Sustainable Reintroduction of the Nitrogen Cycle Post Coal Mining Utilizing the Legume-Rhizobia Symbiosis

Nigel Fisher BSc (Hons)

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Declaration

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Nigel Fisher
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Abstract

Mining in general, and open cut mining in particular, is an inherently destructive process. Mining occupies a very small area of the Australian continent, but causes intense disruption to the environment when concentrated in relatively small regions where minerals outcrop, or are close to the surface. In the upper Hunter River Valley region of New South Wales, open cut coal mining currently directly covers 500 km$^2$ or $1/6$ of the upper valley. Revegetation strategies have moved from rehabilitation to past land use, usually grazing in the Hunter Valley, to reconstruction of native ecosystems, whether wooded or native grasslands. At the Mount Owen mine, mining consent requires reconstruction of approximately 220 ha of the Ravensworth State Forest, a dry sclerophyll forest ecosystem dominated by trees from the genus *Eucalyptus*. Additionally the mine owner and operator have decided to revegetate to forest or woodland the entire disturbance area of about 960 ha.

Topsoil transfer is the usual method of re-establishing native vegetation, but forest topsoil supply is inadequate to fulfil the stated goals. Spoil, the crushed rock from just below the soil horizons down to, and between, the coal seams, is not a medium conducive to plant growth. It is biologically dead, deficient in nitrogen and other plant available nutrients and not having been subjected to soil building processes, lacks any structure. To support a native forest ecosystem, with all its constituent vegetation layers, spoil needs to be physically amended with soil replacement media, preferably forest topsoil, but other materials such as pasture subsoil, chitter (coal washing waste) and, or biosolids (dried sewerage sludge). Nutrients added as fertilizers have been the traditional method of overcoming soil nutrient deficiencies. But this is an expensive, inefficient practice that can result in pollution from leaching and runoff. One method that can be used to circumvent these problems is the addition of soil microbes that mediate the input of plant available nutrients from the soil medium. As nitrogen has been identified as being particularly deficient in this system, the re-introduction of native legumes with their rhizobial symbionts was seen as a sustainable method of supplying nitrogen to the reconstructed forest ecosystem.

To this end, root nodules from native legumes growing in the Ravensworth State Forest, Mount Owen mine rehabilitation areas and Donaldson mine, further down the valley, were collected, sterilized and cultured. Thirty-four bacterial cultures were
subjected to culture-dependent and molecular identification. Twenty-three isolates were confirmed as nodulators by their ability to re-nodulate *Macroptilium atropurpureum*.

Using PCR and targeting a 500bp fragment of the 16s rRNA gene seven members of the genus *Rhizobium* were identified, including three of the same strain, and two from the genus *Agrobacterium*. Seven isolates were identified as not having been previously described. Three of these exhibited high homology to the genus *Pseudomonas* and four were identified from the genus *Burkholderia*. The isolate that was chosen for pot trials and the field inoculum was later identified as belonging to the genus *Burkholderia*, which at the time of identification had not been previously reported in Australia.

These nodulating cultures were inoculated onto seedlings of six legumes that were to be used in the revegetation efforts at Mount Owen mine and tested for specificity and effectiveness in promoting growth in a series of controlled environment trials. The legume species included the dominant middle storey small tree, *Acacia parvipinnula*, four shrubs, *Indigofera australis*, *Daviesia ulicifolia*, *Acacia amblygona*, *Pultenaea retusa*, and a creeper or twiner, *Hardenbergia violacea*. Specificity was determined by the ability to nodulate, with functional nodules scored by observation of a rust red colour indicating the presence of leghaemoglobin. Effectiveness of growth promotion was determined by shoot dry weight and its ratio with nodule dry weight. Results showed marked variation in host-symbiont specificity and significant differences in the ability to promote growth of the legume hosts. *A. parvipinnula* proved to be the least specific host (nodulated by 22/23), while *D. ulicifolia* (nodulated by 13/23) and *P. retusa* (nodulated by 8/23) were the most specific. Bacteria also displayed specificity with six isolates able to form functional nodules on all six hosts. The remaining isolates were either unable to nodulate all of the hosts or formed non-functional nodules. No single isolate proved to be a highly effective growth promoter on all host legumes. For reasons of expediency in progressing the project, the first isolate that formed functional nodules on all hosts was chosen as the field trial inoculum.

The ability of the selected inoculating isolate to promote the growth of a simulated community of plants, including the six test legumes used for specificity testing, found in the Ravensworth State Forest when grown in a range of soil replacement media was then tested in shade house and field trials. The shade house trial demonstrated the ability of the inoculum to successfully increase the growth of the plant community, including in media with a resident rhizobia population, one potential
obstacle to successful inoculum establishment. Under field conditions at the Mount Owen mine, inoculation of the six host legumes with the selected isolate successfully increased survival and dry weight of five of the species, albeit with some interaction between host legumes and soil replacement media.

In conclusion, a number of new soil dwelling bacteria have been cultured and warrant further investigation, including a novel strain of nodulating *Burkholderia*. It was demonstrated that specificity exits between the native legumes and nodulating symbionts tested, with significant differences in ability to nodulate and promote plant growth. Further testing of specificity for other commonly found legumes is warranted. Investigation of the efficacy of multi-isolate inocula versus single isolate inoculum trialled here to increase effectiveness and to facilitate the re-introduction of native nodulators is also suggested. Successful inoculation was shown to enhance revegetation efforts by increasing survival and plant growth in a range of soil replacement media.
Chapter 1.

General Introduction
1.1 Introduction

Humanity has always modified the environment. From hunter-gatherers and early tool-makers leading to the extinction of mega-fauna, right through to the extraction of stone for houses, monuments, cities and minerals for our modern industrial society, slash and burn agriculture and use of fire to maintain grasslands, to the monocultures of modern industrial farming, we constantly change and modify the environment around us to suit our purposes. Conversion of natural ecosystems into human dominated ecosystems is currently the biggest threat to biodiversity (Dobson et al., 1997) and the ecosystem functions that they provide such as water filtration, carbon storage and pollution remediation (Crawford et al., 2005). Not only do human activities impact on all ecosystems world wide, especially terrestrial ecosystems, but the impact and the rate of impact is increasing. Estimates of human impact 15 years ago placed between one-third and one-half of the Earth’s land surface as having been modified by human activity (Vitousek et al., 1997). More recent literature estimates between 75% and 83% of the land surface has been impacted to some degree (Hobbs et al., 2009). In Australia approximately 54% of the land surface is currently affected by a single land use, grazing (Bell, 2001). The total human impact on continental Australia is therefore higher still.

Arguably the most visible impact of these modifications is upon vegetation, while equally importantly, but less visible is the impact upon the soil. Soil is both the basis of terrestrial life and the largest reservoir of biodiversity in terrestrial ecosystems if not on the planet (Doran and Zeiss, 2000; Crawford et al., 2005). Increasingly, there is recognition that the vegetation and soil communities are intimately linked, providing both positive and negative feedbacks on growth, nutrient and energy flows (Wardle et al., 2004).

