thus was deemed as one population. Any potential local adaptation of the host species to its own rhizobial strains can be masked by such scale inadequacy of the sampling design, as mentioned by Thompson (2005).

Competition between the potentially vast number of mutualistic and parasitic rhizobial strains and the accompanying variance under each of the four host species could also mask the probable effects of adaptation of rhizobia to *Acacia* species. The research reported in this chapter investigates and compares the effect of different sources of rhizobial communities on the survival rate and growth of some inland and coastal *Acacia* species. It differs from many other investigations of legume-rhizobium symbiosis which inoculate one or a mix of rhizobial

strains into the host plants. The isolation and inoculation of specific strains allows the determination of the potential fitness of each strain or the relative fitness of different strains. Without such isolation and inoculation, the results should be interpreted carefully.

Soil often contains a diverse mix of rhizobia (Martins *et al.* 1997; Burdon *et al.* 1999; Thrall *et al.* 2000). These rhizobia compete for infection of the host plant's root hairs. Several rhizobia can be found in one nodule (Mita 2012). However, it is well known that the competitive rhizobia are not necessarily the most effective rhizobia for the host plants. Thus in the root system of each seedling, one could find many rhizobial isolates of different effectiveness in the root nodules. The effect on the host is logically a net effect of all the mutualistic and any parasitic (cheater) symbionts inside the nodules of the whole root system. Symbiotic effectiveness indicates the absolute net symbiotic effect of the rhizobial community from a site.

In the current experiment, the effect of a rhizobial community is measured instead of individual rhizobial isolates because revegetation projects with limited time and budget may not be able to identify and inoculate the best rhizobial strains onto seedlings. The source of soil (local vs foreign soil sourced from other species) may become their main consideration instead. As such, the source of soil (rhizobial community) was hypothesised to cause a difference in the survival and growth of different *Acacia* species. The difference may be attributed to co-evolution and hence co-adaptation of rhizobial communities and *Acacia* on the same sites.

4.5 Conclusion

To conclude, no signs of local adaptation of symbiosis were observed in the four selected *Acacia* species. *A. aulacocarpa* established and grew best with inland rhizobia. *A. crassicarpa* established best with coastal rhizobia but grew best with inland rhizobia. *A. elachantha* established and grew best with *A. ramiflora* rhizobia. *A. ramiflora* grew best with *A. aulacocarpa* rhizobia. The increase in survival of *A. ramiflora* under *A. aulacocarpa* rhizobia, among other rhizobial sources, was also one of the highest. Such results would imply that species could grow successfully in soil outside their current distribution ranges. Thus natural range shifts of the four tropical *Acacia* species under climate change or assisted range shifts through revegetation might not be constrained by mutualism.

The results also indicated that inland rhizobia were probably more effective in eliciting net mutualistic responses in the hosts. In addition, the nationally threatened *A. ramiflora* had a higher survival in the presence of coastal rhizobia and grew much faster with *A. aulacocarpa*

rhizobia than with other sources of rhizobia. It therefore appears to be a more specialized host, a trait that can limit the expansion of its restricted distribution range in Burra Range.

Rhizobial diversity, abundance and/or the type of host (fast-growing vs slow-growing) may help explain the patterns in biomass and nitrogen contents of different species grown with different sources of rhizobial communities. Future experiments should isolate individual rhizobial strains from each of the four species, quantify their abundances, identify the most effective rhizobial strains of each species and cross-inoculate into different species in order to study the cause.

5 Effect of drought on the growth, nodulation and nitrogen contents of tropical *Acacia* species

5.1 Introduction

This chapter details the findings of a shadehouse experiment that investigates the effects of drought (absence of watering) on soil moisture and hence the growth, nodulation and nitrogen contents of inland and coastal tropical *Acacias*. Drought in the Australian tropics is expected to be increasingly severe under climate change (CSIRO 2007; OCC 2008). As the nitrogen in tropical savanna ecosystems is replenished mainly through symbiotic nitrogen fixation (Section 1.2), understanding the effect of drought on the nodulation and symbiotic nitrogen fixation of *Acacias* commonly found in tropical savannas is crucial to deducing climate change effects on nitrogen cycling in this important ecosystem. The nitrogen demands of *Acacias* are expected to change with their growth rates so learning the growth responses of *Acacias* to drought is also important. Species from two bioclimatic regions are employed for this experiment to test for different adaptive responses. This introductory section provides an overview of the effect of water stress on the growth, morphological and stomatal responses of plants, in particular *Acacias*. The morphological traits of mesic and semi-arid *Acacias* are also contrasted. The soil moisture effect on nodulation and symbiotic nitrogen fixation is also briefly reviewed.

5.1.1 The effect of drought and water stress on plants

In this thesis, 'drought' refers to the absence of watering or meteorological drought but not necessarily soil moisture deficit or agricultural drought (CSIRO 2007). Water stress occurs as soil moisture falls below a threshold level (s*) when plants initiate stomatal closure (Fig. 5.1). When soil moisture is less than s*, a drop in soil moisture results in a linear decrease in evapotranspiration as a result of continuous stomatal closure and the corresponding decrease in evaporation from the soil. The linear decrease continues until the permanent wilting point of soil (s_w) is reached when all stomata are closed and transpiration stops (Fig. 5.1). Severe water stress is apparent when leaves wilt. Epidermal, palisade and spongy mesophyll cells lose turgor pressure, in turn causing the cell wall and cell membrane to deform.

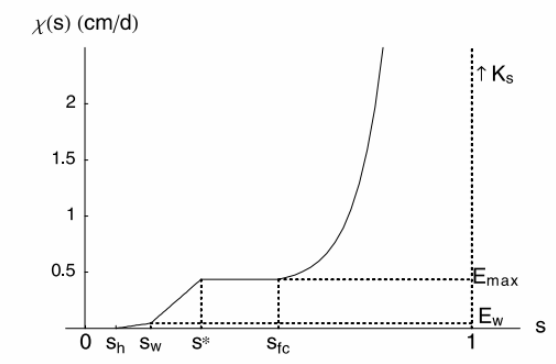


Figure 5.1. The relationship between soil water loss 'χ(s)' and soil moisture 's'. 's*' is the soil moisture level below which plants begin closing their stomata. 's_w' is the permanent wilting point below which plants stop transpiring and begin to wilt. 's_{fc}' is the soil field capacity. 's_h' is the soil moisture level below which water cannot be extracted from the soil through evaporation. E_{max} is the maximum evaporation rate. The non-linear drop in soil water loss when soil moisture is beyond field capacity originates from seepage of excessive water from soil by gravity. Adapted from Rodriguez-Iturbe and Porporato (2006).

Whether seedlings subject to drought in the current experiment experienced water stress depended on the s* which varies with species and soil type. But species adapted to semi-arid ecosystems are more likely to have a lower permanent wilting point than species adapted to a more mesic environment, because the roots of many semi-arid species have higher physiological plasticity by being more capable of lowering cellular solute potential. This physiological adaptation increases the osmotic gradient between root cells and soil water so that water can still be drawn into the roots by osmosis, even though the water potential of drying soil is already very negative (Rodríguez-Iturbe and Porporato 2006; Craine 2009).

Water is absorbed from the soil and then distributed via vascular transport systems and then through osmosis from xylem to surrounding cells. Water absorption by roots is either caused by positive hydrostatic pressure or negative pressure built up in xylem (Taiz and Zeiger 2002). The former is caused by solute accumulation that lowers the water potential of root cells, thus triggering water to move from soil into root cells by osmosis. The latter occurs when water is pulled up the stem and root xylem through transpiration. Due to strong cohesion between water molecules, water will also be drawn into root xylem from surrounding cells in the root stele and cortex. The negative pressure built up in root cells eventually draws water from surrounding soil into the roots.

The rates of water loss and absorption are closely related to soil water content, physiological responses of the plants and/or abiotic factors such as air temperature and relative humidity

(which is directly proportional to the vapour pressure in the atmosphere). In particular, transpiration is regulated through changing stomatal apertures and plasticity of foliage anatomy and morphology, while water absorption by roots at low soil water content is often associated with reduced shoot to root ratio (shootTRR). The relationship between these processes is discussed below in the context of soil water deficit.

5.1.2 The morphological responses of plants to water stress

As vascular plants take up water through their root systems, fine roots and root hairs can increase the surface area in contact with soil water. In drier climates with irregular rainfall, a deep tap root system provides access to groundwater (Virginia ***et al.*** 1986). An example is the woody legume ***Prosopis glandulosa*** in the Sonoran Desert in Southern California of the USA, whose roots can extend to 5.6 m. Root nodules of this legume are also more abundant near the groundwater than the surface, probably because of higher soil moisture and lack of temperature extremes deeper in the soil. Also, roots extending to 53 m below the surface, were found in Arizona, which likely belonged to ***Prosopis*** (Phillips 1963).

A greater root surface area may be produced in dry soil compared to wet soil (Craine 2009). As soil dries up, the air-water interface retreats closer and closer to surfaces of the soil particles. The retreat causes the soil solution which is interconnected between pore spaces to build up negative pressure known as surface tension. As this thin soil water network is pulled towards the roots by transpiration pull, the fine water column within this network may snap (Craine 2009). In addition, soil pores are increasingly filled up with air when soil moisture content decreases. As a result, fewer channels are available for soil water movement, causing a decrease in the soil hydraulic conductivity (Taiz and Zeiger 2002). The interaction between soil water, air particles and soil particles causes plant roots to source water in dry soil by using extensive networks.

5.1.3 Changes in biomass allocation in response to water stress

Apart from deeper roots, a reduced shootTRR is commonly observed in drier conditions. Assuming that the biomass per unit root length remains unchanged, the root biomass for a deeper root system will increase. The increased biomass allocation to roots requires assimilation of more carbon and nutrient into root tissue formation. Because of slower leaf expansion and

lower photosynthetic rate during water deficits (Taiz and Zeiger 2002), the growth of the shoots would become relatively slower. The disproportionate developments of shoots and roots results in a reduced shootTRR.

5.1.4 Morphological traits of semi-arid and mesic Acacias

In a 12-week glasshouse experiment, mesic *Acacia* species were found to have higher specific foliage area (SFA) than semi-arid *Acacia* species (Atkin *et al.* 1998). This result is due to the different foliage type between mesic and semi-arid species and the SFA of each foliage type. The mesic species tested were *A. mearnsii, A. saligna*, *A. melanoxylon, A. irrorata, A. implexa* and *A. dealbata,* while the semi-arid species were *A. colei, A. tetragonophylla, A. aneura* and *A. coriacea* (Atkin *et al.* 1998). Three of the mesic species have only leaflets throughout their life history and three mesic species have both phyllodes and leaflets. *A. colei, A. tetragonophylla, A. aneura* and *A. coriacea* possess leaflets only at the early seedling stage and phyllodes throughout the rest of their life histories. Phyllodes have lower foliage area to foliage mass ratio than leaves. All semi-arid species in this study had both leaflets and phyllodes while mesic species had mostly leaflets, so logically mesic *Acacia* species have higher SFA than semi-arid *Acacia* species. Lower SFA generally means thicker foliage and/or denser tissues according to equations 5.1, 5.2 and 5.3:

$$SFA = \text{foliage area} / \text{foliage mass} \qquad \ldots. (5.1)$$

Substituting, we have:

$$SFA = 1 / [(\text{foliage WW} / \text{foliage area}) * (\text{foliage DW} / \text{foliage WW})] \qquad ..... (5.2)$$

where WW is the wet weight and DW is the dry weight.

It should be noted that foliage tissue density is equal to DW divided by volume, and volume is equal to the area multiplied by tissue thickness. Substituting, we have:

$$SFA = 1 / (\text{foliage tissue density} * \text{foliage thickness}) \qquad ..... (5.3)$$

At the anatomical level, foliage thickness is due to the presence of a unique thick, central parenchymatous mesophyll layer in phyllodes, not found in leaflets. Such a layer is present in the phyllodes of 144 Australian *Acacia* species (Boughton 1986). Thicker leaves are a common

physiological adaptation of plants to drier climatic zones. Transpiration rate is reduced by increasing diffusion distance of water vapour from the intercellular spaces to the atmosphere. As shown in equation 5.4, a doubling of the diffusion distance can increase the diffusion time by four times.

$$t = L^2 / D_s \qquad \text{..... (5.4)}$$

where t is the time required for a water vapour molecule to diffuse from point A to point B, L is the distance between point A and B, and D_s is the diffusion coefficient of water vapour in air which is 2.4×10^{-5} m^2 s^{-1}(Taiz and Zeiger 2002).

A higher SFA was responsible for the higher RGR of mesic species (Atkin *et al.* 1998). Net Assimilation Rate (NAR), which is the net photosynthetic rate per unit leaf area per day, was not the cause and, in fact, NAR did not differ significantly between mesic and semi-arid species. Higher SFA means greater interception of light which results in a greater total net photosynthetic rate per plant, thus causing more carbon to be fixed into the dry mass of the plant.

5.1.5 Phenotypic plasticity of Acacias in response to water stress

In another glasshouse experiment, only mesic species were grown for 16 to 17 weeks and plants received either frequent or less frequent watering (Pohlman *et al.* 2005). Similar to the Atkin's (1998) experiment, some of the species tested had only phyllodineous leaves and some only had bipinnate leaves. In this experiment, there was no significant difference in SFA between watering regimes. The photosynthetic capacity (A_{max}), i.e. net photosynthetic rate measured at saturated light intensity, also did not differ significantly between treatments. The study did not compare the RGR between water treatments. The tested species were *A. melanoxylon, A. implexa, A. dealbata, A. mearnsii, A. cincinnata, A. fulva, A. trachyphloia* and *A. silvestris.* Only *A. melanoxylon, A. implexa* and *A. cincinnata* have phyllodes while the remainder have leaflets.

In addition, Pohlman *et al.* (2005) were specifically interested in how the soil water treatments and an additional factor 'distribution' (widespread versus restricted) interacts in phyllodineous species. They concluded that widespread phyllodineous species had greater specific leaflet area (SLA) plasticity than restricted species. This conclusion was drawn from two lines of evidence. The first is that with infrequent watering, both widespread and restricted species had similar SLA but with frequent watering, the SLA of widespread species increased significantly but that

of restricted species remained similar. The second line of evidence is that with frequent watering, widespread species showed a significantly higher SLA than restricted species. This conclusion has two implications: phyllodineous *Acacia* do not necessarily adjust their SLA to soil water availability; rather SLA sometimes depends on their distribution.

Apart from thicker leaves, plant species from drier areas usually have a smaller leaf size compared to mesic species. This helps to reduce the surface area per leaf for transpiration. However, the total surface area of leaflets or phyllodes is even more important. Otherwise, a greater number of small leaves actually have a greater surface area than a small number of large leaves. Semi-arid or arid zone *Acacia* species such as *A. cambagei, A. aneura, A. peuce* and *A. ramiflora* have relatively small leaves/phyllodes compared with coastal species such as *A. crassicarpa, A. flavescens, A. auriculiformis, A. cincinnata* and *A. aulacocarpa* (pers. observ. 2010).

5.1.6 Stomatal responses to water stress

Apart from leaf area adjustment, stomatal closure is an important physiological mechanism for reducing water loss. Woody plants usually have elliptic guard cells while herbaceous plants, such as grasses, usually have dumbbell-shaped guard cells (Taiz and Zeiger 2002). Both types of cells require adequate turgor pressures via water influx to cause differential arching of the cell wall in order to open the pores. To stimulate water influx, guard cells are believed to pump protons out of their cytoplasm in exchange for the passive uptake of potassium cations and chloride anions. The amino acid, malate, is also produced from phosphoenolpyruvate (Taiz and Zeiger 2002). Concentrating cations and malate inside the vacuole of the guard cells increases the negativity of the osmotic potential, drawing down their water potential. Water thus enters the guard cells via osmosis.

Water deficit can cause stomatal closure by hydropassive means, i.e. passive evaporation of water from the guard cells at a very fast rate that exceeds the rate of replenishment from the nearby epidermal cells, and this usually occurs at a very low relative humidity (Taiz and Zeiger 2002). The low boundary resistance of the leaves (under very windy conditions) may also contribute to hydropassive stomatal closure. Another mechanism of stomatal closure involves hydroactive means that trigger a series of metabolic processes (Taiz and Zeiger 2002; Hopkins and Hüner 2009). These processes involve the effect of abscissic acid (ABA) on the guard cells. When foliage experiences water deficit, ABA is usually released by the cytoplasm of the foliage

mesophyll cells to the surrounding apoplast (cell walls) and eventually reaches the guard cells under transpiration pull. ABA may interfere with the proton pumps in the cell membrane of the guard cells, thus causing either the efflux of potassium cations or inhibiting the uptake of potassium cations. The interference causes the turgor pressure of the guard cells to decrease and hence stomatal closure. Guard cells also respond to the ABA produced from the roots before the foliar cells start losing their turgor pressure. How foliage responds to chemical signals from the roots is important to stomatal response to drought stress. This early detection by roots of a decrease in soil water and the chain of responses that result are important for a plant to defend against or delay water stress.

5.1.7 Interpreting the growth dynamics of inland and coastal Acacia species

At the whole plant level and in a time period of a few months to years, the seedlings of inland *Acacia* species grow slowly, as indicated by their smaller RGR (Atkin *et al.* 1998). The height and diameter of the inland trees are also smaller than those of other inland trees of the same age. However, at the leaf/phyllode level and at a temporal scale of seconds or days, the NAR of their seedlings can be similar to coastal *Acacia* species. RGR is a complex concept because it is determined by *"differences in physiology, morphology and biomass partitioning"* (Shipley 2006). As shown in the equations below, RGR is the result of a change in photosynthetic and/or respiration rate and, in the case of long-term acclimation, altered biomass allocation (also called phenotypic plasticity) (Atkin *et al.* 2006):

$$RGR = NAR * SFA * FMR \quad \text{..... (5.5)}$$

$$NAR = (Pa - Ra) / CC \quad \text{..... (5.6)}$$

$$SFA = \text{total foliage area / total foliage dry mass} \quad \text{..... (5.7)}$$

$$FMR = \text{total foliage mass / plant mass} \quad \text{..... (5.8)}$$

where a) RGR = relative growth rate. Unit: mg carbon gain g^{-1} PB d^{-1} ;

b) NAR = net assimilation rate. Unit: g carbon gain m^{-2} foliage d^{-1} ;

c) SFA = specific foliage area. Unit: m^2 foliage kg^{-1} foliage DW ;

d) FMR = foliage mass ratio ;

e) Pa = daytime net photosynthetic rate of shoot per unit area. Unit: mmol CO_2 gain m^{-2} foliage d^{-1} ;

f) Ra = respiration of roots for 24 hours and of shoots at night. Unit: mmol CO_2 lost m^{-2} foliage d^{-1} ;

g) CC = whole plant carbon concentration. Unit: mmol C g^{-1} PB.

See Atkin *et al.* (2006) and Loveys *et al.* (2002) for details.