1.2 Restoration Ecology

Recognition of the damages inflicted by human activity has lead to increasing efforts to rehabilitate, reclaim or restore the ecosystems in question. This has lead to a new branch of science, restoration ecology. This new discipline is the integrated science that seeks to inform the practice of landscape and ecosystem repair with scientific principles leading to self-sustaining ecosystems (McDonald and Williams, 2009). As an integrated science, restoration ecology uses many principles that have been tested elsewhere, especially ecology and applies them to reclaiming, rehabilitating or restoring an area,
landscape or ecosystem. While terms such as reclamation, rehabilitation and restoration have in the past been used interchangeably, or had different connotations depending on author (Hobbs and Norton, 1996), definitions have become settled with debate and time. Each of these terms now represents a level of intervention that reflects the damage sustained by the target system, the cost of intervention (Holl and Howarth, 2000) and the goals of the intervention as they sit within the wider framework of social, political, economic and cultural aspects (Higgs, 2005).

Reclamation revegetates an area, but not necessarily to any pre-existing condition (Bradshaw, 1997). This may be the only option for a severely damaged area. Rehabilitation aims to improve the condition of a selected area, but not necessarily to the original state. Restoration can be broadly defined as the manipulation of a disturbed habitat or ecosystem to a desired condition (Walker et al., 2007), which may also include reclamation or rehabilitation. But in its strictest sense, restoration aims to return the selected area to a state pre-disturbance based upon a reference site, accurate historical records or inferred from fragmentary data (Bradshaw, 1997; Lake, 2001). The system then becomes self-sustaining, without requiring further manipulation (Zink and Allen, 1998). This is best achieved with ecosystems that are not greatly damaged and have nearby areas that are pristine or near pristine that can act as a source for flora and fauna.

Whatever the starting point for intervention, whether a totally bare landscape such as occurs post mining, or repairing damaged ecosystems where vegetation is pre-existing (eg. grazed woodlands), time is required. If left to natural processes, primary and secondary successions can take long periods of time, from decades to centuries to return a landscape to a state pre-disturbance (Dobson and Bradshaw, 1997). The aim of restoration ecology is to accelerate the processes of natural succession and place the landscape in question on a trajectory that allows it to build towards a self-sustaining and mature ecosystem. However, the conditions that formed the original ecosystem may not be replicated in the future and the resultant ecosystem may not exactly match the system pre-disturbance. The original ecosystem may have been an area of contiguous forest or grassland that when restoration is attempted is a fragment in a mosaic of agricultural and urban land uses. This will alter hydrology and migration of animals and plants into, and out of, the restored fragment. Larger environmental factors such as climate change may alter rainfall regimes representative of the original ecosystem. Many of these factors, including knowledge of the full complement of species that comprised the
original ecosystem as alluded to above, are beyond the control of the restoration practitioner. But, by ensuring that key ecological functions (rather than specific organisms) are restored by introduction or re-introduction of organisms that perform these functions, thresholds that would otherwise prevent succession from proceeding along a desired trajectory can be overcome (Suding and Hobbs, 2009). One key area where this is applicable is biogeochemical cycling. Knowledge of the full extent of soil micro-biodiversity and their interactions with plants and other soil dwelling microbes might not be fully understood at present, but players in key functions such as the nitrogen and phosphorus cycles have been explored and can be introduced, if needed in restoration projects.

1.3 Impacts of Mining

Mining on a global scale occupies 1% of the land surface (Walker and del Morale, 2003), while in Australia it is reported to occupy only a much smaller percentage, namely, less than 0.05% (Bell, 2001). However, mineral ore bodies are not distributed evenly and outcrop in relatively localised areas, where mining becomes concentrated.

Open cut mining is by nature a destructive process, resulting in total devastation of the area subjected to mining and severe disturbance to the surrounding areas needed to support the mining operations. Large volumes of overburden (the material that must be excavated before the economic ore is reached) are created resulting in spoil heaps consisting of crushed rock that has not been subjected to the normal processes of soil formation (Charnock and Grant, 2005). With mining increasingly being recognised as a temporary use of the land (Dobson et al., 1997; Evans, 2006) there is a requirement, in developed nations at least, that a revegetation or restoration programme be included as an integral part of the mining process.

1.3.1 Characteristics of Spoils

Spoils have a number of physical impediments to successful plant establishment including coarse particle sizes, increased bulk density, lack of particle aggregation, surface crusting, unusual pH (acid or alkali), toxic levels of elements, inability to hold water, lack of water penetration, and low organic matter content (Bell and Ungar, 1981; Anderson et al., 1989; Bradshaw, 1997; Rokich et al., 2001; Maiti, 2006). Which of these factors or combination of factors affect the revegetation effort are largely
determined by the geology of the overburden, and can be highly variable between mining provinces, or even within the one mine. For instance, overburden at a single open cut coalmine in Ohio, USA, had a pH of 5.6 (Anderson et al., 1989), while another mine in West Virginia spoil had a pH of 3.3 (Ning and Cumming, 2001). In Wales, UK, pH varied from 4.5 to 8.2 over 10 sites (Bending and Moffat, 1999) and overburden in Hunter Valley mines in New South Wales, Australia had reported values of 8.5 to 9.0 (Charnock and Grant, 2005), to as low 2.5 to 3.0, leading to spontaneous combustion of sulphides (M. Cole pers comm.)

Amelioration of the physical obstacles can be achieved by the addition of suitable soil amendments and various materials have been trialled. Extremes of pH can be overcome using lime and gypsum (Fisher et al., 2000; Wilden et al., 2001), flyash (Blechschmidt et al., 1999), pulverised refinery fines (Chu and Bradshaw, 1996) and chitter (Charnock and Grant, 2005). Chitter will also help prevent surface crusting and assist in water penetration and seedling emergence.

Increasing the productivity of mine spoils has been trialled by the incorporation of organic treatments that attempt to increase soil organic carbon, improve water retention and lower bulk densities, all serious impediments to plant growth. Organic amendments primarily used have been sewage sludge (also called biosolids) due to their availability in bulk (examples of the many papers dealing with this aspect in the literature: Borgegård and Rydin, 1989; Brown et al., 2003; Halofsky and McCormick, 2005; Mercuri et al., 2006). Other treatments have included various mulches and composts composed of plant litter (Owen and Harris, 2001), sawdust and manures (Hetrick et al., 1994; Coyne et al., 1998), straw and bitumen (Reynolds and Lang, 1979).

1.3.1.1 Nutrient Status of Spoils

One of the major obstacles to establishing vegetation on mine spoils are the low levels of plant available nutrients, especially nitrogen (Bloomfield et al., 1982; Bradshaw, 1997; Chu and Bradshaw, 1996; Coyne et al., 1998; Singh et al., 2000; Tordoff, 2000). Newly created primary substrates such as spoils, glacial debris, dunes and volcanic material, contain most of the rock-derived nutrients that they ever will such as phosphorus, magnesium, calcium and potassium (Vitousek, 1997). Given sufficient time, in the order of several decades to centuries, weathering will release the labile components of these minerals and make them biologically available (Vitousek, 1997).
With time the main sources of natural nitrogen input, atmospheric deposition (20 million T yr\(^{-1}\) globally) and biological nitrogen fixation (when suitable organisms colonise the new substrates, 140 million T yr\(^{-1}\) globally) will increase the available nitrogen (Vitousek and Farrington, 1997; Galloway et al., 2004).

Mine operators have attempted to overcome this limitation to successful revegetation by applying fertilisers and seeding directly into spoil heaps with variable results (Brown and Grant, 2000; Mercuri et al., 2006). Even if revegetation attempts are successful, and often using introduced plant species, the downside of fertiliser use is high nutrient runoff; up to 98% depending on soil substrate and rainfall (Wilden et al., 2001) that may lead to eutrophication of surrounding waterways and groundwater and the acidification of the soil itself (Crews and Peoples, 2004). Using fertilisers also has the added costs of on-going application given that the positive effects are often short lived.