Some evidence suggests that SFA is the most important factor for determining the variation in RGR for *Acacia* species. This factor helps to distinguish the slow-growing inland *Acacia* species from the fast-growing *Acacia* species (Atkin *et al.* 1998). One may ask whether the RGR of other plants also vary mainly with SFA. By comparing a total of 24 species of herbaceous monocots and dicots collected from Western Europe, all with C_3 type of photosynthesis, SFA was shown to be the main source of RGR variation (Poorter and Remkes 1990). A review of growth studies of herbaceous species reached the same conclusion (Poorter and van der Werf 1998). Two review studies of woody species concluded that either SFA or NAR is the primary factor for RGR differences between species (Cornelissen *et al.* 1998). A comprehensive meta-analysis of the relative growth rate of 614 species from 83 experiments published in 37 studies concluded that NAR was the most important source of variation of RGR, and that FMR was never important (Shipley 2006). Another main conclusion was that, for herbaceous species, the proportional influences of different factors changed with light intensity. At lower quantum input, the importance of SFA increases while that of NAR decreases, and vice versa. In a field survey, the NAR was much higher for drier zone *Eucalyptus* species than those in the wetter zones, indicating that photosynthesis becomes more efficient on a leaf area basis (Mooney *et al.* 1978).

5.1.8 Soil moisture effect on nodulation and nitrogen fixation

Similar to the effect of temperature, there are sub-optimal and optimal ranges of soil moisture for nodulation of *Acacia* spp. It was found that 15% to 22.5% soil moisture facilitates better nodulation in terms of dry weight and the number of nodules than 7.5% soil moisture for *A. mellifera* (Habish 1970). The occurrence and peak in the number of nodules and nitrogenase activity in the wet season in *Acacia* spp., compared to the disappearance of nodules and low level of nitrogenase activity in the dry season, indicate that adequate soil moisture is an important factor for root nodulation (Langkamp *et al.* 1982; Danso 1995). In a tropical dry forest in Mexico which has a high percentage of leguminous trees, five species from Mimosoideae and four species from Fabaceae were surveyed in dry (April), rainy (July and August), and towards the end of the dry season (October and November) (Gonzalez-Ruiz *et al.* 2008). Four species from Mimosoideae and three species from Fabaceae had indeterminate (branched) nodules within a 50 cm radius from the trunk. The numbers of nodules, and their specific nitrogenase activities, as measured by the acetylene reduction method, were much higher in the wet season than in the dry season. Gravimetric soil moisture of the sandy clay

loam reached 20 to 24% in the wet season, dropped to 10% by October and less than 5% in November and April. Thus, adequate soil moisture is important to nodulation.

The number of nodules, nodule size and the amount of nitrogen fixed per unit nodule weight also decreases with decreasing soil moisture in the common bean *Phaseolus vulgaris* cv. Glamis (Sprent 1976) and the horse bean *Vicia faba* (Gallacher and Sprent 1978). Both species are of agricultural importance and belong to the Fabaceae (formerly Papilionoideae). In the latter study, some of the plants subject to water stress at the beginning were then subject to a normal watering treatment two weeks later. The change in the treatment significantly increased nodule size, number and the amount of nitrogen fixed per unit nodule weight, suggesting the effect of water stress on nodule development is reversible. Larger nodules of *Vicia faba* under the excess water treatment than the normal water treatment do not necessarily mean heavier nodules. Scanning electron microscopy showed that there were more intercellular spaces within larger nodules, lowering their density. The surfaces of nodules subject to excess water treatment had more ridges, leading to an increased surface area (Gallacher and Sprent 1978). This may help gaseous exchange within a growth medium of limited oxygen supply.

Decreasing soil moisture causes physiological and ecological stress. Plants will have to spend more resources to develop fine roots and root hairs to increase contact with soil water (Craine 2009). Plants also have to actively generate and pump solutes across vacuoles and cell membranes to lower the water potential of their own cells compared to the surrounding soil water, to draw in water by osmosis (Taiz and Zeiger 2002). In an ecological context, water stress may intensify the competition between plant species. The root biomass densities of four temperate forests in Netherlands were modelled and compared under 'competition present' and 'competition absent' scenarios (Van Wijk and Bouten 2001). In the presence of competition, root biomass closer to the surface increased while the root biomass in deeper soil decreased. As shallow roots of competitors absorb water and reduce the amount percolating to deeper soil, an increase in shallow root biomass can be seen as an adaptive response to increasing competition for soil water. However, the study did not further examine how water stress interacts with the root competition.

Soil moisture does not necessarily correlate closely with precipitation. It depends on soil percolation rate which varies with soil type (Rodríguez-Iturbe and Porporato 2006). For example, clay soil which has very fine particles has a great surface area to hold water, but sand which has much larger particles is low in water holding capacity. Clay soil may thus have lower seasonal variation in soil moisture than sandy soil. On soil types with relatively high percolation

rates, water can be a scarce resource in the dry season. Such a phenomenon results in competition between individuals.

5.1.9 Aims

This study is the first to investigate how drought will affect the nodulation, growth and water use efficiency of *Acacia* species from tropical coastal and inland areas. By comparing differences in phenotypic plasticity of *Acacia* spp. under drought conditions, this study sought evidence of the strategies that may be employed by inland and coastal species in adapting to climate change.

It is hypothesised that drought would lead to a reduction in:

a) biomass and total nitrogen content of stems, roots, foliageand nodules ;
b) the proportion of nitrogen acquired from symbiotic nitrogen fixation ;
c) foliage water content ; and
d) shootTRR and stemTRR

It is also hypothesised that the semi-arid / inland *Acacias* would adapt to drought better than mesic / coastal *Acacias* by having smaller percentage changes in item (a) to (c) and greater percentage changes in item (d).

Thirdly, it is expected that inland species would have a lower growth rate than coastal species because inland species have fewer leaflets, smaller sized leaflets or only have leaflets for a short duration of their life cycle. Leaflets have been shown to fuel RGR better than phyllodes do because the total surface area of leaflets is higher than the total surface area of phyllodes, resulting in greater net photosynthetic rate per plant per day (Atkin *et al.* 1998).

5.2 Materials, methods and data analysis

5.2.1 Species chosen

Coastal *A. crassicarpa* and *A. holosericea* Cunn. ex Don 1832, and inland *A. elachantha* and *A. ramiflora* were selected. Seeds were collected during the field study. *A. aulacocarpa* was not

used because there were not enough seeds. Seeds of *A. holosericea* were collected from the Town Common Conservation Park in 2011 (same location as *A. crassicarpa*). By using similar species to investigate different aspects of this research, different factors affecting nodulation and symbiotic effectiveness could be better compared.

5.2.2 Seeds and soil sampling

Seeds were collected from at least ten individuals which were at a distance of twice the average height of trees or at least 100 m apart (Gunn 2001). Surface soil (about 20 cm) in the immediate vicinity of the trunks of 10 to 15 individuals was collected from each species.

5.2.3 Shadehouse experiment

5.2.3.1 Experimental design

The experiment consisted of 16 factorial combinations (four species x "inland vs coastal soil" x "8-week drought vs normal watering") in a shadehouse. Each treatment had five replicates at most and three replicates at least. Some replicates did not survive transplantation stress. For *A. ramiflora* x coastal soil x normal watering, only one replicate was available while for *A. ramiflora* x coastal soil x drought, only two replicates were available. Soil from *A. crassicarpa* and *A. holosericea* were mixed into composite coastal soil while those from *A. elachantha* and *A. ramiflora* were mixed to form inland soil. Each pot received 100 g of either coastal or inland soil. The experiment commenced on 21 December 2011 and lasted until 15 February 2012 (56 days).

5.2.3.2 Experimental procedure

The experimental procedure included the preparatory and the experimental phases. The preparation phase was further divided into pre-transplantation and post-transplantation stages. The pre-transplantation stage lasted from mid-September until the first week of December 2011, while the post-transplantation stage lasted from the first week of December 2011 until 21 December 2011. Transplantation occurred on 2 to 4 December 2011.

The pre-transplantation stage involved preparation of all four species x two sources of rhizobia. Two seedlings were grown in each pot which had been filled with vermiculite and perlite in 1 : 1 ratio, as well as 100 g of the corresponding coastal or inland soil. The main purpose of this stage was to cultivate the seedlings to an adequate size before subjecting them to drought. Tap water or non-sterilised nutrient solution was added once every two to three days until the field capacity of the growth medium was reached. Nutrient solution was largely similar to that used in the provenance experiment (Table 4.2), except with the addition of nitrogen. In the first one and a half months, 0.2 g KNO_3 mL^{-1} was added to the nutrient solution. In the two weeks that followed, only 0.1 g KNO_3 mL^{-1} was added. For another three weeks, the nutrient solution was nitrogen-free. The gradual reduction in KNO_3 forced seedlings to rely less on soil nitrogen and more on symbiotic nitrogen fixation to acquire nitrogen.

At the end of the pre-transplantation stage, one seedling per pot was harvested while the other seedling was transferred to a long PVC tube (75 cm tall and 22 cm in diameter). The tubes were filled to 65 cm high with sandy loam sourced by Flintstones Landscape Pty Ltd from Rollingstone, Queensland. The bottom of each tube was sealed with heavy duty polythene bags to contain the soil. Each bag had 10 to 12 small holes punched in them for draining excess water. Seedling transplantation was done carefully to prevent over-compaction of soil and hence root suffocation.

Sandy loam was chosen as the growth medium for the experiment to facilitate soil moisture manipulation. The proportion of sand : silt : clay was approximately 50-70% : 0-50% : 15-20% (Brady and Weil 2010). The large proportion of sand particles results in fast drainage and evaporation. This is helpful as it often takes less time to increase soil moisture than lower it.

During the first week of the post-transplantation stage, about 200 to 500 mL tap water was added once every two to three days in the first week up to the field capacity of sandy loam. In the second week, the same nutrient solution (Table 4.2), together with 0.25 g KNO_3 mL^{-1}, was added because signs of stress were observed on transplanted seedlings. In the second and third weeks, seedlings were observed to have minimal growth in stem and phyllodes. This minimal growth was apparently caused by the transplantation stress. Therefore, the biomass of seedlings harvested before transplantation could well represent the condition of seedlings at the beginning of the experiment, i.e. the condition on day zero. The average maximum and minimum temperatures in the shadehouse throughout the preparation phase were 37.5 and 20.1^{o}C (Fig. 5.2).

After about 3 weeks of acclimation the drought experiment started on 21 December 2011 and lasted until 15 February 2012. Every plant was watered on the first day until water started to drain from the tube's bottom (soil field capacity reached). From then on, tap water was added once every two to three days to the normal watering group (control). For the 8-week drought treatment, no water was added until signs of wilting began to appear in week six. Another two weeks were allowed to confirm that the wilting was a sign of water stress. The average maximum and minimum temperatures in the shadehouse throughout the eight weeks of the drought experiment were 38.2 and 23.3^{o}C (Fig. 5.2).

As the experiment consisted of many seedlings, they were arranged randomly in two separate blocks. Two to three replicates of each treatment were randomly allocated into each block. Repositioning of tubes was not undertaken throughout the experiment as long soil columns could easily 'snap' during movement. Roots in the soil column may break, disrupting any potential morphological adaptations of roots to drought. The two blocks were put adjacent to one another to reduce any difference in light, temperature and wind movement.

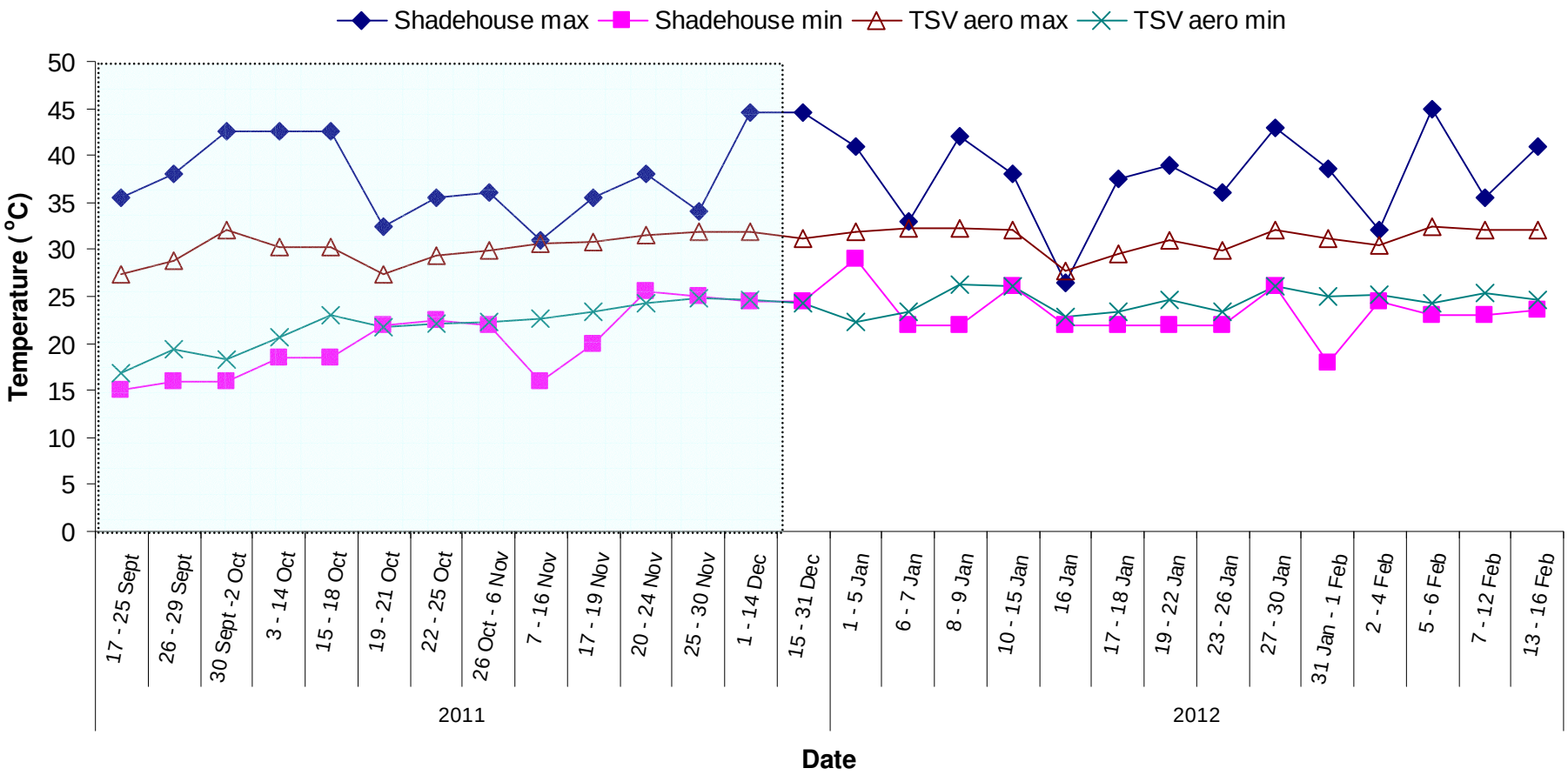


Figure 5.2. The maximum and minimum temperatures inside the shadehouse in the preparatory phase (in hatched rectangle) and experimental phase of the drought experiment. The temperature recorded at the Townsville airport weather station is also provided for comparison (BOM 2012). The experimental period was from 21 December 2011 to 15 February 2012.

5.2.4 Measurements

Foliage area was measured with the computer program ImageJ. Prior to that, fresh phyllodes and leaflets were scanned at a resolution of 300 dpi. The wet weights of phyllodes and leaflets were also measured using a balance with a sensitivity of 0.001 g. Stems, roots, phyllodes, leaflets and nodules were dried at 70^{o}C for 24 to 48 hours. Their biomasses were measured with the same balance. For individual plant organs, all replicates of the same treatment were ground and homogenised with a mill. The homogenised samples were then subjected to nitrogen and isotope analyses (Rayment and Higginson 1992) (Section 2.2.2.3). The gravimetric moisture content of soil at 0 to 25 cm, 25 to 45 cm and 45 to 65 cm depths was determined separately.

5.2.5 Data analysis

5.2.5.1 Biomass

The effects of watering regimes, rhizobial sources and species on the changes in biomass, growth, biomass allocation and foliage morphological traits were tested with ANOVA or Kruskal-Wallis (K-W) tests. K-W tests were undertaken if the assumption of residual normality and/or homogeneity of residual variances could not be met after data transformation.

Since inland and coastal species were known to exhibit different growth rates, to allow a fair species comparison, the effect of drought was normalised with reference to the control (normal watering regime) using equation 5.9:

$$\text{Normalized “X”} = [\ (\text{“}X_{drought}\text{”} - \text{“}X_{normal}\text{”})\ /\ \text{“}X_{normal}\text{”}\ ] * 100 \quad \ldots. (5.9)$$

where “X” could be substituted with variables in Table 5.1 or “α” nitrogen content in Table 5.2

Each normalized variable was then modelled on species, rhizobial sources and their interactions.

Soil moisture, root and nodule dry weights were separately modelled on depth, watering regimes, species and their interactions using ANOVA. The modelling examined whether the effect of drought on these variables changed with depths.

Table 5.1 lists the variables that were related to biomass and the relevant measurements undertaken on harvested seedlings.

Table 5.1.Variables and measurements related to biomass in the drought experiment

RGR, relative growth rate. NAR, net assimilation rate. SFA, specific foliage area. FMR, foliage mass ratio. PB – plant biomass. DW – dry weight. WW – fresh weight.

Variable (units)	Formula	Measurement
Change in "α" biomass (g)	"α" $biomass_{day\ 56}$ – "α" $biomass_{day\ 0}$ • "α" could be stem, foliage, root or nodule.	Biomass of root, stem, phyllode and/or leaflet and nodule at day 0 and 56.
RGR_{mean} (mg DW gain g^{-1} PB day^{-1})	[Ln ($PB_{day\ 56}$) – Ln ($PB_{day\ 0}$)] / 56 days	Same as the above
FMR_{mean} (g foliage DW g^{-1} PB)	($FMR_{day\ 0}$ + $FMR_{day\ 56}$) / two harvests • FMR = (phyllode biomass + leaflet biomass) / PB	Same as the above
SFA_{mean} (m^2 foliage kg^{-1} foliage DW)	($SFA_{day\ 0}$ + $SFA_{day\ 56}$) / two harvests • SFA = (phyllode area + leaflet area) / (phyllode biomass + leaflet biomass)	The area and biomass of phyllode and leaflet
NAR_{mean} (mg DW gain m^{-2} foliage d^{-1})	RGR_{mean} / (SFA_{mean} * FMR_{mean})	See RGR_{mean}, SFA_{mean} and FMR_{mean}
Stem to root ratio	Stem biomass / root biomass	Stem and root biomass
Shoot to root ratio	(Stem biomass + foliage biomass) / root biomass	Stem, foliage and root biomass
Specific foliage weight (SFW) (g foliage WW m^{-2} foliage)	Foliage WW / foliage area • This ratio was shown to be positively correlated with direct measurements of foliage thickness (Dijkstra 1989)	The area and WWs of phyllode and leaflet
Foliage water content	Foliage DW / foliage WW	Phyllode or leaflet WW Phyllode or leaflet DW

5.2.5.2 Nitrogen

The next part of the analysis focused on the effects of watering regimes, the source of rhizobia, species and their interactions on the change in the nitrogen content of stems, roots, foliage and nodules, the $\delta^{15}N_{foliage}$, $\delta^{13}C_{foliage}$ and nitrogen use efficiency (NUE). The effects of species, the source of rhizobia and their interactions on normalized nitrogen contents were also examined. However, due to inadequate time, all samples of individual plant organs of each treatment were pooled for nitrogen analysis. It is thus impossible to compare treatments statistically. $\delta^{15}N$ refers to the proportion of ^{15}N to ^{14}N of a sample with reference to the atmospheric baseline. The equation for calculating $\delta^{15}N$ is:

$$[(R_{sample} / R_{atmosphere}) - 1] * 1000 \quad ..... \quad (5.10)$$

where R = (Percentage of ^{15}N atoms / percentage of ^{14}N atoms) and the percentage of ^{15}N atoms in the air is 0.3663, a global constant. $\delta^{15}N$ unit: parts per thousand (‰).