### 1.3.2 Topsoil Transfer to Improve Spoil Suitability for Vegetation

A commonly used method that overcomes both the physical and nutrient limitations of mine spoils is topsoil transfer and this has been applied to mine site rehabilitation for a considerable period of time (Bradshaw, 1997).

This process removes and separates the topsoil from the remaining overburden that will comprise the spoil heap. Once the spoil heap has been created, topsoil is then respread. This provides a layer of material containing organic matter, microbes and a seed bank that greatly enhances the revegetation (Hannan, 1995; Huxtable et al., 2005; Nussbaumer, 2005). When suitable topsoil is available, this method is the most successful for the reintroduction of native seed, soil fauna and flora and microbes, including nitrogen-fixing rhizobia. Topsoil may need to be stockpiled before spreading, especially early in the life of a mine for up to five years in some cases (Kundu and Ghose, 1997). This can have detrimental effects on the health of the soil leading to changes in physical soil characteristics, loss of seed viability and a reduced soil microbial community (Newman, 1996; Kundu and Ghose, 1997; Huxtable et al., 2005). These effects can be apparent even after as little as six months of stockpiling (Harris and Rengasamy, 2004).

Availability, and or quality of topsoil can be an issue. Often, the topsoil that has been excavated is itself not of a high quality for the purposes of revegetation to a native ecosystem. The land use prior to mining, such as agriculture, may have resulted in
extensive weed infestation or erosion may have removed much of the actual A horizon. In such cases alternatives must be found. Stripping off the “topsoil” and using the B or C horizons as capping material (Kundu and Ghose, 1997), or transferring in topsoil from another site are examples of alternative options (Hözel and Otte, 2003). Subsoils, that is B and C horizons, may differ substantially both chemically and physically from the A horizon. For instance, they may be dispersive in nature, that is, have a high clay and sodium content, making them easily eroded, poorly drained and poorly aerated (Young and Young, 2001). In addition, these soil layers may be biologically impoverished in comparison to the topsoil layer (Will et al., 2010).

### 1.3.3 Legumes and Rhizobia

Restoration of any damaged ecosystem must begin with the primary producers, the plant community (Davy, 2008), and intrinsic to this community, plant-soil interactions must be taken into account (Eviner and Hawkes, 2008). Ecological science has begun to realise that plant and soil microbial communities have a profound influence upon each other, with positive and negative feedbacks between the two (Wardle et al., 2004; Eviner and Hawkes, 2008; Lambers et al., 2009). Soil bacterial communities vary with soil type and pH but also with plant species, age and spatially in relation to root structure (Marschner et al., 2004). Plants themselves act as soil conditioners, reducing erosion, lowering soil bulk density, adding organic matter via litter and turnover of roots and exudation of organic compounds (Walker and del Morale, 2008).

Legumes have been cultivated for their own intrinsic food value for millennia, and the significance of their nitrogen-fixing capabilities have been recognised for over 150 years. The use of legumes and rhizobia for nitrogen accumulation on nutrient deficient mine spoils is itself not new, going back at least to the late 1950’s (Jefferies et al., 1981). What has changed are the intended outcomes for restoration of mined land. Until more recently, when restoration was practised at all, land was restored to agriculture or for some other economic benefit and in many cases pasture legumes such as *Trifolium* spp. were planted to build nitrogen capital in nitrogen impoverished spoils (Bradshaw, 1997; Dobson et al., 1997). If post mining, future land use is to be agriculture, then commonly used pasture or crop legumes can continue to be used. But if the future land use is legislated to be restoration or reconstruction of native vegetation, then more appropriate legume species must be used.
Aside from the primary role of increasing nitrogen within the soil, legumes and their rhizobial symbionts improve soil condition and promote the growth of non-leguminous plants and soil flora and fauna (Antoun et al., 1998; Dakora, 2003). The exudation of carbon compounds directly increases soil microbe activity, while organic acid and proton production enhances mineralization of nutrients directly from the soil (Dakora, 2003). Legumes produce phenolic compounds, flavonoids that suppress microbial pathogens and further mineralize nutrients (Aoki et al., 2000; Dakora, 2003). Rhizobia, both as free-living saprophytes and in their symbiotic form also produce siderophores, suppress pathogens and have been reported to solubilize phosphorus (Rodríguez and Fraga, 1999; Dakora, 2003).

Australian soils are known for their relatively low levels of nitrogen due in part to their comparatively old age (Young and Young, 2001). Native legumes have come to be one of, if not the dominant component of the vegetation, whether in terms of numbers of species or of biomass (Groves, 1994). Increasingly the recognition of the importance and, or desirability of conservation of native vegetation systems has lead to the requirement for restoration of these systems and, given their importance in native ecosystems, the use of native legumes. Concomitant with this is the realisation that as with agricultural legumes, native legumes can have specific symbiotic requirements, and that growth and survival will be improved by use of effective rhizobial symbionts (Murray et al., 2001; Thrall et al., 2005).

Rhizobia have been introduced into agricultural soils as inoculants for over 100 years (Stephens and Rask, 2000; Herridge et al., 2002). Agriculturally important legumes such as Trifolium spp. have been introduced to Australia since European settlement and with the specific nature of the symbiosis between Trifolium and R. leguminosarum, as examples, presumably no compatible rhizobia were resident in Australian soils prior to this introduction (Drew and Ballard, 2010). Since then, many inoculant strains have become naturalised in agricultural soils (Denton et al., 2002), and with clearing of native vegetation, the extent of land area that can be definitively said to harbour only native rhizobia is decreasing.

Clearance of native vegetation and establishment of introduced legumes for pasture improvement has implications for the survival and persistence of native rhizobia. The ability of rhizobia to survive for extended periods as free-living saprophytes appears to be species related. Nodulating rhizobia have been found in soils where their host has not been cultivated for over 30 years, and in some cases with no
known record of their host having been cultivated in that soil (Bloem and Law, 1999; Mendes et al., 2004). In Australia, native rhizobia have not been recovered from soils that have had native vegetation cleared and used for grazing for extended periods, that is since European settlement (Thrall et al., 2001 and 2005).

To the best knowledge of this author, there is only one rhizobial inoculant for native legumes commercially available, Wattle Grow™, and this has been formulated for *Acacia* species native to South-Eastern Australia. Therefore, acquiring native rhizobia for a wider variety of native legumes, especially Fabaceae, requires sourcing from remaining areas of native vegetation, such as national parks, or wilderness reserves. In terms of revegetation programs, whether for mining or other areas, proximity to such areas may well determine the feasibility of this approach. In the absence of locally available rhizobia, the use of species from outside the area may then be required.

1.4 The Hunter Region and Mount Owen Mine

1.4.1 Location

The Hunter region (also referred to as the Hunter Valley) is located 150 km north of Sydney, extending approximately 200 km inland from Newcastle on the coast, New South Wales’ second largest city (Fig 1.1).

In the central Hunter region, there are more than 15 open cut coalmines or mining complexes (Fig 1.2) occupying approximately 500 km² or 1/6 of the central Hunter Valley. Mining Complexes are mines grouped together by the mine owners or operators and referred to by one name, for instance Coal & Allied’s Hunter Valley Operations No. 1 consists of four open cut pits, that were originally managed as four separate mines. Mining leases are held over a further 110 km² (Evans, 2008).