Soil generally has a greater proportion of ^{15}N than air (Boddey *et al.* 2000). If plants acquire a greater proportion of their nitrogen from the soil, their plant tissues should have a greater proportion of ^{15}N to ^{14}N, leading to either more positive or less negative values of $\delta^{15}N$. $\delta^{15}N$ could also vary with plant tissues due to isotopic discrimination in metabolic processes (Section 2.1.7).

Table 5.2 lists the nitrogen-related variables and the relevant measurements undertaken on harvested seedlings.

Table 5.2. Nitrogen-related variables and measurements in the drought experiment

PB –plant biomass. PN –plant nitrogen. NUE – nitrogen use efficiency.

Variables	Formula	Measurement
$\delta^{15}N_{foliage}$ and $\delta^{13}C_{foliage}$ after 56 days (‰)	NA	See Section 2.2.2.3 for detailed methodology
Change in "α" nitrogen content (mg N)	"α" $N_{day\ 56}$ – "α" $N_{day\ 0}$ • "α" could be stem, root, foliage or nodule	The biomasses of stem, root, foliage and nodule were multiplied by their corresponding nitrogen per unit dry weight to obtain their total nitrogen content.
NUE after 56 days (mg PB mg^{-1} PN)	$PB_{day\ 56}$ / $PN_{day\ 56}$	Same as the above

5.3 Results

5.3.1 The effect of species, rhizobial sources and watering regimes on biomass

A general trend towards significantly smaller increase in biomass and reduced shoot to root ratio was observed in almost all species except *A. ramiflora.* The plant biomass of most species-rhizobia combinations under normal watering regime was two to three times that under the drought regime.

Table 5.3. Mean (± 1 S.E.M.) plant biomass changes within 56 days of experiment and shoot to root ratio at the end of the experiment under normal watering and drought

Δ refers to changes from day 0 to 56. Values are measured means. Each variable was subject to a separate ANOVA. EL – *A. elachantha*. Rm – *A. ramiflora*. Cr – *A. crassicarpa*. Ho – *A. holosericea*. *** $P < 0.001$ ** $P < 0.01$ * $P < 0.05$. NS – not significant.

Variables	Species	Rhizobial source	Watering regime - Normal	Watering regime - Drought	Significance
Δ Plant biomass (g)	Cr	Coastal	20.236 ± 1.293 (4)	8.804 ± 1.293 (4)	***
		Inland	22.496 ± 1.157 (5)	10.640 ± 1.157 (5)	***
	Ho	Coastal	20.766 ± 1.293 (4)	7.954 ± 1.157 (5)	***
		Inland	21.405 ± 1.157 (5)	10.252 ± 1.157 (5)	***
	EL	Coastal	12.096 ± 1.493 (3)	3.821 ± 1.493 (3)	***
		Inland	11.928 ± 1.293 (4)	6.003 ± 1.293 (4)	***
	Rm	Coastal	1.970 (1)	1.430 ± 1.829 (2)	NS
		Inland	7.615 ± 1.157 (5)	3.059 ± 1.157 (5)	NS
Shoot to root ratio at day 56	Cr	Coastal	2.741 ± 0.299 (4)	1.806 ± 0.299 (4)	**
		Inland	3.417 ± 0.267 (5)	2.598 ± 0.267 (5)	**
	Ho	Coastal	2.760 ± 0.299 (4)	2.322 ± 0.267 (5)	**
		Inland	2.281 ± 0.267 (5)	1.977 ± 0.267 (5)	**
	EL	Coastal	2.507 ± 0.345 (3)	2.591 ± 0.345 (3)	**
		Inland	2.754 ± 0.299 (4)	2.154 ± 0.299 (4)	**
	Rm	Coastal	4.435 (1)	3.088 ± 0.423 (2)	**
		Inland	2.770 ± 0.267 (5)	2.367 ± 0.267 (5)	**

The plant biomass (PB), stem biomass, foliage biomass, root biomass and nodule dry weight (NDW) of all species increased after 8 weeks of experiment. The increases in PB, stem biomass and foliage biomass were all significantly higher with inland rhizobia than coastal rhizobia (Table 5.4). The effect of rhizobia on these variables did not change with species or watering regimes. The mean values of PB, stem biomass and foliage biomass across all species with inland rhizobia were respectively 11.8 g, 1.8 g and 6.3 g. The same variables with coastal rhizobia were smaller by 0.6 g, 0.2 g and 0.4 g.

The biomass of all plant organs in all species (except *A. ramiflora*) under drought were significantly lower than those under normal watering regime (Table 5.4). It should be noted that the species x watering interaction was significant for the changes in PB ($F_{3, 48} = 8.51$; $P < 0.001$), stem biomass ($F_{3, 48} = 5.50$; $P < 0.01$), foliage biomass ($F_{3, 48} = 11.02$; $P < 0.001$) and NDW ($F_{3, 48} = 7.01$; $P < 0.01$). A significant interaction occurred largely because the absolute changes in biomass differed between mesic and semi-arid species, and that the biomass of only certain species under drought was lower than those under normal watering regime. For example, the differences in PBs between normal watering and drought treatment of *A. crassicarpa* and *A. holosericea* were -11.7 and -12.0 g while the difference for *A. elachantha* was -6.9 g. The differences in terms of foliage biomass for *A. crassicarpa* and *A. holosericea* were -6.69 and -6.36 g while for *A. elachantha*, this was -3.48 g.

Table 5.4. Biomass changes of four *Acacia* species, and under two rhizobial sources and two watering regimes

Δ refers to changes from day 0 to 56. Each variable was subject to a separate ANOVA. Only significant factors are shown. EL – *A. elachantha*. Rm – *A. ramiflora*. Cr – *A. crassicarpa*. Ho – *A. holosericea*. *** $P < 0.001$ ** $P < 0.01$ * $P < 0.05$. NS – not significant.

Biomass (g)	Factor	F-ratio	P-value	Drought minus normal watering and the corresponding P-value[A]			
				Cr	Ho	EL	Rm
Δ Plant biomass	Species	$F_{3, 48} = 58.861$	***	-11.7	-12.0	-6.9	-4.1
	Rhizobia	$F_{1, 48} = 8.269$	**	***	***	***	NS
	Watering	$F_{1, 48} = 137.491$	***				
	Species x watering	$F_{3, 48} = 8.510$	***				
Δ Shoot biomass	Species	$F_{3, 48} = 69.3$	***	-9.08	-8.52	-4.82	-2.90
	Rhizobia	$F_{1, 48} = 16.188$	***	***	***	***	*
	Drought	$F_{1, 48} = 154.9$	***				
	Species*Drought	$F_{3, 48} = 4.38$	***				
Δ Stem biomass	Species	$F_{3, 48} = 23.923$	***	-2.38	-2.16	-1.34	-1.25
	Rhizobia	$F_{1, 48} = 11.693$	**	***	***	***	**
	Watering	$F_{1, 48} = 195.49$	***				
	Species x rhizobia	$F_{3, 48} = 3.525$	*				
	Species x watering	$F_{3, 48} = 5.499$	**				
Δ Foliage biomass	Species	$F_{3, 48} = 70.181$	***	-6.69	-6.36	-3.48	-1.65
	Rhizobia	$F_{1, 48} = 9.266$	**	***	***	***	NS
	Watering	$F_{1, 48} = 141.766$	***				
	Species x watering	$F_{3, 48} = 11.024$	***				
Δ Root biomass	Species	$F_{3, 48} = 25.272$	***	-2.01	-2.16	-1.87	-1.25
	Watering	$F_{1, 48} = 39.685$	***	***	***	**	NS
Δ Nodule dry weight	Species	$F_{3, 48} = 51.619$	***	-0.59	-0.39	-0.25	-0.14
	Watering	$F_{1, 48} = 71.820$	***	***	***	***	NS
	Species x watering	$F_{3, 48} = 7.010$	**				

[A] – values are measured mean differences.

5.3.2 The effect of species, rhizobial sources and watering regimes on growth

The RGR_{mean} and NAR_{mean} for all species under drought were lower than those under normal watering regime ($F_{1, 48} = 21.227$; $P < 0.001$ and $F_{1,48} = 10.274$; $P < 0.05$ respectively) (Table 5.5). The differences in RGR_{mean} between the two watering regimes were -11.4 and -16.1 mg DW gain g^{-1} PB d^{-1} for *A. crassicarpa* and *A. holosericea* while the differences in RGR_{mean} for *A. elachantha* and *A. ramiflora* were -13.1 and -17.1 mg DW gain g^{-1} PB d^{-1}. The differences in NAR_{mean} between the two watering regimes of *A. crassicarpa* and *A. holosericea* were -531 and

-2 090 mg DW gain m^{-2} foliage \d^{-1} while those of *A. elachantha* and *A. ramiflora* were -3 212 and -5 421 mg DW gain g^{-1} foliage d^{-1}. The differences between the two watering regimes in terms of SFA_{mean} and FMR_{mean} were not significant but both growth traits appeared to be higher in coastal species than in inland species.

Table 5.5. The growth traits of four *Acacia* species, and under two rhizobial sources and two watering regimes

RGR – relative growth rate; NAR – net assimilation rate; SFA – specific foliage area; FMR – foliage mass ratio. Each trait was subject to a separate ANOVA or a Kruskal-Wallis test. Only significant factors are shown. EL – *A. elachantha*. Rm – *A. ramiflora*. Cr – *A. crassicarpa*. Ho – *A. holosericea*. *** $P < 0.001$ ** $P < 0.01$ * $P < 0.05$

Growth trait	Factor	F-ratio	P-value	Multiple comparison[A]			
RGR	Species	$F_{3, 48} = 3.017$	*	Drought minus normal watering[B]			
(mg DW gain g^{-1} PB	Rhizobia	$F_{1, 48} = 15.933$	***	Cr	Ho	EL	Rm
d^{-1})	Watering	$F_{1, 48} = 21.227$	***	-11.4	-16.1	-13.1	-17.1
				Species comparison			
				Ho = EL > (Cr) > Rm			
				65.9	65.1	60.3	57.7
NAR_{mean}	Species	$F_{3, 48} = 9.562$	***	Drought minus normal watering[B]			
(mg DW gain m^{-2}	Rhizobia	$F_{1, 48} = 9.071$	**	Cr	Ho	EL	Rm
foliage d^{-1})	Watering	$F_{1, 48} = 10.274$	**	-531	-2 090	-3 212	-5 421
				Species comparison			
				EL = Ho > Rm > Cr			
				12 807	12 223	11 653	8 184
SFA_{mean}	Species	$H_3 = 24.891$	***	Species comparison			
(m^{-2} foliage kg^{-1}				Cr > Ho = Rm = EL			
foliage DW)				12.2	10.4	10.2	10.0
FMR_{mean}	Species	$F_{3, 48} = 12.475$	***	Species comparison			
(g foliage DW g^{-1} PB)	Species x	$F_{3, 48} = 5.867$	**	Cr > Ho > (Rm) > EL			
	rhizobia			0.60	0.55	0.51	0.52

[A] Values are measured means (species comparison) or measured mean differences (Drought minus normal watering).

[B] No further test between normal watering and drought was undertaken for each species because species x watering was not significant.

5.3.3 The effect of species, rhizobial sources and watering regimes on biomass allocation

The stemTRR and shootTRR after 56 days under drought were significantly lower than those under normal watering regime ($F_{1, 48}$ = 28.94; $P < 0.001$ and $F_{1, 48}$ = 13.18; $P < 0.01$ respectively) (Table 5.6). The stemTRR of inland species under drought (0.513 to 0.730) were greater than those of coastal species (0.346 and 0.356) under drought. This contrast between mesic and semi-arid species was not observed under the normal watering regime and in shootTRR in both watering regimes.

Table 5.6. The stem to root and shoot to root ratios of four *Acacia* species, and under two rhizobial sources and two watering regimes

StemTRR – stem to root ratio. ShootTRR – shoot to root ratio. Only significant factors were shown. Each plant trait was subject to a separate ANOVA. EL – *A. elachantha*. Rm – *A. ramiflora*. Cr – *A. crassicarpa*. Ho – *A. holosericea*. *** $P < 0.001$ ** $P < 0.01$ * $P < 0.05$

Plant trait	Factor	F-ratio	P-value	Drought minus normal watering per species [A, B]			
				Cr	Ho	EL	Rm
StemTRR after	Species	$F_{3, 48}$ = 12.251	***	-0.32	-0.19	-0.18	-0.37
56 days	Watering	$F_{1, 48}$ = 28.941	***				
ShootTRR	Species	$F_{3, 48}$ = 3.880	*	-0.87	-0.34	-0.31	-0.47
after 56 days	Watering	$F_{1, 48}$ = 13.181	**				
	Species x rhizobia	$F_{3, 48}$ = 5.658	**				

[A] No further test between normal watering and drought was undertaken for each species because species x watering was not significant.

[B] Values are measured mean differences.

5.3.4 The effect of species, rhizobial sources and watering regimes on foliage morphology

The foliage under drought was thinner than the foliage under normal water regime, as shown by the SFW ($F_{1, 48}$ = 190.13; $P < 0.001$) (Table 5.7). But the interspecific variation in SFW under normal watering differed from that under 8-week drought ($F_{3, 45}$ = 20.408; $P < 0.001$). With normal watering, the SFW of *A. crassicarpa, A. holosericea A. elachantha* and *A. ramiflora* after 56 days were 278, 325, 372 and 271 g foliage WW m^{-2} foliage. Under drought, the SFW of these four species became 159, 139, 218 and 258 g foliage WW m^{-2} foliage. Thus inland *A. elachantha* and *A. ramiflora* had higher SFW than coastal *A. crassicarpa* and *A. holosericea* under drought.

In addition, the DW to WW ratios of foliage of *A. crassicarpa*, *A. holosericea* and *A. elachantha* under drought was higher than those under normal watering regime by 0.373, 0.578,

0.231 (Table 5.7). A higher ratio indicated that foliage water content was lower under drought. However, compared to the normal watering regime, drought failed to affect the DW to WW ratio of *A. ramiflora* foliage significantly.

Since the differences between normal watering regime and drought treatment in terms of biomass, the SFW and foliage water content of *A. ramiflora* were not significant, it appears that *A. ramiflora* was the most drought-tolerant species.

Table 5.7. The specific foliage weight and foliage dry weight to wet weight ratios in four *Acacia* species, and under two rhizobial sources and two watering regimes

SFW – specific foliage weight. FoliageWR – foliage dry weight to wet weight ratio. Traits were subject to ANOVA or Kruskal-Wallis tests. EL – *A. elachantha*. Rm – *A. ramiflora*. Cr – *A. crassicarpa*. Ho – *A. holosericea*. *** $P < 0.001$ ** $P < 0.01$ * $P < 0.05$. NS – not significant.

Plant trait	Factor	F-ratio	P-value	Multiple comparison[A]
$SFW_{day\ 56}$	Species	$F_{3, 46} = 19.575$	***	Normal watering minus drought per species
(g foliage WW	Watering	$F_{1, 46} = 190.13$	***	Cr Ho EL Rm
m^{-2} foliage)	Species x watering	$F_{3, 46} = 20.408$	***	-119 -186 -154 -13
				*** *** *** NS
				Species comparison (normal watering)
				EL > Ho > Cr = Rm
				372 325 278 271
				Species comparison (drought)
				Rm > EL > Cr = Ho
				258 218 159 139
$FoliageWR_{day\ 56}$	Watering (Cr)	$H_1 = 12.803$	***	Normal watering minus drought per species
	Watering (Ho)	$H_1 = 12.015$	**	Cr Ho EL Rm
	Watering (EL)	$H_1 = 5$	*	-0.373 0.578 0.231 0.014
	Watering (Rm)	$H_1 = 0.619$	NS	*** ** * NS
	Species (normal watering)	$H_3 = 20.280$	***	Species comparison (normal watering)
				Ho > Cr = Rm = EL
				0.390 0.332 0.319 0.296
	Species (drought)	$H_3 = 25.535$	***	Species comparison (drought)
				Ho > (Cr) > EL > Rm
				0.968 0.705 0.527 0.305

[A] Values are measured means (species comparison) or measured mean differences (normal watering minus drought)

5.3.5 Drought effects on normalized biomass

The biomass of all plant organs under drought was lower than those under norml watering regime by 28% to 100% (Table 5.8). Recall that the normalized variable is the difference in that variable between drought treatment and normal watering regime divided by the control group (normal watering) and then multiplied by 100% (Equation 5.9). The normalized PB, stem biomass and root biomass were -55%, -67% and -45% respectively (Table 5.8). They did not vary significantly with species or rhizobia.

The foliage biomass and NDW under drought were also lower than those under normal watering regime (Table 5.8). The normalized foliage biomass with coastal rhizobia was -62% which was 13% more negative than that with inland rhizobia ($F_{1, 24} = 5.262$; $P < 0.05$). The normalized NDW with coastal rhizobia was -72% while that with inland rhizobia was only -53% ($F_{1, 24} = 9.236$; $P < 0.01$). Differences in symbiotic nitrogen fixation rates between the two sources of rhizobia were thus reflected in these two normalized biomasses, which were never significantly different between species.

Table 5.8. Variation of normalized biomass with four *Acacia* species and two rhizobial sources

Values are measured means ± 1 S.E.M. Each variable was subject to a separate ANOVA. Only significant factors are shown. In – inland rhizobia. Co – coastal rhizobia. EL – *A. elachantha*. Rm – *A. ramiflora*. Cr – *A. crassicarpa*. Ho – *A. holosericea*.