The Ravensworth State Forest is one of the largest surviving remnants of the Central Hunter Ironbark-Spotted Gum-Grey Box Forest (*Eucalyptus crebra* - *Corymbia maculata* - *Eucalyptus molucanna*) and has been classified as an endangered ecological community by the New South Wales Department of Environment & Climate Change (DECC) since 2001. Land clearing mainly for agriculture and grazing has resulted in a reduction in the extent of forest to about 18,000 ha or 29% of its pre-European settlement distribution. Its occurrence in the region includes 34 remnants over 100 ha or more and 1000 very small patches of less than 10 ha in size, indicating that the
remaining distribution is very fragmented (Peake, 2006). The increase in coal mining, particularly open cut mining has placed remnants under further pressure.

Floristically, the Central Hunter Ironbark-Spotted Gum-Grey Box Forest is described as an ecological community that grades from forest to open woodland found on soils derived from Permian sediments. The dominant canopy species name the community, but co-dominants include Broad-leafed Ironbark (E. fibrosa), and Forest Red Gum (E. tereticornis).

An upper middle storey of small trees generally consisting of Allocasuarina luehmannii or Acacia parvipinnula may be found in some remnants. The middle, shrub layer may grade from totally absent to sparse through to moderately dense, depending on the impact of human activity. Important shrub species include Acacia falcata, Daviesia ulicifolia, Pultenaea spinosa, Breynia oblongifolia, Hakea sericea and Bursaria spinosa (Peake, 2006). Other commonly found (leguminous) shrubs include Acacia amblygona, Indigofera australis and Hardenbergia violacea.

Ground cover can also be sparse to moderately dense, and includes numerous forbs, some grass species and fewer ferns, sedges and other herbs. Selected examples include Glycine clandestine, G. tabacina, Dianella revoluta, Desmodium varians, Cymbopogon refractus, Dichondra repens and Cheilanthes sieberi (Peake, 2006). A species list (from the DECC website based upon Peake, 2006) is included in Appendix 1.

This Endangered Ecological Community grades into the Lower Hunter Spotted Gum-Ironbark Forest to the south and southeast of its distribution. This vegetation community differs in aspects of its floristic composition, but contains considerable overlap in middle and understory species. Major remnants of the Lower Hunter Spotted Gum-Ironbark Forest occur around the Ashtonfield-Beresfield areas where supplemental collections of rhizobia were undertaken.
Fig 1.1 Location of the Mount Owen Mine in the Hunter coalfields of New South Wales, Australia. The mine is located approximately 100 km north-west of Newcastle, which is itself about 150km north of Sydney.
Adjacent to the Ravensworth State Forest, the Mount Owen mine is located in the central Hunter valley, 20km northwest of Singleton (Fig 1.2). Before mining commenced in 1994, the forest covered an area of about 450 ha. Mining consent has allowed mining operations to pass through and destroy about half or 220 ha of forest. As one condition of the mining consent, the owners and operators of the mine must reconstruct and restore the original area of forest with the aim to “maintain the diversity and genetic resource of the flora currently existing within the locality” (Plan of Management, Mount Owen Mine, 1995).

**Fig 1.2** Aerial photograph of the central Hunter Valley, bounded by Singleton to the south, Muswellbrook to the north and Denman to the west. Mount Owen mine and Ravensworth State Forest are located approximately half way between Singleton and Muswellbrook (red circle). Grey areas are other open cut coalmines. Photo courtesy of Google Earth.
This was the first time such consent conditions had been imposed upon a mine in the Hunter Valley (Plan of Management, Mount Owen Mine, 1995). Additionally, the current owners of the mine, Xstrata Coal NSW P/L, have undertaken to revegetate the entire disturbance area of 960 ha to woodland or forest ecosystem (Fig 1.3).

As stated in Section 1.3.2, topsoil transfer is the most effective method of revegetating spoil heaps, amending the physical conditions of the spoil and reintroducing native vegetation through the seed bank and soil microbes necessary for nutrient cycling. The entire area of forest topsoil must be used for the restoration of the spoil heaps in accordance with the mining consent. Thus an additional area of approximately 700 ha must be revegetated without the benefit of forest topsoil. This has led to investigations into the efficacy of alternative soil replacement media as substrates for a forest ecosystem in a project funded by the Australian Coal Association Research Council (ACARP), ACARP C12033 “Topsoil Substitutes and the Sustainability of Native Forest Ecosystem in the Hunter Valley”. It was specified that any such soil replacement medium must be readily available in bulk, and if not available on site, able to be transported economically to site. Consequently mine spoil, pasture subsoil, chitter and biosolids and combinations thereof were trialled, giving a number of treatments plus forest topsoil as a reference.

1.4.2 Soil Conditions at the Mount Owen mine

Spoil at the Mount Owen mine consists of interbedded sandstones and mudstones. These layers have low levels of some essential plant nutrients and can have an alkaline pH, up to 9.7. As with all spoils and new substrates, these bedrock materials are biologically dead, initially lacking in soil microbes such as fungi and rhizobia necessary for the acquisition of phosphorus and nitrogen respectively.

Subsoils in general have not been used extensively in mine rehabilitation, probably due to the mechanical extraction of subsoil and its inclusion in either overburden or topsoil fractions. It can have advantages as a medium for revegetation as it will not contain a seed bank, but will still have some characteristics of topsoil, including soil microbes. At Mount Owen, pasture subsoil was used in rehabilitation trials to avoid the weed and pasture species that dominated the flora of the pasture areas and consisted of the material contained in a layer 10cm to 20cm under the pasture topsoil.
Chitter is a waste product of the coal washing process whereby substandard material is removed from the coal. It consists of crushed rock strata immediately above, below and imbedded within the coal seams as well as coal of inferior quality that has been industrially washed and is a material readily available at all coal mines. Generally it is very coarse in nature leading to poor consolidation and air voids. This can allow oxidation, high acidity and salt levels. However, it has been reported to reduce the formation of surface crusting that occurs on the spoil and subsoil after wetting.

Fig 1.3 Aerial photograph of the Mount Owen Mine and Ravensworth State Forest. Illustrated is the pre-mining forest area (green outline) and the entire mining disturbance area (red dotted line).
Biosolids, or sewage waste, is another readily available waste product sourced from the nearby Branxton plant of the Hunter Water Corporation that provides a rich source of organic nutrients and has been used as a soil ameliorant in both mine rehabilitation and agriculture in the past (Brown et al., 2003; Cooper, 2005; Halofsky and McCormick, 2005; Paschke et al., 2005).

With the exception of the pasture subsoil, these materials are not soils and have not been subjected to soil forming processes such as weathering and the accumulation of organic matter. As a consequence there are abiotic factors that include but are not restricted to pH, structure and mineral content that may influence how these materials support revegetation efforts. Additionally, there are biotic factors that will impact plant growth. Without suitable soil microbes, many of the biogeochemical cycles responsible for releasing or acquiring the mineral elements from the soil, or in this case soil replacement media, necessary for sustainable plant growth will not occur at all, or will only be partially present. Thus inoculation with suitable rhizobia symbionts will be necessary to ensure the successful revegetation of spoil dumps and ensure a sustainable nitrogen cycle is established.

1.5 Objectives of this Study

Amelioration of mine spoil, a medium not readily conducive to supporting the full number of plant species found in the forest ecosystem used as a reference, requires both physical and biological amendment. Physical amendment was investigated in ACARP C12033, and this study seeks to follow on from that study and establish sustainable nitrogen cycles in soil replacement media.