*** $P < 0.001$ ** $P < 0.01$ * $P < 0.05$

Normalized variable	Factor	F-ratio	P-value	Multiple comparison
Δ Foliage biomass	Rhizobia	$F_{1, 24} =$ 5.262	*	Co vs In Co (↓62 ± 4%) > In (↓49 ± 3%) Co vs In in each species EL: Co (↓71 ± 7%) > In (↓43 ± 6%) Rm: Co (↓53 ± 13%) > In (↓48 ± 6%) Cr: Co (↓59 ± 6%) > In (↓54 ± 6%) Ho: Co (↓61 ± 6%) > In (↓51 ± 6%)
Δ Nodule dry weight	Rhizobia	$F_{1, 24} =$ 9.236	**	Co vs In Co (↓72 ± 7%) > In (↓53 ± 5%) Co vs In in each species EL: Co (↓90 ± 13%) > In (↓28 ± 11%) Rm: Co (↓100 ± 22%) > In (↓76 ± 10%)

Table 5.8 (Cont'd)

		Cr: Co (↓67 ± 11%) > In (↓51 ± 10%) Ho: Co (↓59 ± 10%) > In (↓52 ± 10%)
Δ Plant biomass	No factors were significant	Mean: ↓55 ± 3%
Δ Stem biomass	No factors were significant	Mean: ↓67 ± 3%
Δ Root biomass	No factors were significant	Mean: ↓45 ± 4%

5.3.6 Drought effect on normalized growth

The RGR_{mean} of all species subjected to drought were less than the RGR_{mean} under the normal watering regime. The normalized RGR_{mean} was significantly more negative with coastal rhizobia than inland rhizobia ($F_{1, 24} = 6.516$; $P < 0.05$), which were respectively -31% and -13% (Table 5.9). The NAR_{mean} under drought was lower than that under normal watering regime by 20% while SFA_{mean} and FMR_{mean} were not significantly affected by drought.

Table 5.9. Variation of normalized growth traits with four *Acacia* species and two rhizobial sources

Values are measured means ± 1 S.E.M. RGR – relative growth rate. NAR – net assimilaton rate. SFA – specific foliage area. FMR – foliage mass ratio. Each trait was subject to a separate ANOVA. Only significant factors are shown. In – inland rhizobia. Co – coastal rhizobia. EL – *A. elachantha*. Rm – *A. ramiflora*. Cr – *A. crassicarpa*. Ho – *A. holosericea*. *** $P < 0.001$, ** $P < 0.01$ * $P < 0.05$

Normalized variable	Factor	F-ratio	P-value	Multiple comparison
RGR_{mean}	Rhizobia	$F_{1, 24} = 6.516$	*	<u>Co vs In</u> Co (↓31 ± 6%) > In (↓13 ± 4%) <u>Co vs In in each species</u> EL: Co (↓31 ± 10%) > In (↓9 ± 9%) Rm: Co (↓33 ± 18%) > In (↓20 ± 8%) Cr: Co (↓27 ± 9%) > In (↓13 ± 8%) Ho: Co (↓33 ± 8%) > In (↓10 ± 8%)
NAR_{mean}	No factors were significant			Mean: ↓20 ± 5%
SFA_{mean}	No factors were significant			Mean; ↑3 ± 3%
FMR_{mean}	No factors were significant			Mean; ↓1 ± 2%

5.3.7 Drought effects on normalized shoot to root and stem to root ratios

StemTRR under the drought treatment was less than that under normal watering regime. The normalized stemTRR varied with the rhizobial source ($F_{1, 25} = 4.827$; $P < 0.05$) (Table 5.10). The effect of rhizobial source on the normalized stemTRR also changed with species ($F_{3, 25} = 6.798$; $P < 0.01$). The normalized stemTRRs for *A. elachantha* with coastal rhizobia or with inland rhizobia were +8% and -67% respectively. For the other three species, regardless of the rhizobial source, the stemTRR under drought was always lower than that under normal watering regime. Neither species nor rhizobial source was a significant factor in altering the normalized shootTRR.

Table 5.10. Variation of normalized stem to root and shoot to root ratios with four *Acacia* species and two rhizobial sources

Values are measured means ± 1 S.E.M. Each plant trait was subject to a separate ANOVA. In – inland rhizobia. Co – coastal rhizobia. EL – *A. elachantha*. Rm – *A. ramiflora*. Cr – *A. crassicarpa*. Ho – *A. holosericea*. *** $P < 0.001$ ** $P < 0.01$ * $P < 0.05$. NS – not significant.

Normalized variable	Factor	F-ratio	P-value	Multiple comparison
Stem to root ratio after 56 days	Species	$F_{3, 25}$ = 1.220	NS	<u>In vs Co</u> In (↓46 ± 5%) > Co (↓32 ± 6%)
	Rhizobia	$F_{1, 25}$ = 4.827	*	<u>In vs Co in each species</u> EL: In (↓67 ± 10%) ; Co (↑8 ± 12%) Rm: In (↓47 ± 9%) = Co (↓39 ± 15%) Cr: In (↓40 ± 9%) = Co (↓56 ± 10%)
	Species x rhizobia	$F_{3, 25}$ = 6.798	**	Ho: In (↓35 ± 9%) = Co (↓36 ± 9%) <u>Species comparison within each rhizobia</u> Co: EL (% increase) Cr = Rm = Ho (% decrease) In: EL = Rm = Cr = Ho (% decrease)
Shoot to root ratio after 56 days	Species	$F_{3, 25}$ = 0.849	NS	<u>In vs Co in each species</u> EL: In (↓33 ± 11%) ; Co (↑3 ± 12%) Rm: Co (↓30 ± 15%) = In (↓19 ± 10%)
	Rhizobia	$F_{1, 25}$ = 0.129	NS	Cr: Co (↓34 ± 11%) = In (↓22 ± 10%) Ho: Co (↓15 ± 10%) = In (↓13 ± 10%) <u>Species comparison within each rhizobia</u>
	Species x rhizobia	$F_{3, 25}$ = 1.975	NS	Co: EL (% increase) Cr = Rm = Ho (% decrease) In: EL = Cr = Rm = Ho (% decrease)

5.3.8 *Drought effects on normalized specific foliage weight and foliage dry weight to wet weight ratio*

The normalized SFW varied significantly with species (H_3 = 13.924; P < 0.01) (Table 5.11). It was highest in coastal *A. holosericea* (-47.7%) and *A. crassicarpa* (-43.2%). *A. elachantha* was intermediate between *A. crassicarpa* and *A. ramiflora* with its SFW under drought lower by 39.5% than that under normal water regime. The normalized SFW of *A. ramiflora* was the minimal (-1.9%).

The normalized foliage DW to WW ratio) differed between species ($F_{3, 24}$ = 3.529; P < 0.05) (Table 5.11). The normalized foliage DW to WW ratios of both *A. holosericea* and *A. crassicarpa* were +113% and +112% while those of *A. elachantha* and *A. ramiflora* were +78% and -2.9% respectively.

Table 5.11. Variation in normalized specific foliage weight and foliage dry weight to wet weight ratio with four *Acacia* species and two rhizobial sources

Values are measured means. Foliage thickness was subject to a Kruskal-Wallis test. Folaige dry weight to wet weight ratio was subject to ANOVA. EL – *A. elachantha*. Rm – *A. ramiflora*. Cr – *A. crassicarpa*. Ho – *A. holosericea*.

*** P < 0.001 ** P < 0.01 * P < 0.05. NS – not significant.

Normalized variable	Factor	F-ratio	P-value	Species comparison
Specific foliage weight after 56 days	Species	H_3 = 13.924	**	Ho ↓47.7% = Cr ↓43.2% > (EL) ↓39.5% > Rm ↓1.9%
	Rhizobia	H_1 = 0.230	NS	
Foliage dry weight to wet weight ratio after 56 days	Species	$F_{3, 24}$ = 3.529	*	Ho ↑113% = Cr ↑112% > (EL) ↑78% = Rm ↓2.9%
	Rhizobia	$F_{1, 24}$ = 0.664	NS	
	Species x rhizobia	$F_{3, 24}$ = 1.030	NS	

5.3.9 *Root biomass, nodule dry weight and soil moisture at different soil depths*

Soil moisture at the end of the experiment varied significantly between species ($F_{3, 131}$ = 42.008; P < 0.001) and between watering regimes ($F_{1, 131}$ = 54.958; P < 0.001). The effect of watering regimes on soil moisture changed with soil depth ($F_{2, 131}$ = 3.189; P < 0.05). While the differences between watering regimes were significant at each soil depth regardless of species,

the difference was greater at 0 to 25 cm than that at 45 to 65 cm soil depth (Fig. 5.3). Soil moisture levels were significantly higher in the *A. ramiflora* treatments. For example, soil moisture under normal watering and drought ranged from about 7.1 to 9.8% and 4 to 5.6% respectively.,but the soil moisture of *A. elachantha* under normal and drought treatments were slightly lower and ranged from 3.1 to 4.6% and 2.1 to 2.8% respectively. The soil moisture levels of *A. crassicarpa* under normal watering and drought ranged from 2.8 to 3.3% and 1.1 to 2.0% respectively, and hence were even less.

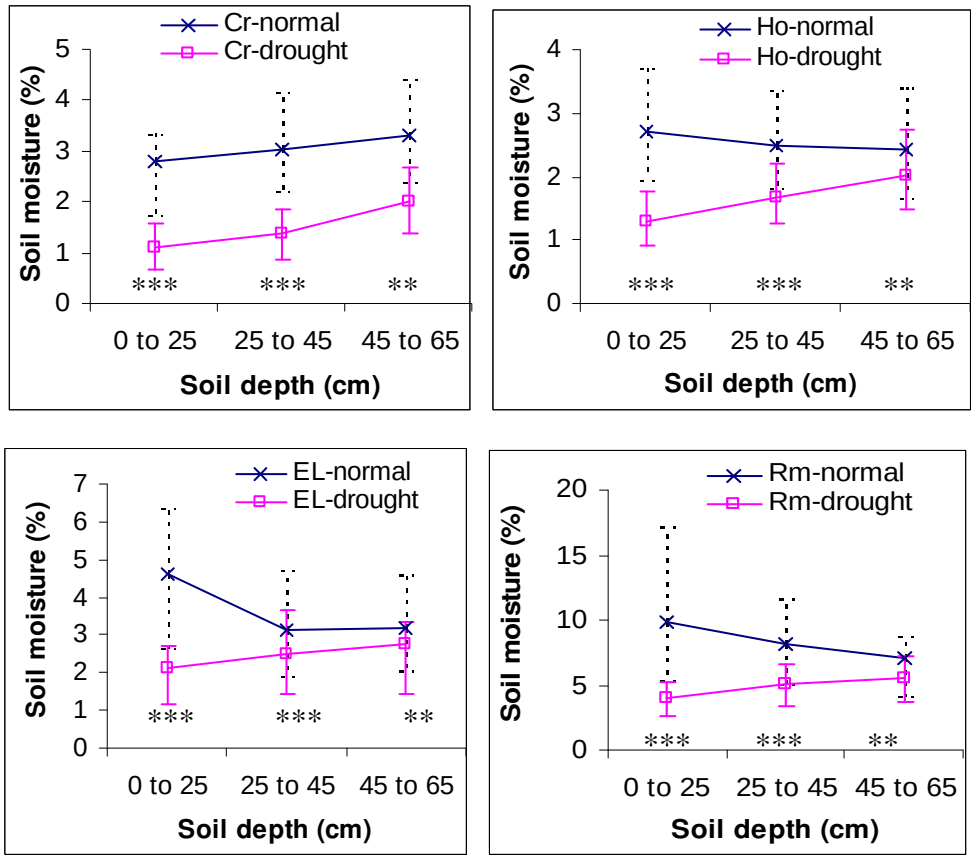


Figure 5.3. Measured mean soil moisture in which *A. crassicarpa* (Cr), *A. holosericea* (Ho), *A. elachantha* (EL) and *A. ramiflora* (Rm) grew, under two watering regimes and three soil depths after 56 days of experiment. Error bars are 95% confidence intervals. Significant differences between watering regimes at each depth are marked with asterisks. Depth x watering regimes was significant ($F_{2, 131} = 3.19$; $P < 0.05$), thus many pairwise comparisons between watering regimes were run. *** $P < 0.001$ ** $P < 0.01$ * $P < 0.05$.

The root biomass of each species in the top 25 cm of soil was significantly greater than in deeper soil ($F_{2, 168} = 339.706$; $P < 0.001$). The variation of root biomass with depths differed significantly between species ($F_{6, 168} = 11.342$; $P < 0.001$). For *A. crassicarpa*, the root biomass decreased from the top 25 cm to 25 to 45 cm and finally to 45 to 65 cm (Fig. 5.4). For the other

three species, the root biomass in 25 to 45 cm was similar to that in the 45 to 65 cm depth, but the root biomass in both depths were lower than that in the top 25 cm.

The root biomass under drought was alwys significantly lower than that under normal watering regime, regardless of soil depths or species ($F_{1, 168} = 78.32$; $P < 0.001$). The difference in root biomass between watering regimes in the top 25 cm soil was the greatest in coastal species, intermediate in *A. elachantha* and lowest in *A. ramiflora* (Fig, 5.4)

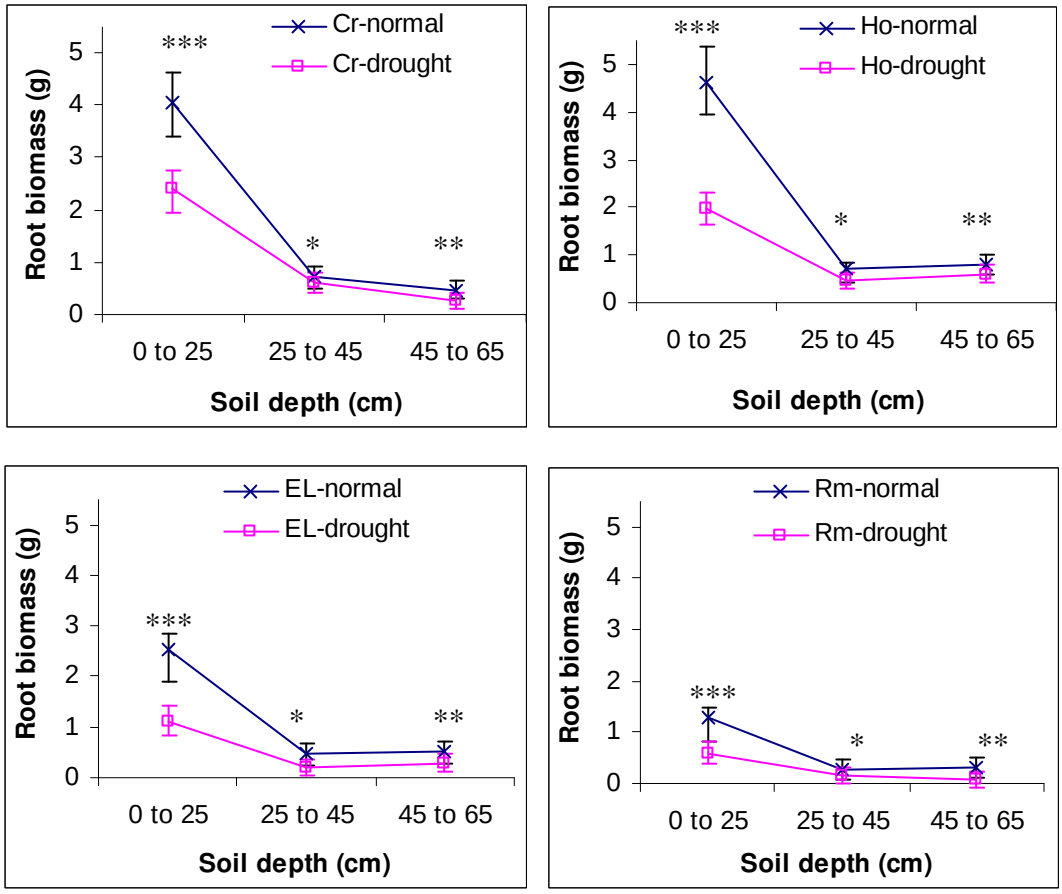


Figure 5.4. Measured mean root biomass of *A. crassicarpa* (Cr), *A. holosericea* (Ho), *A. elachantha* (EL) and *A. ramiflora* (Rm) under two watering regimes and three depths after 56 days of experiment. Error bars are 95% confidence intervals. Depth x watering regimes is significant ($F_{2, 168} = 15.37$; $P < 0.001$), thus many pairwise comparisons between watering regimes were run. *** $P < 0.001$ ** $P < 0.01$ * $P < 0.05$.

The drought treatment x depth interaction was significant in influencing the NDW ($F_{2, 168} = 12.573$; $P < 0.001$). Thus the negative effect of drought on the NDW varied significantly between depths. For instance, the negative effect of drought was significant only in the top 25 cm and 25 to 45 cm soil depths but not at the 45 to 65 cm soil depth (Fig. 5.5). The species x

depth interaction was also significant ($F_{6, 168} = 30.677$; $P < 0.001$). The change of NDWs with depths therefore varied with species. For example, while the mean NDWs of *A. crassicarpa*, *A. holosericea*, and *A. elachantha* decreased with soil depth, the mean NDWs in the 25 to 45 cm depth of the latter two species at (0.001 to 0.03 g) were higher than that of *A. crassicarpa* (0.0004 g to 0.004 g).

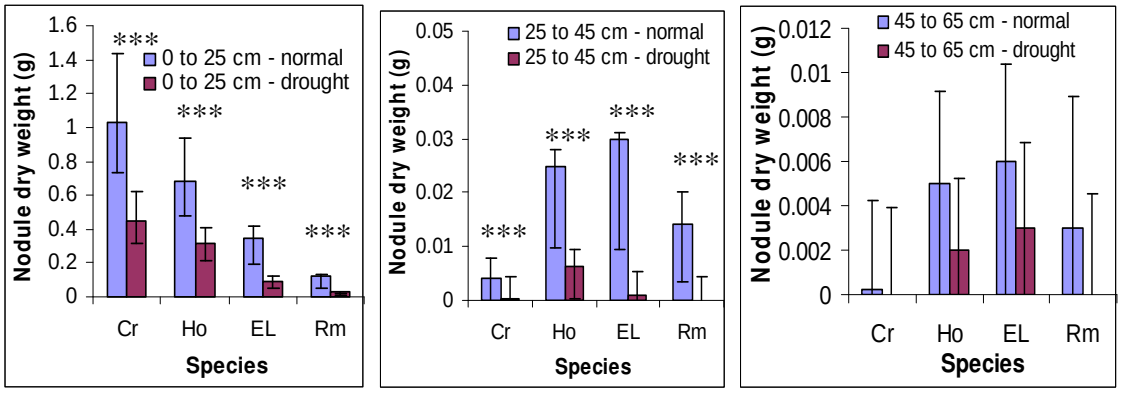


Figure 5.5. Measured mean nodule dry weights of *A. crassicarpa* (Cr), *A. holosericea* (Ho), *A. elachantha* (EL) and *A. ramiflora* (Rm) under two watering regimes and three soil depths after 56 days of experiment. Error bars are 95% confidence intervals. Significant differences between watering regimes at each depth are marked with asterisks. Depth x watering regimes was significant ($F_{2, 168} = 12.573$; $P < 0.001$) and the effect of drought is only significant at 0 to 25 cm and 25 to 45 cm depths. *** $P < 0.001$ ** $P < 0.01$ * $P < 0.05$.

The changes in NDW and root biomass could be explained by the combined effects of species, watering regimes and depths together (Fig. 5.6). The clear trend of decreasing soil moisture in treatments involving *A. crassicarpa* / *A. holosericea*, *A. elachantha* to *A. ramiflora* likely reflected the inherent water use requiements of mesic and semi-arid species. The mesic species always had higher NAR and greater foliage area. Their transpiration rates were likely higher. The soil moisture measured at day 56 thus varied with species under both watering regimes.

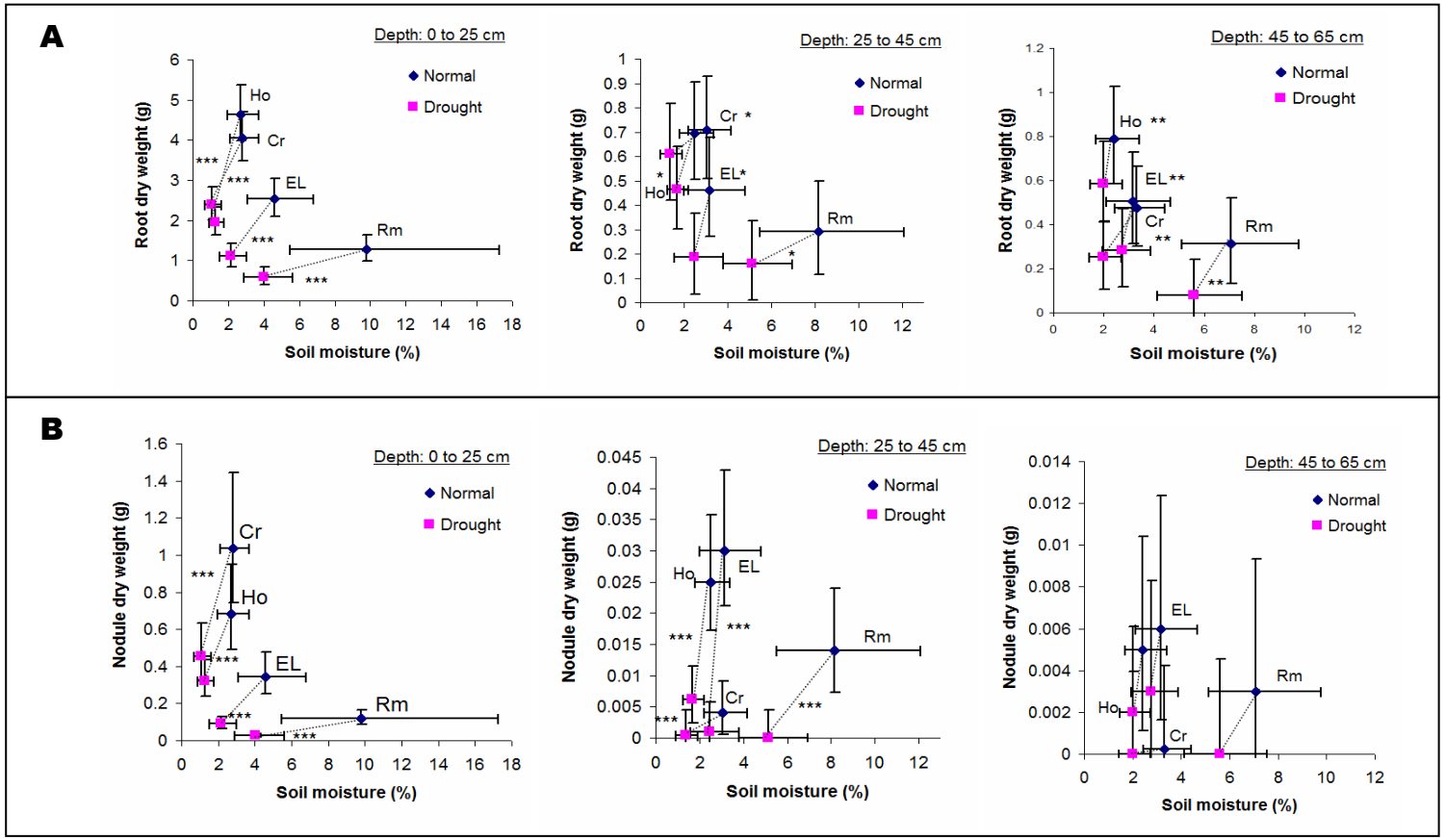


Figure 5.6. The interspecific differences in soil moisture and root dry weight (A) and nodule dry weight (B) at three depths and two watering regimes. Values are measured means ± 95% confidence intervals. Asterisks indicate significant differences in root or nodule dry weights between normal watering and drought regimes in each species. *** $P < 0.001$ ** $P < 0.01$ * $P < 0.05$. Soil moisture between normal watering and drought always differed significantly, regardless of species or depth (Fig. 5.3). Cr, *A. crassicarpa*. Ho, *A. holosericea*. EL, *A. elachantha*. Rm, *A. ramiflora*.

5.3.10 Drought effects on nitrogen contents

The replicates of each plant organ in every treatment were homogenised into one composite sample for nutrient analysis. Thus the nitrogen contents could not be compared statistically between treatments.

The nitrogen contents of stems, roots, foliage and nodules of all species under drought were lower than those under normal watering regime except the nodules of *A. holosericea* in which a slight and probably insignificant higher nitrogen content under drought was recorded. The normalized nitrogen contents ranged from +12 to -100% (Fig. 5.7 and 5.8). In *A. crassicarpa*, *A. holosericea* and *A. elachantha*, the normalized nitrogen content of every plant organ in the presence of coastal rhizobia was more negative than there was in the presence of inland rhizobia. This situation was reversed in the case of *A. ramiflora*.

Most of the normalized stem and foliage nitrogen contents with either coastal or inland rhizobia ranged from -40% to -66% (Fig. 5.7 and 5.8). The normalized foliage nitrogen content of *A. ramiflora* under coastal rhizobia was only -21% and hence was an exception. On the other hand, the normalized root nitrogen content ranged from -20% to -43%. Only in *A. elachantha* with coastal rhizobia and *A. ramiflora* with inland rhizobia did the normalized root nitrogen content reach as negative as -66 and -67% respectively. A relative higher allocation of nitrogen to root than shoot (stem plus foliage) under drought was thus generally observed.

The nodule nitrogen contents under drought were lower than those under normal watering regime. The differences between the two watering regimes were greater with inland species and with coastal rhizobia. In the presence of coastal rhizobia, the normalized nodule nitrogen contents of inland *A. elachantha* (-90%) and *A. ramiflora* (-100%) were more negative than those of *A. crassicarpa* (-47%) and *A. holosericea* (-56%) (Fig. 5.7). In the presence of inland rhizobia, the normalized nodule nitrogen contents of *A. crassicarpa*, *A. elachantha* and *A. ramiflora* were -12%, -32% and -73% respectively. However, a +2% was recorded for the normalized nodule nitrogen content of *A. holosericea*.

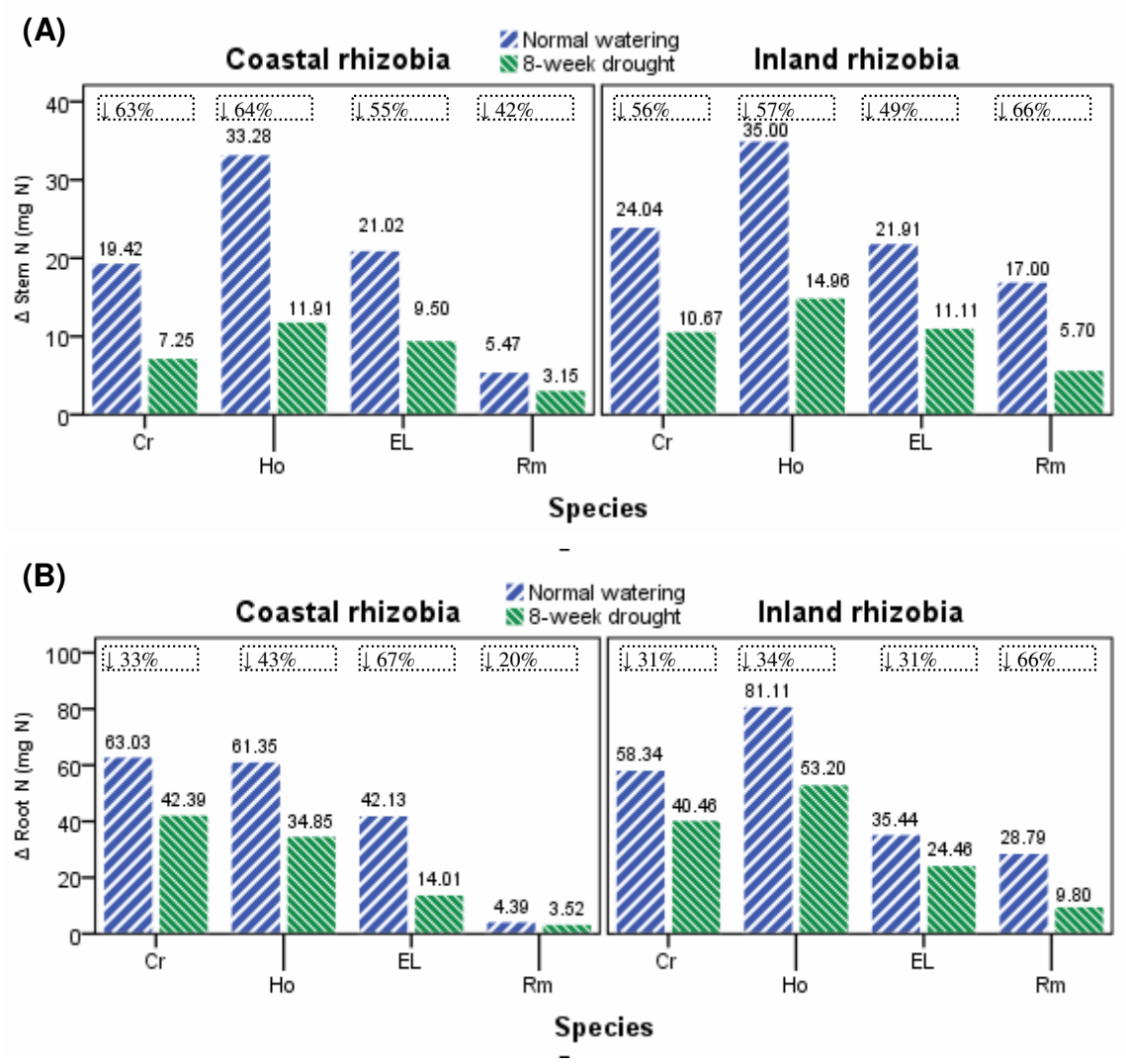


Figure 5.7. Changes in the nitrogen contents of stems (A) and roots (B) between day 0 and day 56 in *A. crassicarpa* (Cr), *A. holosericea* (Ho), *A. elachantha* (EL) and *A. ramiflora* (Rm) with two sources of rhizobia under two watering regimes. The nitrogen content of a specific plant organ equals the product of nitrogen per unit dry weight and the dry weight of that plant organ. Due to inadequate time, all samples of individual plant organs of each treatment were pooled for nitrogen analysis. It is thus impossible to compare treatments statistically. Each number immediately above a bar indicates the change in nitrogen content of a specific treatment. The number in each rectangle indicates the normalized nitrogen content of each species-rhizobia combination.

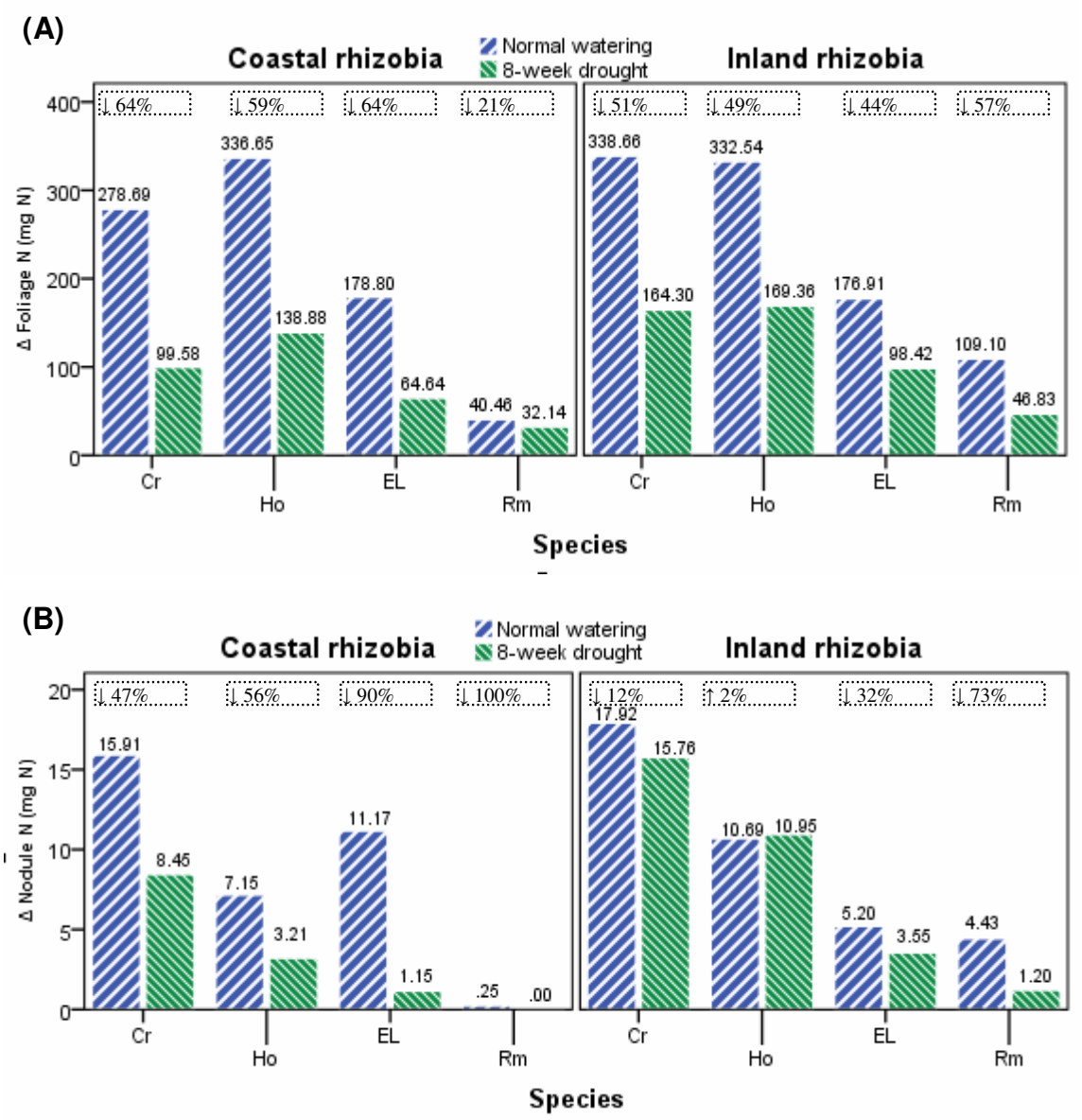


Figure 5.8. Changes in the nitrogen contents of foliages (A) and nodules (B) between day 0 and day 56 in *A. crassicarpa* (Cr), *A. holosericea* (Ho), *A. elachantha* (EL) and *A. ramiflora* (Rm) with two sources of rhizobia under two watering regimes. The nitrogen content of a specific plant organ equals the product of nitrogen per unit dry weight and the dry weight of that plant organ. Due to inadequate time, all samples of individual plant organs of each treatment were pooled for nitrogen analysis. It is thus impossible to compare treatments statistically. Each number immediately above a bar indicates the change in nitrogen content of a specific treatment. The number in each rectangle indicates the normalized nitrogen contents of each species-rhizobia combination.

5.3.11 Drought effects on nitrogen use efficiency

The NUE was defined as the amount of PB produced per unit PN. The response to drought found in this experiment was largely species-specific. For example, in *A. holoserciea* and *A. elachantha*, drought resulted in lower NUE by about 4.4 to 5.1 and 5.3 to 6.4 mg DW mg^{-1} N (Fig. 5.9). However, drought caused the NUE of *A. crassicarpa* with coastal rhizobia to increase by 2 mg DW mg^{-1} N; with inland rhizobia, a decrease of 4.9 mg DW mg^{-1} N was recorded. The change in the NUE of *A. ramiflora* due to drought, in the presence of either source of rhizobia, was about 1 mg DW mg^{-1} N only.

It should be noted that with coastal rhizobia, the mesic *A. crassicarpa* had the highest NUE (normal watering: 54.6 mg DW mg^{-1} N and drought: 55.6 mg DW mg^{-1} N) while semi-arid *A. ramiflora* had the lowest NUE (normal watering: 39.6 mg DW mg^{-1} N and drought: 38.7 mg DW mg^{-1} N) (Fig. 5.9). *A. holosericea* and *A. elachantha* were similar to each other and could be ranked intermediate. With inland rhizobia, the trend became different. Given adequate watering, the NUE in decreasing order was *A. crassicarpa*, *A. elachantha*, *A. ramiflora* and *A. holosericea*. When subject to drought stress, *A. ramiflora* was ranked first, giving 48.8 mg DW mg^{-1} N. *A. crassicarpa*, *A. elachantha* and *A. holosericea* were ranked second, third and fourth, giving 46.1, 43.5 and 41.3 mg DW mg^{-1} N respectively.

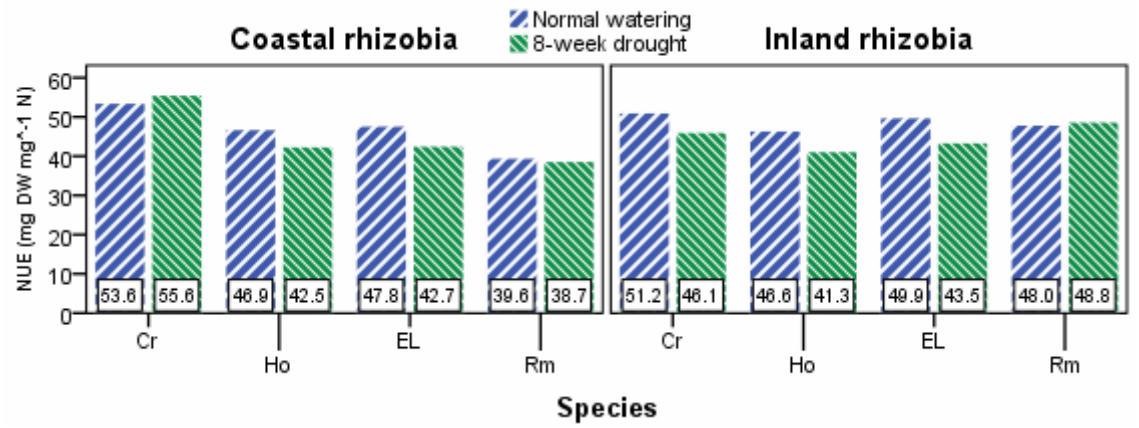


Figure 5.9. The nitrogen use efficiency (NUE) of *A. crassicarpa* (Cr), *A. holosericea* (Ho), *A. elachantha* (EL) and *A. ramiflora* (Rm) with two sources of rhizobia under two watering regimes after 56 days of experiment. Due to inadequate time, all samples of individual plant organs of each treatment were pooled for nitrogen analysis. It is thus impossible to compare treatments statistically. Each number at the bar bottom is the NUE value of a particular treatment.