Specific objectives, therefore are:-

1) To collect, culture and identify a library of native rhizobial bacteria from areas of native remnant vegetation. Rhizobia will be harvested from root nodules from a wide variety of native legumes that are commonly found within firstly, the Mount Owen mine and Ravensworth State Forest, and expanded to include other areas of native vegetation if required.

Culturing will be performed using standard practices devised for rhizobial bacteria, that is, plating on yeast mannitol agar (Vincent, 1970).
Identification will be performed using culture-dependent methods (for example colony morphology and generation time), intrinsic antibiotic resistance to distinguish between cultures and finally use of 16S rRNA gene and PCR to identify the cultured bacteria.

2) To identify any specificity issues and determine if there are differences in efficiency in terms of nitrogen fixation between the native rhizobia and selected native legumes that will be used in the restoration of the native forest ecosystem at Mount Owen and within the Hunter Valley.

This will be performed through a series of growth room experiments under controlled conditions of light, temperature, nutrient and water supply, where individual cultures will be inoculated onto the root systems of the selected native legumes. Specificity will be determined by the formation of functional root nodules. The efficiency of nitrogen fixation will be determined by a destructive harvest and subsequent dry weight of shoots.

3) From the above trials, a suitable rhizobium or rhizobia will be selected for use as an inoculum and to determine the efficacy of the combination in promoting plant growth in the soil replacement media identified in ACARP C12033 under controlled (shadehouse) and field conditions.

Shade house trials will be conducted at the University of Newcastle plant growth facility, with the field trials being undertaken at the Mount Owen mine. Results will be determined by destructive harvest of shoots for both inoculated and uninoculated plants in fully replicated, randomised experiments.
Chapter 2.

Identification of Bacterial Isolates
2.1 Introduction

The identification and differentiation of bacterial isolates collected from any complex environmental sample, whether airborne, aquatic or soil has always proved crucial to understanding bacterial ecology. The study of rhizobial symbionts is somewhat simplified because they can be collected from a distinctive plant organ, the root nodule.

Nodulating, nitrogen-fixing bacteria were first isolated and cultured in 1888 by M. Beijerinck, and the term rhizobia was coined by B. Frank in 1889 with the publication of the name *Rhizobium leguminosarum* (Young and Haukka, 1996). For the remainder of the 19\textsuperscript{th} and the majority of the 20\textsuperscript{th} century, all such nitrogen-fixing nodulating bacteria were grouped into the genus *Rhizobium*. By the early 1930’s, Fred et al., (1932) had classified rhizobia based upon their selective ability to nodulate various members of the legumes, their symbiotic partners. As more investigations were performed, this classification based upon cross-inoculation was shown to be inadequate in describing rhizobial diversity. In the early 1980’s, it was recognised that further subdivision was required, resulting in the genus *Bradyrhizobium* (Jordan, 1982) being established to distinguish the faster growing *Rhizobium* from the slower growing *Bradyrhizobium*. Since then a growing research effort has resulted in the establishment of several more genera, including *Mesorhizobium* (Jarvis et al., 1997), *Sinorhizobium* (Chen et al., 1988), *Azorhizobium* (Dreyfus et al., 1988) and *Allorhizobium* (de Lajudie et al., 1998) all of which belong to the α-proteobacteria subdivision of the prokaryotes. The ability to form effective N-fixing nodules with legumes has also been reported in another genus within α-proteobacteria, *Methyllobacterium* (Sy, et al., 2001) as well as other genera that belong to the β-proteobacteria subdivision including *Ralstonia* (Chen, et al., 2001; Rasolomampianini, et al., 2005) (note, the nodulating members of the genus *Ralstonia* has since been renamed and transferred to the genus *Cupriavidus* Vandamme and Coeyne, (2004)) and *Burkholderia* (Moulin, et al., 2001; Barrett and Parker, 2005; Rasolomampianini, et al., 2005; Garau, et al., 2009), and the genus *Pseudomonas* from the γ-proteobacteria (Benhizia, et al., 2004).

Identification of the new genera and species that have been recognised as root-nodulating symbionts largely has been achieved with the advent of molecular biology and the ability to target and sequence specific genes. The most commonly used gene for identification of bacteria is the 16S ribosomal RNA gene. This gene has been widely used because it appears to be present in all cellular based life and its function, and hence
its DNA sequence, has largely remained unchanged for billions of years making it equally as useful for ancient and more recently evolved organisms. The gene has a relatively small size of approximately 1540 base pairs allowing easy analysis. The DNA sequence is composed of regions that range from very highly conserved, less conserved to highly variable in nature (Stackebrandt, 2001).

These highly conserved regions of the 16S rDNA make it possible to utilise a small number of primers to identify many bacterial taxa (Stackebrandt, 2001). Many other genes such as the 23S and 5S rRNA genes, genes involved in DNA repair such as recA, or other DNA fragments that do not necessarily encode genes such as intergenic spacer regions, can and have been used for bacterial identification. The number of sequences from single gene types available for comparison do not match the thousands of 16S rDNA sequences that have been submitted to Genbank (run by the National Centre for Biotechnology Information), the EMBL (European Molecular Biology Laboratory), or the DDBJ (DNA Data Bank of Japan) (Salemi and Vandamme, 2003).

While molecular techniques have lead to culture-independent methods of bacterial identification, especially from environmental samples, there is still a need for culture dependent techniques. These make it possible to isolate, grow and maintain examples of pure cultures to study bacteria-bacteria or bacteria-plant interactions, or phenotypic features of bacterial colonies/cells. Indeed many of the newer genera of rhizobia were first investigated based upon observations of cell morphological characteristics. For instance members of the genus Bradyrhizobium were further investigated based on their slower growth on yeast mannitol mineral salts media, flagella arrangement and genera of host plants nodulated (Jordan, 1982).

The use of in vitro characteristics, while not usually diagnostic in and of themselves, can identify important secondary characteristics that aid in delineation between rhizobial isolates, or identification of non-rhizobial isolates. For instance, rhizobia are recognised as slow growing in relation to many common soil bacteria and form small round colonies. However, individual isolates of Rhizobium, Sinorhizobium and Mesorhizobium cannot be distinguished from one another on this basis alone. Gram staining separates bacterial isolates into Gram-negative and -positive groupings, and aids in ascertaining purity of a culture. This test will not however, separate rhizobia, nor will it distinguish between Gram-negative bacteria that may have been co-cultured.

Use of Intrinsic Antibiotic Resistance (IAR) to characterise different rhizobial strains has a long history, dating back to the early 1960’s (Davis, 1962; Graham, 1963
(cited in Josey et al., 1979)), and may be considered a classic method of characterisation.

The Antimicrobial disc Susceptibility Test (AST) method is a variation in antibiotic delivery, where instead of incorporating one or a few antibiotics into agar media in a series of Petri plates, paper discs with a known concentration of antibiotics are dispensed onto a lawn of the target isolate. The antibiotic diffuses into the agar creating a zone of inhibition (or not) dependent on the susceptibility or resistance of the isolate (Bauer et al., 1966). This technique tests a large number of antibiotics with a minimal number of plates. Intrinsic antibiotic resistance and the derivative antimicrobial disc susceptibility test have been utilized in numerous studies to demonstrate isolate heterogeneity in field studies examining soil microbial diversity, with different species of rhizobia including *Rhizobium leguminosarum* and *R. phaseoli* (Josey, et al., 1979; Benyon and Josey, 1980), *R. meliloti* (Antoun, et al., 1982; Jenkins and Bottomley, 1985) and *Bradyrhizobium* species (Date and Hurse, 1991). While this technique has not been employed as extensively in recent years due to the increased and more definitive use of molecular biology, there still remains a place for the use of IAR as it is relatively simple and inexpensive. Recently IAR has been used to characterise different strains of African *Bradyrhizobium* (Abaidoo, 2002) and Tunisian *Rhizobium* (Labidi, et al., 2003).