5.3.12 Drought effects on $\delta^{15}N_{foliage}$ and $\delta^{13}C_{foliage}$

In general, $\delta^{15}N_{foliage}$ was raised under drought (Fig. 5.10). The increases in $\delta^{15}N_{foliage}$ of *A. crassicarpa* with coastal and inland rhizobia were 0.3 and 0.5‰ respectively (Fig. 5.10). These two increases were the lowest among the four species. In the presence of coastal rhizobia, the $\delta^{15}N_{foliage}$ of mesic *A. crassicarpa* and *A. holosericea* (normal watering: 0 to 0.5‰ and drought: 0.3 to 1.8‰) were less than those of inland *A. elachantha* and *A. ramiflora* (normal watering: 0.6 to 3.3‰ and drought: 2.4 to 3.5‰). In the presence of inland rhizobia, the $\delta^{15}N_{foliage}$ of coastal *A. crassicarpa* and *A. holosericea* (normal watering: 0.3 to 0.4‰ and drought: 0.8 to 1.1‰) were also less than that of inland *A. elachantha* and *A. ramiflora* (normal watering: 1.6 to 2.6‰ and drought: 2.6 to 3.7‰).

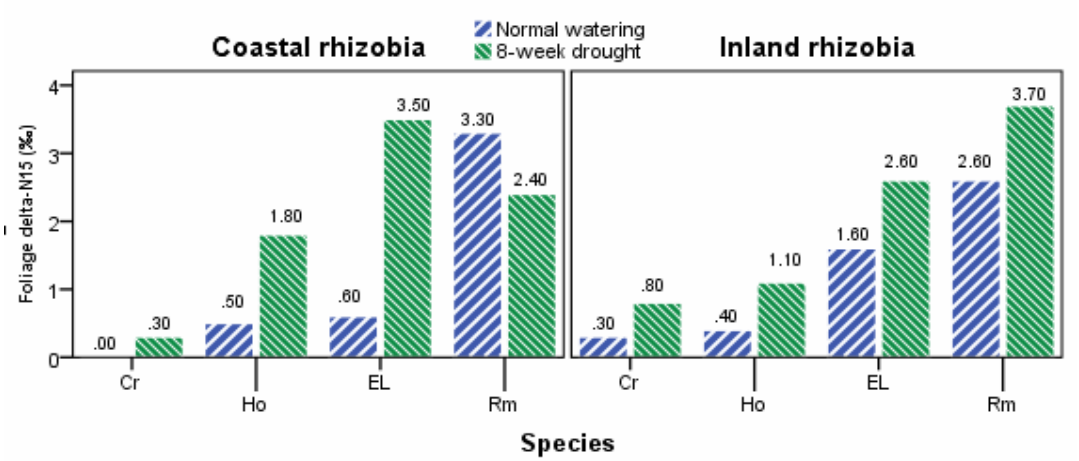


Figure 5.10. The $\delta^{15}N_{foliage}$ of *A. crassicarpa* (Cr), *A. holosericea* (Ho), *A. elachantha* (EL) and *A. ramiflora* (Rm) with two sources of rhizobia under two watering regimes after 56 days of experiment. Due to inadequate time, all samples of individual plant organs of each treatment were pooled for isotope analysis. It is thus impossible to compare treatments statistically. Each number at the bar top is the $\delta^{15}N_{foliage}$ value of a particular treatment.

$\delta^{13}C_{foliage}$ generally reflects intrinsic WUE of plants under various environmental conditions (Li 1999; Bacon 2004). $\delta^{13}C_{foliage}$ of *A. crassicarpa* and *A. holosericea* ranged from -27.9 to -29‰ while that of *A. elachantha* and *A. ramiflora* ranged from -29.8 to -31.1‰, regardless of watering regimes (Fig. 5.11). As higher or less negative $\delta^{13}C_{foliage}$ indicates higher WUE, inland *Acacias* could be deduced to have a lower WUE regardless of watering treatment.

In the presence of coastal rhizobia, the $\delta^{13}C_{foliage}$ of *A. crassicarpa*, *A. elachantha* and *A. ramiflora* under drought were less negative those under normal watering regime by 0.4, 0.5 and 0.4‰ respectively while the $\delta^{13}C_{foliage}$ of *A. holosericea* was more negative by 0.6‰ (Fig. 5.11).

Negligible changes in $\delta^{13}C_{foliage}$ (by 0.1‰) due to drought were observed in *A. crassicarpa* and *A. elachantha* with inland rhizobia. The $\delta^{13}C_{foliage}$ of *A. holosericea* with inland rhizobia became less negative by 0.5‰ while that of *A. ramiflora* also with inland rhizobia became less negative by 1‰.

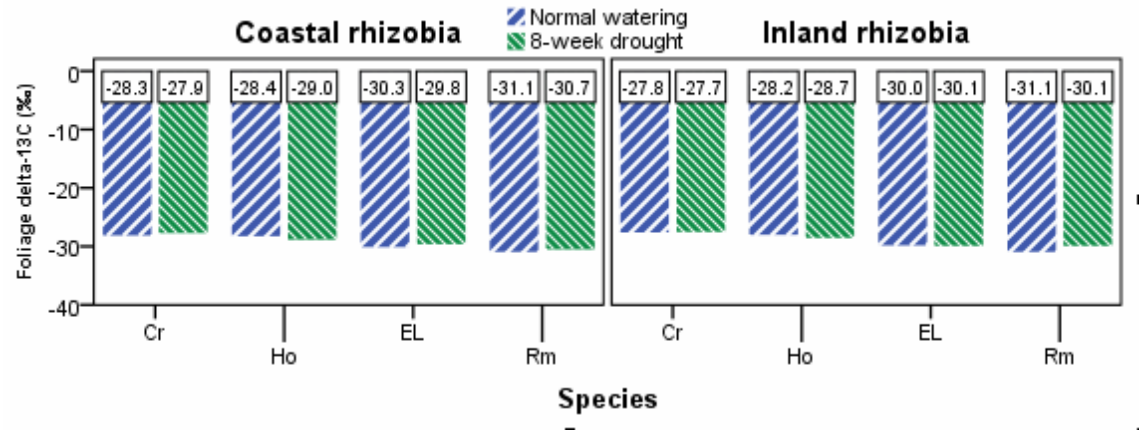


Figure 5.11. The $\delta^{13}C_{foliage}$ of *A. crassicarpa* (Cr), *A. holosericea* (Ho), *A. elachantha* (EL) and *A. ramiflora* (Rm) with two sources of rhizobia under two watering regimes after 56 days of experiment. Due to inadequate time, all samples of individual plant organs of each treatment were pooled for isotope analysis. It is thus impossible to compare treatments statistically. Each number at the bar top is the $\delta^{13}C_{foliage}$ value of a particular treatment.

5.3.13 Growth dynamics

The RGR was significantly correlated with NAR_{mean} (R^2_{adj} = 0.443 ; P < 0.001) (Fig 5.12). RGR had no relationship with either SFA_{mean} or FMR_{mean}. The positive regression was in line with the previous research by Shipley (2006). In other studies, RGR was found to be determined by SFA instead of NAR (Poorter and Remkes 1990; Atkin *et al.* 1998).

There was no clear difference between mesic and semi-arid *Acacias* in NAR (Table 5.5). The normalized NAR also did not significantly differ between species (Table 5.9).

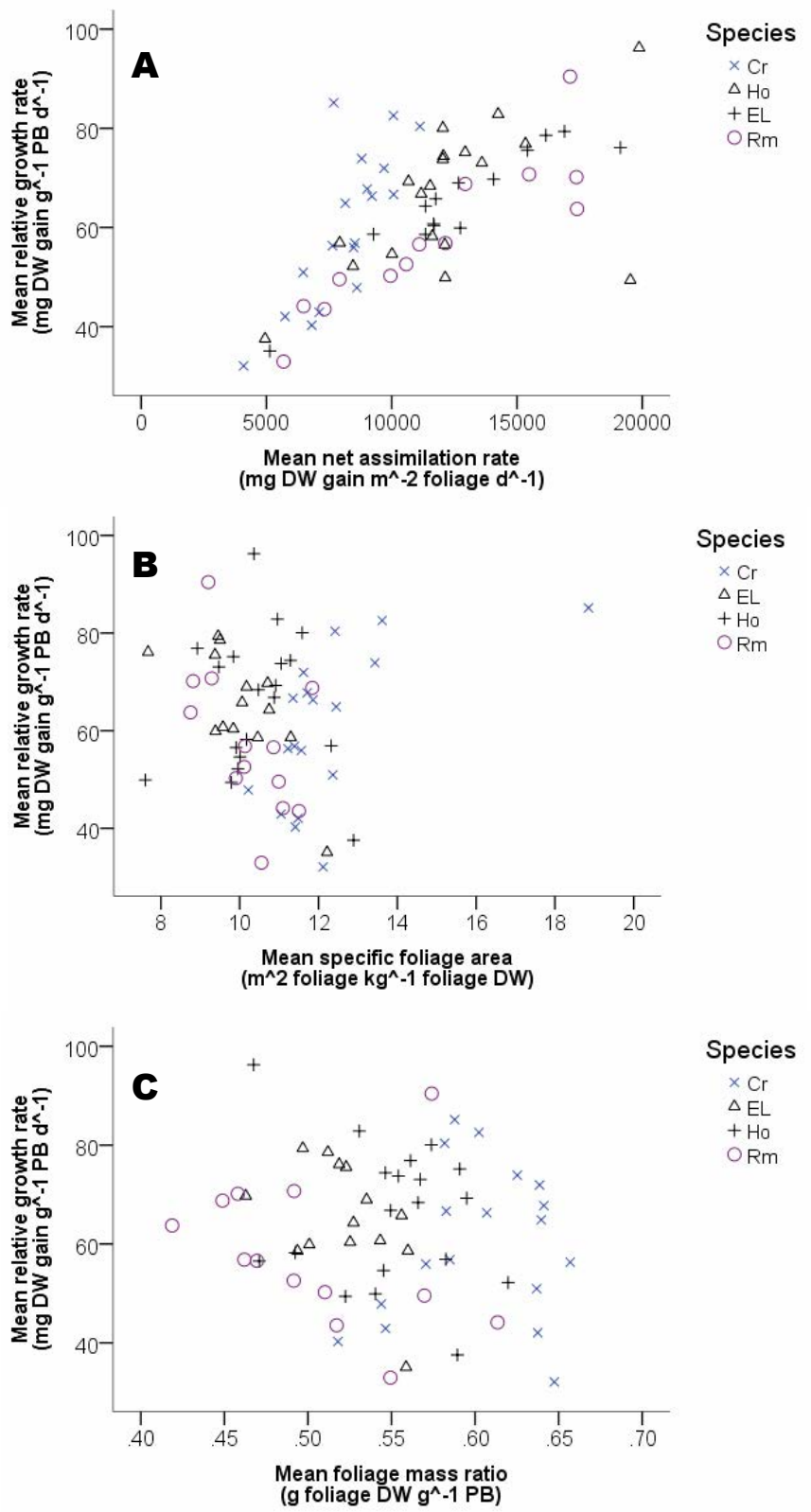


Figure 5.12. The relationship between relative growth rate and mean net assimilation rate (A), specific foliage area (B) and foliage mass ratio (C) of the four studied *Acacia* species. Cr, *A. crassicarpa*. Ho, *A. holosericea*. EL, *A. elachantha*. Rm, *A. ramiflora*.

5.4 Discussion

5.4.1 Indirect evidence of water stress under drought

The normal watering regime was meant to maintain the field capacity of the soil column but the experimenter underestimated the amount of watering required. According to the the model proposed by Rodríguez-Iturbe *et al.* (2006), a drop in stomatal conductance is indicative of water stress. Stomatal conductance, the conductance to water per unit leaf area per second, was not directly measured but deduced in this experiment. It has two components: stomatal aperture and stomatal density. Its variation can be deduced from changes in $\delta^{13}C$ and NAR. Firstly, $\delta^{13}C$ was found to be positively related to intrinsic WUE (Farquar *et al.* 1989; Li 1999). The two inland *Acacia* species and coastal *A. crassicarpa* had either no change or increased $\delta^{13}C$ (and hence WUE) when subject to drought while their NAR always decreased (Fig. 5.11 and Table 5.9). Using equation 5.11, their stomatal conductance must decrease significantly. Whether this was due to smaller stomatal aperture or lower stomatal density is not known. For *A. holosericea,* there was a slight decrease (more negative) in $\delta^{13}C$ (hence WUE) and a decline in NAR. Without quantitative values for WUE, according to equation 5.11, whether its stomatal conductance decreases (indicative of water stress) is unknown.

$$WUE = NAR / \text{stomatal conductance } (g_s) \qquad \ldots\ldots (5.11)$$

Where: a) NAR – g carbon gain m^{-2} foliage d^{-1}

b) g_s – g water transpired m^{-2} foliage d^{-1}

Adapted from Bacon (2004)

It is acknowledged that after 8 weeks of drought, soil moisture ranged from a mean value of 9.7% to 1.1% because the four studied species have different inherent water use and transpiration rates. *A. crassicarpa* and *A. holosericea*, under the normal watering regime might have already been under water stress since their soil moisture ranged from 2.5 to 3% only.

5.4.2 Drought effects on biomass

The biomass of all plant organs under drought were lower than that under normal water regime (Table 5.4). This fits the general vegetation stress pattern observed in other studies. For instance, drought was observed to reduce stem biomass, root biomass, needle leaf biomass and PB of *Pinus pinaster* in localities studied in Spain, France and Morocco that had mean annual rainfalls of 855, 1190 to 1357 and 763 mm respectively (Aranda *et al.* 2010). Desiccation cycles of 2-

days, 4-days and 6-days lowered PB, total leaf area and mean RGRs of *Acacia tortilis* and *A. xanthophloea* in Kenya (Otieno *et al.* 2001). When grown at 20% field capacity instead of 100% in pots, *A. koa* was found to have lower PB and abovegound biomass at high light but not under low light conditions (Craven *et al.* 2010). The current experiment therefore reinforces the general consenus that drought can slow down plant growth (Section 5.1.1).

5.4.3 The effects of drought and rhizobial sources on biomass and growth

The key question of interest is whether the relatively lower growth rate and biomass under drought differed between rhizobial source and/or between inland and coastal species. When data from both the drought and normal watering regimes was pooled, higher PB, stem biomass and foliage biomass were achieved with inland rhizobia than coastal rhizobia (Section 5.3.1). Also, the normalized foliage biomass, NDWs and RGRs were significantly higher with coastal rhizobia than inland rhizobia. There were no significant effects of species on the normalized biomass and growth rates (Tables 5.8 and 5.9).

5.4.4 Drought effects on stem to root and shoot to root ratios

When pooling data from both drought and normal watering and excluding the species outlier *A. elachantha*, the differences between rhizobial sources in terms of stemTRR and shootTRR were not significant. Nevertheless, the two ratios under drought were lower than those under normal watering regime, reflecting a general strategy to allocate proportionally more resources to roots than to shoots when plants were subjected to drought. It has previously been found that decreased nitrogen acquired by symbiotic nitrogen fixation by rhizobia led to decreased shootTRR and vice versa (Kucey and Paul 1982; Requena *et al.* 1997). Since the nitrogen acquired by symbiotic nitrogen fixation under drought was also lower than that under normal watering regime, as indicated by more positive $\delta^{15}N_{foliage}$ under drought (Section 5.3.12), the results of the current experiment were largely consistent with previous findings.

Smaller shootTRR has been identified as an adaptation of plants to drought. A smaller shootTRR under cyclic drought was demonstrated in slow-growing *A. tortilis* but not in fast-growing *A. xanthophloea* in Kenya (Otieno *et al.* 2001). *Pinus pinaster* also had lower shootTRR when soil moisture was set at 40% field capacity, compared to the shootTRR at 100% soil field capacity (Aranda *et al.* 2010). In another study, the root biomass ratio of *A. koa*

receiving high light under drought was higher than *A. koa* receiving intermediate and low light (Craven *et al.* 2010). The foliage mass ratio was always lower under drought, regardless of the light intensity experienced by *A. koa* seedlings. The shootTRR of *A. koa* could thus be deduced to be smaller under drought than under normal watering regime.

An exception to the lower stemTRR and shootTRR under drought was observed in *A. elachantha* with coastal rhizobia whose stemTRR and shootTRR under drought were higher than those under normal watering regime by 8% and 3% respectively, although this was not statistically significant. However, when grown with inland rhizobia, the stemTRR and shootTRR of *A. elachantha* under drought were lower than those under normal watering regime by 67% and 33% respectively.

5.4.5 Drought effects on specific foliage weight and water content

Examination of foliage thickness (inferred from SFW) and water content (DW to WW ratios) indicates that the two inland *Acacias* stored more water than the two coastal *Acacias*. The stomatal conductance of the two inland *Acacias* was deduced to be more responsive to drought so the two species might have greater control over transpiration (Sections 5.4.8 and 5.4.10). Their smaller total foliage area would have also contributed to lower transpiration and the rates of water uptake by transpiration pull, thereby conserving soil water (as indicated by higher soil moisture in treatments with inland *Aacias* than with coastal *Acacias*) (Fig. 5.12). The average increases in total foliage area of *A. crassicarpa*, *A. holosericea*, *A. elachantha* and *A. ramiflora* under the normal watering regime from day 0 to day 56 were 1 289, 884, 545 and 328 cm^2 respectively. When under drought, they were 485, 386, 235 and 163 cm^2 respectively. Thicker foliage of inland *Acacias,* than coastal *Acacias* under drought, also meant a greater diffusion distance for water vapour to reach the air and hence a slower rate of transpiration (Section 5.1.4).

5.4.6 Root biomass at different depths

There was no evidence of increasing root depth under the drought treatments. Small drought-induced differences in root depth (e.g. 10 cm) were observed in another drought experiment with herbaceous legumes (Amar 1996). Such differences, even if present, could not be detected by dividing the soil column into three 20 to 25 cm depths.

There was no evidence of increasing root DW at greater depths. Sandy loam textured soil has efficient drainage and would dry quickly under high temperatures in the shadehouse (mean maximum temperature 38.2°C). Under normal watering, the mean soil moisture on the upper surface of *A. holosericea*, *A. elachantha* and *A. ramiflora* soil columns was the highest (Fig. 5.3). Surface soil was aggregrated by high root biomass and hence retained more soil water than the looser soil at greater depths. On the other hand, when subject to drought, evaporation in the deeper soil was less than that at the surface soil, resulting in increasing soil moisture with depth. However, regardless of watering regimes, lower root biomass and NDW were recorded in deeper soil than the upper soil. The root biomass and NDW under normal watering were always higher than those subject to drought.

5.4.7 Nitrogen investment in roots and shoots under drought

The generally greater decline in stem and foliage nitrogen content than the decrease in root nitrogen content is adaptive to drought (Fig. 5.7 and 5.8). As drought might threaten plant survival, nitrogen was diverted to roots proportionally more than the aboveground plant parts to support the root's function of water acqusition.