The definitive test of whether a bacterial isolate or culture can be classified as rhizobial is the ability to nodulate a suitable host. To this end, Vincent (1970) recommended re-inoculating any such culture or isolate onto a host legume (preferably the same one that the isolate was collected from to eliminate specificity issues) in a Koch’s Postulate style test of infectivity. This not only proves that a component of a bacterial culture is able to nodulate, it also allows for the re-isolation of the rhizobia responsible for nodulation.

For the purposes of this study, root nodules were collected from native legume hosts and investigated using a series of more traditional culture dependent methods before identification by molecular methods. The tests began with an examination of *in vitro* characteristics of cultures of root nodulating bacteria before investigating antibiotics as a method of separating duplicate species. Identification of cultures was performed by PCR of a 16S rRNA gene fragment. These were undertaken to identify indigenous rhizobia for preservation and re-introduction to soils via revegetation efforts. On the basis of these tests, isolates were selected for specificity testing in Chapter 3.
2.2 Materials and Methods

2.2.1 Isolate Collection and Growth

With the stated aim of the project being the re-introduction of native rhizobia, legume species native to the Ravensworth State Forest-Mount Owen Vegetation Complex were targeted for collection of root nodules. Collections were made from understorey and mid-storey species of the genus *Acacia* and the family Fabaceae.

Collections were made from three sites.

1) The Ravensworth State Forest, and

2) Rehabilitated areas of the Mount Owen open cut coalmine, both located approximately 100 km north west of Newcastle, in the upper Hunter Valley (32° 21’S 151° 06’E).

3) The Donaldson open cut coalmine, (32° 48’S 151° 36’E) approximately 30 km west of Newcastle in the lower Hunter Valley.

Root nodule bacteria were collected from 22 different species of native legumes from the three sites, with some duplication of host species between sites (Table 2.1). Nodules were collected from six species of native legumes at the Donaldson coalmine (designated as “B”), from 15 species from the Mount Owen mine rehabilitation sites (designated as “MO”), and from five species from Ravensworth State Forest (designated as “RSF”). One nodule from the Mount Owen rehabilitated site was collected from roots that became detached before being able to trace them back to the host plant, and was labelled MOX. The most likely hosts were *Glycine clandestina*, *Acacia decora* or *Acacia implexa* (Table 2.1).

Host species for nodule collection were chosen opportunistically by a random walk. Nodules were collected as host species were identified. Initial attempts made to collect from the Ravensworth State Forest proved to be of limited success. Much of the middle- and understorey were in poor condition due to grazing by native herbivores. Further, the soil conditions were extremely compacted making recovery of roots from the plants identified difficult.

Consequently, efforts at collection were made on a section of the spoil dump that had been rehabilitated in 1999 (4 years previously) with forest topsoil as part of experimental work performed by Nussbaumer (2006), for her PhD. The vegetation in this area consisted of a mixture of species derived from the residual seed bank within the topsoil, and seeded species. The seeded species included seven species of *Acacia* (*A.*
amblygona, A. decurrens, A. falcata, A. implexa, A. myrtifolia, A. parvipinnula and A. suaveolens) and five species of Fabaceae (Daviesia ulicifolia, Hardenbergia violacea, Indigofera australis, Jacksonia scoparia and Kennedia prostrata). The decompaction of this soil due to its excavation and subsequent respreading facilitated the excavation and collection of roots and root nodules.

An opportunistic collection was made at the Donaldson Coal Mine, near Beresfield. The vegetation surrounding this mine is classified as Lower Hunter Box-Ironbark Forest (see Chapter 1). As the middle- and understorey contains many species common to the two vegetation communities, that is the Lower Hunter Box-Ironbark Forest surrounding the Donaldson coal mine and the Central Hunter Ironbark-Spotted Gum-Grey Box Forest that the Ravensworth State Forest is a part of, it was decided to collect from this area from species found in both communities and from any other species identified.

Root nodules were located by excavating soil from around the base of each host legume. One root nodule per host plant was collected from identified species. Opportunistic collections were made from more than one plant for some hosts, at some sites (Table 2.1). When roots with nodules attached were located, they were collected, stored in plastic bags, kept moist with filter paper moistened with distilled water, and then transported to the laboratory. Nodules were then surface sterilised using the following procedure, modified from Marsudi et al., (1999).

Nodules were first washed in tap water to remove any obvious adhering soil transferred from the field and then placed into 1.5 mL Eppendorf tubes to which 500 μL of 5% H₂O₂ (v/v) was added and vortexed for 1 min. The H₂O₂ was removed before adding 500 μL of 70% ethanol and vortexed for 3 min. This step was repeated once before five consecutive washes in sterilized Milli-Q water.

Nodules were then either stored in a 1:1 glycerol:Yeast Mannitol Broth (YMB) solution pH 7, at -80°C or crushed using a glass or steel rod and the exudate streaked onto Yeast Mannitol Agar (YMA) plates, pH 7 and cultured at 28°C (Vincent, 1970). This process was performed under strict aseptic conditions (see Appendix 2 for YMA formula). Repeated subculturing on YMA plates was undertaken to obtain pure isolates. When pure isolates were determined to have been obtained, samples of each were grown for 36-48 h in YMB, pH 7, and were then stored at -80°C in a 1:1 glycerol: YMB solution.
In vitro Characterisation of Isolates

Several in vitro characteristics were studied to distinguish between isolates. Growth form of individual colonies was employed as an initial indication of the presence of rhizobia (Fig 2.1). Often colony growth form is not definitive, but can serve to eliminate cultures that are not of the desired type. Rhizobia as a group produce small, circular colonies with smooth edges, usually colourless to white, sometimes opaque. Rhizobia can produce mucilaginous, exopolysaccharide slime, especially when grown on YMA. Rhizobia in general are Gram-negative, straight rods that range in size from 0.5-1.0µm by 1.2-3.0µm (Garrity et al., 2001).

### Table 2.1 Host species and sites of root nodule collection. Ravensworth State Forest = RSF, Mount Owen mine rehabilitated site = MO, B = Donaldson coal mine located at Beresfield. Asterisks denote isolates that were lost in culture due to fungal infection.