5.4.8 Adaptation of A. ramiflora to drought

A. ramiflora adapts to drought better than other species with the least decreases in water content and foliage thickness (Table 5.11). Under drought in the presence of inland rhizobia, the NUE and $\delta^{13}C_{foliage}$ were higher (or less negative for $\delta^{13}C_{foliag}$) than those under normal watering regime while the NUE and $\delta^{13}C_{foliage}$ of other species either decreased or remained unchanged (Fig. 5.9 and 5.11). $\delta^{13}C_{foliage}$ was found to be positively correlated with WUE in previous studies (Farquar *et al.* 1989; Li 1999).

Data on the growth of *A. ramiflora* under drought in the presence of coastal rhizobia was contradictory. For instance, the NUE under drought was lower than that under normal watering. Seedlings also had no nodules so nitrogen could not be fixed via symbiotic nitrogen fixation. Given limited nitrogen supply under drought, low NUE was undesirable as nitrogen could be depleted quickly by maintenance. On the other hand, the $\delta^{13}C_{foliage}$ (and hence WUE) of *A. ramiflora* in the presence of coastal rhizobia under drought was less negative that that under

normal watering. Also, the decreases in the nitrogen contents of stem, foliage and roots were smaller than those in the presence of inland rhizobia (Fig. 5.7 and 5.8). This unexpected result suggests that many nodules of *A. ramiflora* occupied by coastal rhizobia might be parasitic. Failure to nodulate under drought might thus relieve the host plant from extra nutrient stress.

In the current experiment, reduced stomatal conductance may be the primary reason for the increased WUE of *A. ramiflora* after 56 days without water (Fig. 5.11). To explain this, recall a definition of WUE widely adopted by plant physiologists (equation 5.11). The WUE of plants can be increased by higher NAR and/or lower g_s. Note that the diffusion gradient of water vapour from the intercellular spaces to the atmosphere is about 50 times higher than the diffusion gradient of CO_2 from atmosphere to intercellular spaces (Bacon 2004). Therefore, when the stomatal aperture and/or density is reduced, the proportional reduction in NAR is higher than that in g_s, leading to increased WUE. According to Table 5.5, the NAR of *A. ramiflora* under drought was calculated to be lower than that under normal watering by 5 421 mg DW gain m^{-2} foliage d^{-1}. With reference to equation 5.11, the proportional reduction in g_s had to be high to compensate for this decrease of NAR.

5.4.9 The relative importance of symbiotic nitrogen fixation and nitrogen uptake from soil

The two inland *Acacia* species had more positive $\delta^{15}N_{foliage}$ than the two coastal *Acacia* species (regardless of watering regime) (Fig. 5.10). Such difference reflected the fact that the relative importance of symbiotic nitrogen fixation to the two inland species was less than that to the two coastal species. This deduction is based on the method of measuring the ^{15}N natural abundance in which the proportion of ^{15}N to ^{15}N in air was set as the standard and hence $\delta^{15}N_{air}$ was zero. Plants taking up nitrogen purely from air thus have a negative $\delta^{15}N_{foliage}$ due to isotopic discrimination in the process of symbiotic nitrogen fixation and metabolic assimilation (Shearer and Kohl 1986). For instance, the field collected phyllodes of *A. crassicarpa*, *A. aulacocarpa*, *A. elachantha* and *A. ramiflora* were negative in $\delta^{15}N_{phyllode}$ values. The phyllodes of their seedlings forced to rely on symbiotic nitrogen fixation for 4 months also had negative $\delta^{15}N_{phyllode}$ values (Table 2.7). Should the $\delta^{15}N_{foliage}$ be greater than zero, a larger proportion of the plant nitrogen would have come from the ^{15}N enriched soil than from the ^{15}N depleted air. When more positive $\delta^{15}N_{foliage}$ was observed in some species, one could deduce a higher importance of nitrogen uptake from soil. The ^{15}N natural abundance method is based on the fact that the nitrogen of a tree/shrub as a whole was derived externally such as from soil (direct uptake or through

mycorrhizae) or air although for individual organs, part of their nitrogen came from redistribution such as the resorption from foliage during senescence (Aerts 1996).

Likewise, plants under drought have more positive $\delta^{15}N_{foliage}$ values than those under normal watering and thus are expected to take up nitrogen predominantly from the soil by root instead of through symbiotic nitrogen fixation.

5.4.10 The different water use efficiencies of inland and coastal Acacias under drought

The stomatal conductance (g_s) of *A. elachantha* and *A. ramiflora* was possibly more responsive to drought than the stomatal conductance of *A. crassicarpa* and *A. elachantha*. Results suggest that the reduction in NAR due to drought was much greater in the two inland *Acacia* species than the two coastal *Acacia* species (Table 5.5). Also, the WUE of inland species increased under drought while the WUE of coastal species either decreased or remained unchanged under drought. According to equation 5.11, the reduction of g_s of inland *Acacias* would be greater than the g_s of the coastal *Acacias*.

The WUEs of both coastal species were higher than those of both inland species, probably because the soil moisture in treatments involving the former species are lower than treatments involving the latter species.

5.4.11 Comparative responses of inland and coastal Acacias to drought

The experiment demonstrated that while drought had a negative effect on all four *Acacias* species studied, inland *A. elachantha* and *A. ramiflora* were less vulnerable to drought than coastal *A. crassicarpa* and *A. holosericea*. This may be due to the following adaptive traits:

- More drought-sensitive stomatal conductance was deduced indirectly from the data but direct measurement is required to verify it (Sections 5.4.8 and 5.4.10).
- Foliage thickness, indirectly deduced from its correlated specific foliage weight (Dijkstra 1989), either remained the same or was reduced slightly under drought, in contrast to the substantially reduced foliage thickness in coastal *Acacias*.
- Under drought, foliage water content was reduced by a lesser extent in inland than coastal species.

- Lower water uptake and hence reduced transpiration (higher soil moisture remaining after 56 days of experiment) was indicated in the inland species.
- WUEs of the two inland *Acacia* species were raised under drought while the WUEs of the two coastal *Acacia* species decreased or remained unchanged.

For the four *Acacia* species, the nodule nitrogen contents and the relative importance of symbiotic nitrogen fixation in acquiring nitrogen decreased under drought. The percentage reduction was found to be more severe in the two inland *Acacias* than the two coastal *Acacias*. It is argued that the greater reduction in inland *Acacias* might be of adaptive significance. Inland *Acacias* might have less incentive to nodulate and initiate symbiosis that failed to fix atmospheric nitrogen effectively as a result of drought, thereby avoiding parasitic symbiosis that could reduce the host fitness. This hypothesis should be further tested in the future. The result of the drought experiment did not validate the original hypothesis that any mutualistic symbiosis between inland *Acacias* and inland rhizobia resulted in greater drought tolerance than one between coastal *Acacias* and coastal rhizobia.

6 General discussion

6.1 Summary of findings and their implications for climate change adaptation

6.1.1 Would climate change affect seed germination of the four tropical Acacias?

The seed germination experiment supplements the results of a similar experiment by Congdon *et al*. (in prep.); the results of both studies were used to draw conclusions about the heat tolerance and sensitivitiy of inland and coastal tropical *Acacia* seeds.

In the current experiment, *A. ramiflora* and *A. crassicarpa* exhibited the highest heat tolerance because they achieved the highest germination percentage in the wet heat-5 mins treatment (strong heat). *A. elachantha* displayed only moderate heat tolerance as it experienced some mortality under wet heat-5 mins (strong heat) and hence the germination percentage was less than the maximum. *A. aulacocarpa* had the lowest heat tolerance as it only achieved high germination in the 100^{o}C dry-1 min and 80^{o}C wet-1 min treatments (low and moderate heat).

On the other hand, the seed germination of all the coastal *Acacias* in the study by Congdon *et al*. (in prep.) increased with temperatures from 60^{o}C to 80^{o}C and then to 100^{o}C dry-5 mins. However, the seed germination percentages of inland *Acacias* either increased or remained similar between dry heat-5 mins treatments at 60^{o}C and 80^{o}C, and were significantly less at 100^{o}C (Table 3.4 and Fig. 3.2). Since inland *Acacias* showed optimal germination at a lower temperature than coastal *Acacias* in that experiment, their heat tolerance can be considered as lower than that of coastal *Acacias.*

The heat sensitivities of *Acacia* seeds were deduced from seed germinaton percentages in the low heat treatments (80^{o}C / 100^{o}C dry-1 min). The most sensitive seeds were *A. aulacocarpa* and *A. ramiflora*. Seeds with intermediate and lowest heat sensitivities were *A. elachantha* and *A. crassicarpa* respectively.

Though the seeds of inland and coastal tropical *Acacia* species differ in their heat sensitivity and tolerance levels, it is uncertain if their germination might be affected by climate change. This is because whether new fire regimes will develop remains to be established, and if there are new fire regimes, whether the regimes in semi-arid/arid and coastal sites differ will have to be investigated.

It should be noted that the temperature and duration of the heat exposure matter to seeds with physical dormancy. But so far field data on these two aspects is scarce. Also, the 20^{o}C difference in their dry heat tolerance level seems relatively small compared to the surface soil temperature and the range of subsurface soil temperatures under experimental fire (Williams 2002). Characterising the tolerance level in response to dry heat instead of to wet heat has particular relevance to climate change because the frequency of exceptionally low soil moisture years and the extent of Queensland affected by such extreme events were predicted to increase (OCC 2008). Thus it is likely that the soil seed bank will be increasingly exposed to dry heat rather than wet heat under wildfires. Thirdly, whether climate change shapes new fire regimes in nature is hard to predict. In terms of fire danger index that incorporates temperature, humidity, wind speed and drought factors, fire frequency and severity in Queensland will likely increase in the future (OCC 2008). But when direct factors other than weather variables are considered, the future fire regimes in both the tropical and temperate Australia remain uncertain (Bradstock 2010). These other factors include varying ignition rates from lightning and anthropogenic sources, the effect of changing dominance of the functional vegetation types on fuel types, decomposition of plant debris under increasing aridity and the effect of CO_2 on plant growth (Bradstock 2010). In addition, readers should note that the frequency, seasonality and coverage of fire can affect the recruitment and population dynamics of *Acacia* seedlings (Williams 2002). Remote sensing methods were used to describe these spatial and temporal aspects of fire regimes in tropical savannas (Russell-Smith and Yates 2007). Conceptual models were constructed to summarise the possible trend of these fire properties in Australia under climate change (Bradstock 2010). But these regional or continental studies do not provide adequate information on fire regimes against which future seed germination events should be assessed. These studies have relatively coarse resolution in order to cover the fire responses of broad vegetation types. Secondly, the aspects of the fire regimes they are characterising are not directly addressing the question of whether seed exposure to fire temperature and duration may change under climate change. Even among those variables not directly relevant to the heat tolerance and sensitivity level of seeds, such as fire coverage and seasonality, the level of uncertainty of predictions about these fire properties under climate change remains high.

6.1.2 Factors affecting symbiotic nitrogen fixation of seedlings

Seedling growth and symbiotic nitrogen fixation may be affected by the source of bacteria and meteorological drought. Sites can vary in their microbial communities and certain bacteria may infect *Acacias* more easily and fix atmospheric nitrogen more effectively than others. Assisted migration of *Acacias* may be necessary if climate has become too hostile for their survival but seed dispersal is too slow to cope with the changes. Meteorological drought can be caused by inter-annual variability of rainfall and sometimes El Niño or La Niña phenomena. Under climate change, the symbiotic nitrogen fixatin of *Acacias* would likely be negatively affected by increasingly common and severe soil moisture deficit. It is thus useful to examine how absence of watering may affect the soil moisture, growth and symbiotic nitrogen fixation of the plants.

The provenance experiment indicated that *A. crassicarpa*, *A. aulacocarpa*, *A. elachantha* and *A. ramiflora* were generally promiscuous in nodulation. All four studied species could develop nodules with rhizobia originating from their own soil or from soil sourced from the other three species, when watered with nitrogen-free modified McKnight solution.

The provenance experiment found no evidence that an *Acacia* species adapts to rhizobia found in its natural population. In other words, the changes in survival, biomass or nitrogen contents of an *Acacia* species forming nodules with its own rhizobia were not higher than the same *Acacia* species forming nodules with rhizobia living in the soil that supports the populations of other species. *A. aulacocarpa* had the highest increase in survival when forming nodules with inland rhizobia, i.e. rhizobia from soil supporting the natural populations of either *A. elachantha* or *A. ramiflora*. *A. crassicarpa* and *A. ramiflora* had the highest increase in survival when forming nodules with coastal rhizobia, i.e. rhizobia from soil supporting the natural populations of either *A. crassicarpa* or *A. aulacocarpa*. The survival of *A. elachantha* increased the most with rhizobia from *A. ramiflora*. In addition, *A. crassicarpa*, *A. aulacocarpa* and *A. elachantha* had the highest increases in biomass and nitrogen contents in the presence of inland rhizobia. *A. ramiflora* had the highest biomass and nitrogen contents in the presence of *A. aulacocarpa* rhizobia.

Local adaptation of *Acacia*-rhizobia symbiosis is relevant to climate change adaptation. The habitat range of a species may shift, with or without human assistance (e.g. revegetation or natural seed dispersal and recruitment) in response to climate changes (e.g. changes in temperature, rainfall, evaporation and fire regimes). But the extent of range shift might be constrained by edaphic factors such as substrate type and biotic interactions such as mutualism

(Keith 2011; Stanton-Geddes and Anderson 2011). It was recently shown that the number of nodules and the height of a legume host, *Chamaecrista fasciulata,* planted immediately outside its population range were lower than those at the centre (Stanton-Geddes and Anderson 2011). However, from the results of the current experiment and other studies, the level of host/rhizobial specificity required for nodules to become effective in *Acacia* appeared not as high as *C. fasiculata,* or several cultivated pea lines in the Middle East (Lie *et al.* 1987; Stanton-Geddes and Anderson 2011). So it is possible that the constraint on the range shift of *Acacia* imposed by mutualism might not be as high as expected, but cross-inoculation or provenance experiments involving more *Acacia* species and more populations of each species are required to verify such generalization.

In the drought experiment, the four *Acacia* species studied were *A. crassicarpa*, *A. holosericea*, *A. elachantha* and *A. ramiflora*. While drought had a negative effect on the biomass and nitrogen contents of all four studied species, inland *A. elachantha* and *A. ramiflora* were less vulnerable to drought than coastal *A. crassicarpa* and *A. holosericea*. These differences were accompanied by the following traits in the inland species:

- Foliage thickness, indirectly deduced from its correlated specific foliage weight (Dijkstra 1989), either remained the same or was reduced slightly under drought, in contrast to the substantially reduced foliage thickness in the two coastal *Acacia* species.
- Foliage water content was reduced to a lesser extent than coastal species.
- WUEs of the two inland *Acacia* species were raised under drought while the WUEs of the two coastal *Acacia* species decreased or remained unchanged.
- More drought-sensitive stomatal conductance was deduced indirectly from the data but direct measurement is required to verify it (Sections 5.4.8 and 5.4.10).

For all the four studied species, the nodule nitrogen contents and the relative importance of symbiotic nitrogen fixation in acquiring nitrogen (compared to uptake from soil via roots) decreased under drought. The percentage reduction was found to be more severe in the two inland *Acacias* than the two coastal *Acacias*. The greater reduction of the two inland *Acacia* species might be of adaptive significance. *A. ramiflora* and *A. elachantha* might have less incentive to nodulate and initiate symbiosis that failed to fix atmospheric nitrogen effectively as a result of drought, thereby avoiding parasitic symbiosis that could affect the host fitness. This hypothesis should be tested in the future. The drought experiment did not validate the original hypothesis that the inland *Acacia*-rhizobia symbiosis was more drought tolerant than the coastal *Acacia*-rhizobia symbiosis in terms of biomass and nitrogen contents of foliage, stems and roots.

The decreases in foliage and nodule biomass, stem to root ratio and foliage nitrogen content in all the four *Acacia* species under drought were higher in the presence of coastal rhizobia than in the presence of inland rhizobia, suggesting symbiosis formed with the latter source of rhizobia was less affected by drought.

The apparent higher drought-tolerance of the two inland *Acacia* species and inland rhizobia has implications for climate change adaptation. Inoculation with inland rhizobia might alleviate the effect of drought on some *Acacia* species. Under climate change, prolonged periods of drought interrupted by less frequent but more intense rainfall events are expected (BOM 2010). This can potentially result in drier savanna soil in which mineralization and nitrification could be inhibited (Holt and Coventry 1990; Schmidt and Lamble 2002; Richards *et al.* 2012). Plant-available soil nitrogen and direct uptake of soil nitrogen via roots might be lessened. At the same time, reduced water availability may cause reduced effectiveness of symbiotic nitrogen fixation (Table 5.8). If symbiosis with inland rhizobia helps the host plant to alleviate the negative effect of drought, this trait would be adaptive. In the case of assisted range shift through revegetation, inoculating the soil around the planted seedlings with inland rhizobia might confer the host plant with extra drought-tolerance. However, the inoculated rhizobial population should be significantly greater than the local rhizobial population to avoid outcompetition (Lie *et al.* 1987; Thrall *et al.* 2005; Thrall *et al.* 2007; Heath and Tiffin 2008). The evidence also suggested that the two inland *Acacias* could have a higher chance of surviving a single drought event.

6.1.3 Factors affecting the nodulation of mature Acacias

In the field study, effective nodule density and/or biomass of *A. crassicarpa* were found to increase with increasing tree size, greater root biomass, the change from dry to rainy season, increasing soil depth, decreasing soil moisture and increasing soil conductivity. Negative $\delta^{15}N_{foliage}$ values were recorded for all four *Acacia* species, suggesting that symbiotic nitrogen fixation and/or mycorrhizal uptake of soil nitrogen could be their main source(s) of nitrogen in their natural habitats (Section 2.3.3). However, there were a negligible number of nodules in the top 24 cm of soil for *A. aulacocarpa, A. elachantha* and *A. ramiflora.* If the negative $\delta^{15}N_{foliage}$ values of these three species were caused by symbiotic nitrogen fixation, root nodules might be found deeper in the soil profile. The effect of climate change on rhizobial and mycorrhizal symbiosis probably differs and understanding the cause of negative $\delta^{15}N_{foliage}$ values in these

three species is fundamental to understanding the relative importance of the two types of symbiosis in the nutrition of *Acacia*.

While nodulation effectiveness was found to be the highest at an optimal level of soil moisture or sub-peak/moderate rainfall in previous research (Habish 1970; Lawrie 1981; Langkamp *et al.* 1982), the current study found a negative correlation between soil moisture and nodule density or biomass. This correlation probably indicated that nodulation needed adequate drainage, and hence aeration. However, given that *A. crassicarpa* soil and root nodules were sampled only once in the wet season and once in the dry season, this correlation could be an artefact of temporal variability. Otherwise, low soil moisture might be a result of higher water uptake by greater *Acacia* root biomass, as soil moisture and root biomass were shown to be negatively correlated in the drought experiment (Fig. 5.6). This is because adequate watering in the wet season leads to high root biomass development, drawing down soil moisture in the period between separate rainfall events. At the same time, by acquiring adequate water for growth, plants can form nodules with soil rhizobia. Thus higher nodule biomass can be achieved at the same soil moisture level in the wet season than in the dry season (Fig. 6.1).