<table>
<thead>
<tr>
<th>Host Species</th>
<th>Collection Sites</th>
<th>Host Plants Collected</th>
<th>Resulting Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acacia amblygona</td>
<td>MO</td>
<td>1</td>
<td>MOAamb</td>
</tr>
<tr>
<td></td>
<td>RSF</td>
<td>2</td>
<td>RSFAmb1 &amp; 2</td>
</tr>
<tr>
<td>Acacia cultriformis</td>
<td>MO</td>
<td>1</td>
<td>MOAcul</td>
</tr>
<tr>
<td>Acacia decora</td>
<td>MO</td>
<td>1</td>
<td>MOAdecor</td>
</tr>
<tr>
<td>Acacia implexa</td>
<td>MO</td>
<td>2</td>
<td>MOAimp1 &amp; 2</td>
</tr>
<tr>
<td>Acacia linifolia</td>
<td>B</td>
<td>1</td>
<td>BAli</td>
</tr>
<tr>
<td>Acacia myrtifolia</td>
<td>MO</td>
<td>1</td>
<td>MOAm</td>
</tr>
<tr>
<td>Acacia parvippinnula</td>
<td>B</td>
<td>2</td>
<td>BAp1 &amp; 2</td>
</tr>
<tr>
<td></td>
<td>RSF</td>
<td>2</td>
<td>RSFAp1 &amp; 2</td>
</tr>
<tr>
<td>Acacia saligna</td>
<td>MO</td>
<td>2</td>
<td>MOAsal 1 &amp; 2</td>
</tr>
<tr>
<td>Acacia suaveolens</td>
<td>B</td>
<td>1</td>
<td>BAs</td>
</tr>
<tr>
<td>Daviesia genistifolia</td>
<td>MO</td>
<td>1</td>
<td>MODg</td>
</tr>
<tr>
<td>Daviesia ulicifolia</td>
<td>B</td>
<td>3</td>
<td>BDu1, 2 &amp; 3</td>
</tr>
<tr>
<td></td>
<td>MO</td>
<td>1</td>
<td>MODu</td>
</tr>
<tr>
<td>Desmodium brachypodium</td>
<td>RSF</td>
<td>1</td>
<td>RSFDb*</td>
</tr>
<tr>
<td>Dillwynia retorta</td>
<td>B</td>
<td>1</td>
<td>BDr*</td>
</tr>
<tr>
<td>Glycine clandestina</td>
<td>MO</td>
<td>3</td>
<td>MOGclan1*, 2 &amp; 3</td>
</tr>
<tr>
<td>Hardenbergia violacea</td>
<td>MO</td>
<td>1</td>
<td>MOHv</td>
</tr>
<tr>
<td>Indigofera australis</td>
<td>MO</td>
<td>3</td>
<td>MOIa1, 2 &amp; 3</td>
</tr>
<tr>
<td>Kennedia rubicunda</td>
<td>MO</td>
<td>1</td>
<td>MOKr</td>
</tr>
<tr>
<td>Pultenaea retusa</td>
<td>MO</td>
<td>1</td>
<td>MOPr*</td>
</tr>
<tr>
<td>Pultenaea spinosa</td>
<td>MO</td>
<td>3</td>
<td>MOPspin1, 2 &amp; 3</td>
</tr>
<tr>
<td>Templetonia stenophylla</td>
<td>MO, RSF</td>
<td>2</td>
<td>MOTs, RSFTs *</td>
</tr>
<tr>
<td>Zornia dictocarpia</td>
<td>RSF</td>
<td>1</td>
<td>RSFZd *</td>
</tr>
<tr>
<td>Indeterminate Host</td>
<td>MO</td>
<td>1</td>
<td>MOX</td>
</tr>
</tbody>
</table>
Relative growth speed distinguished between fast growing isolates (colonies appearing within 24 h of plating) not expected to be rhizobia, slower growing isolates of *Rhizobium, Sinorhizobium, Mesorhizobium* which produce colonies 2-3 days after streaking onto YMA plates and the very slow growing *Bradyrhizobium* which produce very small colonies some 5-6 days after plating.

YMA plates impregnated with 0.003% bromocresol purple indicator (yellow at acid pH below 5.2 and purple at more basic pH above 6.8) were used to distinguish isolates that were acid or alkali excreters (Vincent, 1974).

**FORM**

CIRCULAR ELEVATION  IRREGULAR  FILAMENTOUS  RHIZOID

RAISED  CONVEX  FLAT  UMBONATE  CRATERIFORM

MARGIN

ENTIRE  UNDULATE  FILIFORM  CURLED  LOBATE

**Fig 2.1.** Illustration of different colony morphologies from Colome, *et al.* (1987) showing colony form and margin edge, which are descriptors used in Table 2.4.
2.2.3 Gram Staining
Approximately 500 mL of bacterial sample grown in YML was pipetted onto a clean slide. The bacterial cells were fixed to the slide by heating the slide on a heat block at 60°C until liquid had evaporated. The primary stain of crystal violet was added to the slide and incubated for 1 minute and then gently rinsed with deionised water for a maximum of 5 seconds. Gram’s Iodine was then added and incubated for 1 minute. The slide was then rinsed with 90% ethanol for a short period of approximately 3-5 seconds. Safranin was then added and incubated for 1 minute before the slide was rinsed with deionised water for another 5 seconds. The slide was then patted dry with paper towelling and examined under a light microscope at 1000x magnification.

2.2.4 Antimicrobial Disc Susceptibility Test
The antimicrobial disc susceptibility test (AST) provides a quick method of distinguishing between isolates, without providing definitive identification (Antoun et al., 1982; Labidi et al., 2003).

The procedures and antibiotics used were modified from the methods of Labidi et al., (2003). Isolates were grown for 72 h in 50 mL of YMB. The culture was centrifuged for 10 min at 2,700 x g using a Beckman GS-6R bench centrifuge. The supernatant was discarded and the pellet resuspended in sterile 0.09% saline solution to a concentration of between $10^6$ and $10^8$ cells/mL that prevented further bacterial growth. Under strict aseptic conditions, 300 µL of resuspended isolate was placed on YMA plates to form an even lawn of bacterial growth. The plates were allowed to dry for approximately 10-15 min at room temperature, before applying the antimicrobial discs (6 mm diameter paper discs impregnated with a known concentration of antibiotic) of selected antibiotics using an Oxoid multi-disc dispenser. Each isolate was tested against 10 antibiotics arranged on 3 yeast mannitol agar plates; 2 plates had 3 discs/plate while the third plate had 4 discs (Fig 2.2). The ten antibiotics (supplied by Oxoid P/L) and their concentrations were ampicillin 10µg/disc, carbenicillin 100µg/disc, gentamicin 10µg/disc, kanamycin 30µg/disc, nalidixic acid 30µg/disc, novobiocin 30µg/disc, penicillin G 10 IU/disc, rifampin 5µg/disc, streptomycin 10µg/disc, tetracycline 30µg/disc and are shown in Table 2.2 grouped by their mode of action upon bacteria. These concentrations of “µg/disc” are equivalent to mg/L, so that 30µg/disc is equivalent to 30mg/L (Labidi et al., 2003).
**Table 2.2** The antibiotics used in the antimicrobial disc susceptibility test grouped by their mode of action.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Method of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>Inhibits transpeptidation enzymes involved in the cross-linking of the polysaccharide chains of the bacterial cell wall peptidoglycan. Activates cell wall lytic enzymes.</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td></td>
</tr>
<tr>
<td>Penicillin G</td>
<td></td>
</tr>
<tr>
<td>Gentamicin</td>
<td>Binds to 30s subunit of bacterial ribosome to inhibit protein synthesis &amp; cause misreading of mRNA.</td>
</tr>
<tr>
<td>Streptomycin</td>
<td></td>
</tr>
<tr>
<td>Kanamycin</td>
<td></td>
</tr>
<tr>
<td>Tetracycline</td>
<td>Binds to 30s subunit &amp; interferes with aminoacyl-tRNA binding.</td>
</tr>
<tr>
<td>Rifampin</td>
<td>Blocks RNA synthesis by binding to &amp; inhibiting the DNA-dependent RNA polymerase.</td>
</tr>
<tr>
<td>Nalidixic Acid</td>
<td>Inhibits bacterial DNA gyrase or topoisomerase II by binding to the DNA gyrase complex.</td>
</tr>
<tr>
<td>Novobiocin</td>
<td>Blocks energy transduction by competitively inhibiting the ATPase reaction catalysed by GyrB.</td>
</tr>
</tbody>
</table>
2.2.5 Isolate DNA Preparation, 16S rDNA Amplification and PCR Protocol

Bacterial isolates were removed from the -80°C freezer, and under strict aseptic conditions, a loop of culture was placed into a 15 mL Falcon tube containing YML and allowed to grow for 36-48 h at 28°C in a shaking water bath oscillating at 120 rpm. Thereafter each culture was centrifuged for 10 minutes at 2,700 x g using a Beckman GS-6R bench centrifuge. Each resulting bacterial pellet was resuspended in 1 mL of YML and genomic DNA was obtained using a Qiagen DNeasy Blood & Tissue kit following the manufacturer’s instructions.