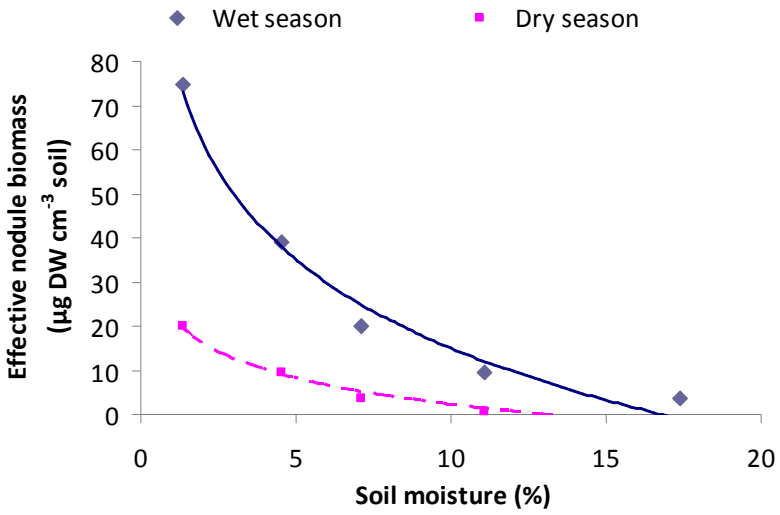


Figure 6.1. Modelled change in effective nodule biomass with seasons and soil moisture of *A. crassicarpa*. See Sections 2.2.3.2 and 2.3.2 for an explanation of how the model was constructed.

The field study also showed that the density of dead nodules of *A. crassicarpa* in the dry season was significantly higher than in the wet season, reflecting the importance of seasonal stress. There was, however, no relationship between total soil nitrogen/phosphorus and the density/biomass of effective nodules.

6.2 Future research

6.2.1 Nodulation of mature individuals

In future field studies, trial sampling up to 1 m soil depth should be undertaken for all four species to understand the change of nodule density/biomass with depth before conducting large-scale sampling. Such trials can also provide evidence on whether symbiotic nitrogen fixation was responsible for the negative $\delta^{15}N_{foliage}$ values of the three species with few nodules in the top 24-cm soil. Previous studies indicate that a sizeable rhizobial population or high number of nodules were found in deeper soil (Dawson ***et al.*** 1989; Evans ***et al.*** 2005). This information is also important as plants may nodulate in deeper soil when surface soil is expected to become increasingly hostile under climate change (e.g. more frequent and intense fires, and longer duration of heat and drought stress) (BOM 2010). Inland and coastal *Acacia* species may display different patterns.

The seasonal differences in nodule density/biomass recorded are not precise because the field sampling was conducted only once per season. The nodules of many *Acacias* were mostly indeterminate (with meristematic tissues and connection to root vascular bundles) and hence potentially perennial (Sprent 2009). However, stress could induce early senescence of nodules in annual or perennial legumes (Pate 1958, 1976; Zahran 1999). Nodules of five *Acacia* species in Victoria were observed to be annual and the number of necrotic nodules rose substantially in the dry summer (Lawrie 1981). In the current study, the density of dead nodules of *A. crassicarpa* in the dry season was also significantly higher than in the wet season, reflecting the importance of seasonal stress. To follow seasonal changes in nodules, nodules should be sampled every month.

The effect of plant available nitrogen and phosphorus, instead of total soil nitrogen and phosphorus, on nodulation should be examined in the future. The field study found no relationship between total soil nitrogen/phosphorus and the density/biomass of effective nodules. Plant-available nitrogen includes ammonium and nitrate while plant-available phosphorus generally refers to dissolved phosphate ions that are most readily available for root absorption (Brady and Weil 2010). Their levels in soil are often only a very small proportion of the total nitrogen/phosphorus but have been found to be correlated with symbiotic nitrogen fixation or

nodulation (Section 2.1.6). Since plant-available nitrogen could vary with fire regime (Richards *et al.* 2012), which is expected to be significantly altered by climate change, characterising its relationship with inland and coastal *Acacia* species should be undertaken in the future.

The field study demonstrated that using non-nodulated seedlings of the same species as a reference species can result in an invalid %Ndfa. Future research should also attempt to identify and sample suitable reference species in the field, preferably more than one, including at least a non-nitrogen fixing legume and a non-legume dicotyledon of similar root system, phenology and tree/shrub size (Shearer and Kohl 1986; Danso *et al.* 1992; Andrews *et al.* 2011). Since some *Acacia* species are known to form mycorrhizae, reference species of a similar mycorrhizal status should also be included (Andrews *et al.* 2011). Through the use of multiple reference species, %Ndfa calculated with the ^{15}N natural abundance method will always be in a range, indicating some degrees of uncertainty.

6.2.2 Provenance effects on symbiotic nitrogen fixation

The lack of local adaptation found in the provenance study is largely consistent with the results of previous provenance experiments on temperate *Acacia* species (Burdon *et al.* 1999; Thrall *et al.* 2000; Barrett *et al.* 2012; Birnbaum *et al.* 2012). Either local adaptation of *Acacia*-rhizobia symbiosis occurred rarely or the experimental design failed to unmask it. For example, it is possible that one population sampled for each of the four *Acacia* species in this experiment is not representative of the whole species. More populations should be sampled in the future. Also, the host plant might form effective nodules with individual rhizobial strains associated with it, but the nodulation by other less effective rhizobial strains also associated with the host might have masked the effect (Barrett *et al.* 2012).

It was shown that a large natural rhizobial population was associated with higher plant height, and nodule number (and size) are important predictors of rhizobia number in the nodule and in the rhizosphere (Lie *et al.* 1987; Thrall *et al.* 2005; Thrall *et al.* 2007; Heath and Tiffin 2008). Symbiotic effectiveness also depends on the extent of local adaptation which hinges on the degree of specificity between a host plant and rhizobial strains, and the net competitive result of mutualistic and parasitic interactions. To investigate the reasons behind the apparent absence of local adaptation of *Acacia* host-rhizobia symbiont, future research should investigate:

- the size of rhizobial populations (Thrall *et al.* 2005; Thrall *et al.* 2007);

- genetic diversities of the host plant and rhizobia (Burdon *et al.* 1999; Barrett *et al.* 2012);
- the correlations between genetic diversities of the host plant and rhizobia (Barrett *et al.* 2012) ; and
- the correlations between environmental variables, the growth responses of host plants and the size of rhizobia populations (Thrall *et al.* 2007; Barrett *et al.* 2012);

Provenance experiments involving more *Acacia* species and more populations of each species are required to verify whether mutualistic symbiosis will become a constraint to natural range shifts of tropical *Acacia* species.

6.2.3 Drought effects on symbiotic nitrogen fixation

In the future, cross-inoculation experiments should also involve different durations of desiccation cycles and different intensities of watering (e.g. watering up to soil field capacity, 50%, 30% and so on) (Otieno *et al.* 2001; Craven *et al.* 2010). Results of experiments incorporating the effect of climatic variability are more applicable. Also, the effect of drought on net assimilation rate and stomatal conductance should be measured directly in the future.

Cross-inoculation experiments of larger spatial scale (e.g. more species and more populations of each species) should be conducted to establish whether *Acacia* species living on coastal rhizobia would be more vulnerable to drought. Results of such experiments would have broad implications for tropical *Acacias* in the region under climate change. In Queensland and the Northern Territory, there are 286 and 180 *Acacia* species respectively (Maslin 2001).

Future cross-inoculation experiments should also be accompanied by studies examining climate effect (e.g. temperature and moisture) on the growth and reproduction of the two sources of rhizobia, nodulation and symbiotic effectiveness. The different influences over symbiotic effectiveness due to inland and coastal rhizobia in the drought experiment possibly reflected the climatic differences between the coast and inland areas, and hence might continue to evolve under climate change. Some research effort has been devoted to examining the effect of climate change on plant-fungal associations and on fungi found in the natural ecosystems but the amount of literature devoted to investigating the effect of climate chagne on rhizobia in the natural ecosystems appears relatively sparse.

6.2.4 Germination response to heat

In the natural environment, the viability, physical dormancy and the germination of seeds of Fabaceae could be affected by fires of different intensities, higher post-fire soil temperatures due to altered soil thermal properties, very high soil temperatures in summer of different durations, different incubation temperatures representing various seasons (after application of dormancy-breaking temperatures), and their interactions with soil depths (Auld and O'Connell 1991; Auld and Denham 2006; Ooi *et al.* 2009; Santana *et al.* 2010). While inland *Acacias* were found to have a lower optimal seed germination temperature than coastal *Acacias* based on the experiment by Congdon *et al.* (in prep.), the treatments applied were steady heat for a few minutes only. To predict how climate change affects seed germination, the aforementioned natural variability must be accounted for in future experiments. The natural temporal and spatial distribution of seeds in various soil depths must also be surveyed to provide the background knowledge of seed bank dynamics (Auld and Denham 2006). To expand the discovery that heat sensitivity and tolerance levels differ between the four inland and coastal *Acacia* species, future fire regime investigations on tropical savannas should also include heat duration and temperature at different soil depths. Such knowledge is vital to understanding the biological significance of the changes in climatic parameters such as fire frequency and intensity, extreme heat and mean seasonal temperature in terms of their effects on seed germination, mortality, and seed bank persistence in a savanna woodland.

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Appendix 1. Mean (± 1 S.D.) biomass of four *Acacia* species, and under two rhizobial sources and two watering regimes in the drought experiment

Δ refers to changes from day 0 to 56. EL – *A. elachantha*. Rm – *A. ramiflora*. Cr – *A. crassicarpa*. Ho – *A. holosericea*. Number of replicates in parentheses.

Species	Rhizobial source	Watering regime	Δ Plant biomass (g)	Δ Stem biomass (g)	Δ Foliage biomass (g)	Δ Root biomass (g)	Δ Nodule dry weight (g)
Cr	Coastal	Normal	20.236 ± 0.946 (4)	3.374 ± 0.330 (4)	10.640 ± 0.507 (4)	3.226 ± 0.843 (4)	0.988 ± 0.059 (4)
		Drought	8.804 ± 0.698 (4)	0.856 ± 0.066 (4)	4.398 ± 0.370 (4)	3.226 ± 0.843 (4)	0.324 ± 0.081 (4)
	Inland	Normal	22.496 ± 3.494 (5)	3.389 ± 0.687 (5)	13.071 ± 1.882 (5)	5.031 ± 1.407 (5)	1.004 ± 0.119 (5)
		Drought	10.640 ± 2.113 (5)	1.115 ± 0.329 (5)	6.008 ± 1.394 (5)	3.035 ± 0.994 (5)	0.482 ± 0.145 (5)
Ho	Coastal	Normal	20.766 ± 0.728 (4)	3.143 ± 0.369 (4)	11.465 ± 1.242 (4)	5.462 ± 0.896 (4)	0.695 ± 0.072 (4)
		Drought	7.954 ± 2.481 (5)	0.839 ± 0.234 (5)	4.425 ± 1.295 (5)	2.411 ± 0.889 (5)	0.279 ± 0.135 (5)
	Inland	Normal	21.405 ± 3.196 (5)	3.035 ± 0.748 (5)	11.211 ± 2.085 (5)	6.480 ± 1.457 (5)	0.680 ± 0.207 (5)
		Drought	10.252 ± 1.951 (5)	1.012 ± 0.257 (5)	5.494 ± 0.808 (5)	3.421 ± 0.864 (5)	0.323 ± 0.078 (5)
EL	Coastal	Normal	12.096 ± 6.244 (3)	1.870 ± 0.808 (3)	6.034 ± 2.691 (3)	3.774 ± 2.382 (3)	0.423 ± 0.378 (3)
		Drought	3.821 ± 2.314 (3)	0.747 ± 0.516 (3)	1.934 ± 1.106 (3)	1.089 ± 0.715 (3)	0.053 ± 0.060 (3)
	Inland	Normal	11.928 ± 3.108 (4)	2.271 ± 0.619 (4)	6.213 ± 1.793 (4)	3.184 ± 1.151 (4)	0.261 ± 0.177 (4)
		Drought	6.003 ± 0.888 (4)	0.775 ± 0.170 (4)	3.199 ± 0.407 (4)	1.923 ± 0.585 (4)	0.107 ± 0.026 (4)
Rm	Coastal	Normal	1.970 (1)	0.452 (1)	1.150 (1)	0.354 (1)	0.014 (1)
		Drought	1.430 ± 0.545 (2)	0.270 ± 0.081 (2)	0.814 ± 0.394 (2)	0.347 ± 0.069 (2)	0 (2)
	Inland	Normal	7.615 ± 3.279 (5)	2.028 ± 0.632 (5)	3.256 ± 1.165 (5)	2.123 ± 1.384 (5)	0.198 ± 0.181 (5)
		Drought	3.059 ± 1.066 (5)	0.616 ± 0.195 (5)	1.430 ± 0.394 (5)	0.976 ± 0.521 (5)	0.036 ± 0.042 (5)

Appendix 2. The mean (± 1 S.D.) relative growth rate, net assimilation rate, specific foliage area and foliage mass ratio of four *Acacia* species, and under two rhizobial sources and two watering regimes between day 0 and day 56 in the drought experiment

RGR – relative growth rate; NAR – average net assimilation rate; SFA – specific foliage area; FMR – foliage mass ratio.

EL – *A. elachantha*. Rm – *A. ramiflora*. Cr – *A. crassicarpa*. Ho – *A. holosericea*. Number of replicates in parentheses.

Species	Rhizobial source	Watering regime	RGR (mg DW gain g^{-1} PB d^{-1})	NAR_{mean} (mg DW gain m^{-2} foliage d^{-1})	SFA_{mean} (m^{-2} foliage kg^{-1} foliage DW)	FMR_{mean} (g foliage DW g^{-1} PB)
Cr	Coastal	Normal	58 ± 6 (4)	8173 ± 1183 (4)	12 ± 0.4 (4)	0.6 ± 0.03 (4)
		Drought	43 ± 3 (4)	7067 ± 1183 (4)	11 ± 0.6 (4)	0.6 ± 0.05 (4)
	Inland	Normal	73 ± 8 (5)	8671 ± 780 (5)	14 ± 3 (5)	0.6 ± 0.02 (5)
		Drought	64 ± 21 (5)	8600 ± 2828 (5)	12 ± 1 (5)	0.6 ± 0.04 (5)
Ho	Coastal	Normal	76 ± 6 (4)	13464 ± 1998 (4)	10 ± 1 (4)	0.6 ± 0.03 (4)
		Drought	51 ± 11 (5)	11319 ± 5406 (5)	10 ± 2 (5)	0.6 ± 0.04 (5)
	Inland	Normal	73 ± 15 (5)	13211 ± 3879 (5)	10 ± 1 (5)	0.5 ± 0.04 (5)
		Drought	65 ± 11 (5)	11149 ± 1812 (5)	11 ± 1 (5)	0.5 ± 0.05 (5)
EL	Coastal	Normal	72 ± 12 (3)	14111 ± 4203 (3)	10 ± 1 (3)	0.5 ± 0.03 (3)
		Drought	51 ± 14 (3)	9390 ± 3687 (3)	11 ± 1 (3)	0.5 ± 0.03 (3)
	Inland	Normal	71 ± 6 (4)	14641 ± 3438 (4)	9 ± 1 (4)	0.5 ± 0.01 (4)
		Drought	64 ± 5 (4)	12560 ± 1116 (4)	10 ± 1 (4)	0.5 ± 0.04 (4)
Rm	Coastal	Normal	50 (1)	7975 (1)	11 (1)	0.6 (1)
		Drought	39 ± 8 (2)	6085 ± 566 (2)	11 ± 0.4 (2)	0.6 ± 0.05 (2)
	Inland	Normal	70 ± 13 (5)	15904 ± 2246 (5)	9 ± 1 (5)	0.5 ± 0.06 (5)
		Drought	54 ± 9 (5)	10378 ± 2041 (5)	11 ± 1 (5)	0.5 ± 0.03 (5)

Appendix 3. The mean (± 1 S.D.) stem to root ratio, shoot to root ratio, specific foliage weight and foliage dry weight to wet weight ratio of four *Acacia* species, and under two rhizobial sources and two watering regimes at day 56 in the drought experiment

EL – *A. elachantha*. Rm – *A. ramiflora*. Cr – *A. crassicarpa*. Ho – *A. holosericea*. Number of replicates in parentheses.

Species	Rhizobial source	Watering regime	Stem to root ratio at day 56	Shoot to root ratio at day 56	Specific foliage weight at day 56 (g foliage WW m^{-2} foliage)	Foliage dry weight to wet weight ratio at day 56
Cr	Coastal	Normal	0.640 ± 0.032 (4)	2.741 ± 0.293 (4)	281 ± 30 (4)	0.327 ± 0.017 (4)
		Drought	0.288 ± 0.091 (4)	1.806 ± 0.540 (4)	150 ± 35 (4)	0.738 ± 0.188 (4)
	Inland	Normal	0.706 ± 0.176 (5)	3.417 ± 0.628 (5)	277 ± 15 (5)	0.337 ± 0.015 (5)
		Drought	0.410 ± 0.167 (5)	2.598 ± 0.800 (5)	166 ± 33 (5)	0.678 ± 0.213 (5)
Ho	Coastal	Normal	0.590 ± 0.129 (4)	2.760 ± 0.651 (4)	321 ± 38 (4)	0.401 ± 0.040 (4)
		Drought	0.376 ± 0.086 (5)	2.322 ± 0.449 (5)	138 ± 5 (4)	0.965 ± 0.038 (4)
	Inland	Normal	0.486 ± 0.144 (5)	2.281 ± 0.569 (5)	329 ± 28 (5)	0.382 ± 0.014 (5)
		Drought	0.316 ± 0.050 (5)	1.977 ± 0.236 (5)	139 ± 8 (4)	0.971 ± 0.045 (4)
EL	Coastal	Normal	0.593 ± 0.248 (3)	2.507 ± 0.995 (3)	358 ± 28 (3)	0.299 ± 0.06 (3)
		Drought	0.647 ± 0.177 (3)	2.591 ± 0.590 (3)	241 ± 63 (3)	0.410 ± 0.160 (3)
	Inland	Normal	0.763 ± 0.278 (4)	2.754 ± 0.540 (4)	382 ± 43 (4)	0.295 ± 0.040 (4)
		Drought	0.413 ± 0.08 (4)	2.154 ± 0.514 (4)	201 ± 32 (4)	0.615 ± 0.125 (4)
Rm	Coastal	Normal	1.25 (1)	4.435 (1)	228 (1)	0.297 (1)
		Drought	0.765 ± 0.092 (2)	3.088 ± 0.738 (2)	261 ± 37 (2)	0.305 ± 0.012 (2)
	Inland	Normal	1.068 ± 0.254 (5)	2.770 ± 0.650 (5)	279 ± 12 (5)	0.324 ± 0.019 (5)
		Drought	0.716 ± 0.215 (5)	2.367 ± 0.653 (5)	257 ± 10 (5)	0.306 ± 0.036 (5)