A fragment of 16S rDNA, approximately 500 bp in length was amplified by PCR and visualised by gel electrophoresis (Fig 2.3).

The primers used correspond to positions 8-28 (forward primer) and 539-560 (reverse primer) of the *Escherichia coli* rDNA (Table 2.3). PCR’s were carried out in a 20µL reaction volume containing 1 µL of template DNA, 10 µL of DNA (2x Fermentas MasterMix), 2 µL of forward primer (20µM), 2 µL of reverse primer (20 µM) (supplied by GeneWorks) and 5 µL of dH₂O. The PCR program from Lafay and Burdon (1998) was performed on an Eppendorf Mastercycler personal thermal cycler and involved an initial denaturation cycle at 95°C for 5 min, followed by 35 denaturation-annealing-extension cycles at 95°C for 30 sec, 52°C for 1 min and 72°C for 1 min, with a final extension step at 72°C for 5 min.

Table 2.3 The primers used for the sequencing of the 16S rDNA for the bacterial isolates.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ – 3’)</th>
<th><em>E. coli</em> Position</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16SF27</td>
<td>AGAGTTTGATCATGGCTCAGA</td>
<td>8-28</td>
<td>Brosius <em>et al.</em>, (1978)</td>
</tr>
<tr>
<td>16SR6</td>
<td>GTATTACGCAGCCTGCTGGCAGC</td>
<td>539-560</td>
<td>Brosius <em>et al.</em>, (1978)</td>
</tr>
</tbody>
</table>

The PCR product obtained was purified using a Promega Wizard kit following the manufacturer’s instructions. PCR product and primers were sent for sequencing using Applied Biosystems 3730xl capillary sequencers and Big Dye Terminator chemistry version 3.1 (Applied Systems). Sequence data returned from Australian Genome Research Facility (AGRF) were aligned and assembled using Sequencher® 4.7 (Gene
Codes Inc) and the resulting sequences compared to the NCBI microbe database Genbank sequences using the Megablast program.

### 2.2.6 Phylogenetic Relationships

A phylogenetic tree was constructed using the Phylogeny.fr platform. The “one-click” option where alignment is performed using MUSCLE3.7 program and phylogeny is determined using PhyML3.0 program (Dereeper et al., 2008). The phylogenetic tree was constructed using Dendroscope 2.5 (Huson et al., 2006) (www-ab.informatik.uni-tuebingen.de/software/dendroscope).

### 2.2.7 Verifying Nodulation

To obtain definitive proof of nodulation ability of a rhizobial isolate, roots of a suitable host were re-infected with the isolate and nodulation assessed (Vincent, 1970). To this end, seeds of *Macroptilium atropurpureum*, a promiscuous nodulator (Sprent, 2001), were grown in 500 mL screw cap jars (Fig 2.4) containing 100 mL of a modified Hoagland’s solution with nitrogen omitted, in 1.5% agar (see Appendix 1). *M. atropurpureum* seeds were surface sterilised by immersion in 70% ethanol in 15 mL Falcon tubes while being vortexed for three min. Once seedlings had germinated, they were inoculated with 1 mL of the bacterial isolate grown in YML for 48 h.

Jars containing seedlings and inoculum were grown in a Thermoline germination cabinet with a 12/12 h day/night illumination cycle and a 25/20°C day/night temperature cycle. Jars were observed over a period of six to 12 weeks and presence or absence of nodules noted.

**Fig 2.3** 1% agarose Gel showing 500 bp fragment of 16S rDNA. Far left lane is Fermentas MassRuler™ DNA ladder.
2.3 Results

2.3.1 *In vitro* Characterisation

*In vitro* characterisation of bacterial isolates was performed to ascertain whether bacteria cultured from root nodules could reasonably be assumed to be rhizobia, based upon observable features as shown in Table 2.4.

2.3.1.1 Growth

Observation of bacterial isolate growth starting from 24 h on YMA plates revealed four basic colony morphologies, three different colony colours and four growth speeds. Isolates are described below after being divided into four groupings based on their colony morphology.

2.3.1.2 Round colonies

Twelve of the isolates produced small, round, individually distinct colonies that varied in colour from clear to cloudy, with the majority of these being cream coloured. Individual colonies were consistent in size for each isolate, and all were very small. A single isolate produced 1-2 mm diameter colonies, five produced colonies under 1 mm
and the remaining four did not produce colonies over 0.5 mm in diameter. All these isolates were judged to be slow growers. Two were judged to be very slow growers, that is, colonies were not visible until after 72 h of growth. Three were rated as slower growing with colonies visible at 72 h and the remaining seven had colonies visible at 48 h.

2.3.1.3 Exopolysaccharide producing colonies
Eleven slow growing isolates did not produce distinct colonies, but instead formed a gummy substance, which was presumed to be exopolysaccharide slime. These isolates were mainly slow growers (growth at 48 h) although four were rated as slower growers. All gum-producing isolates were either cloudy or cream coloured.

2.3.1.4 Filamentous colonies
Nine colonies produced a filamentous growth form with several slight variations from tight curled filaments to loose, less curled sparse filaments. This colony type was always judged to be fast in their growth speed, and always produced cream coloured colonies.

2.3.1.5 Larger round colonies
Finally, two fast growing isolates produced large round colonies or irregularly shaped colonies that were up to 3 mm in diameter.

2.3.2 pH Characteristics
The pH characterisation and ability to grow at low and high pH ranges produced the following results (Table 2.5). None of the 34 isolates tested positive for alkali production when grown on YMA plates that had the pH adjusted to 5.0. Eleven isolates produced an acid reaction when grown on YMA plates that had pH adjusted to 9.0. Eight of these were slow growing isolates, MOTs was classified as slower, with only two fast growing isolates, MODu and MOGclan2. All but one of the isolates, MOGclan3, were able to grow at pH 9.0, whereas six isolates failed to grow at pH 5.0.
**Table 2.4** Morphological characteristics of the isolate colonies after 24 hours growth on YMA plates.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Colour</th>
<th>Colony Morphology</th>
<th>Size (mm)</th>
<th>Growth Speed</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAli</td>
<td>Cloudy</td>
<td>Numerous individual round colonies</td>
<td>1-2</td>
<td>Slow</td>
</tr>
<tr>
<td>BAp2</td>
<td>Clear</td>
<td>Small, round, smooth</td>
<td>&lt; 1</td>
<td>Slow</td>
</tr>
<tr>
<td>BAs</td>
<td>Cream</td>
<td>Small, round, smooth</td>
<td>&lt; 1</td>
<td>Slow</td>
</tr>
<tr>
<td>MOAsal2</td>
<td>Cream</td>
<td>Small, round, smooth</td>
<td>&lt; 1</td>
<td>Slow</td>
</tr>
<tr>
<td>MODg</td>
<td>Cloudy</td>
<td>Small, smooth, round, shiny</td>
<td>&lt; 1</td>
<td>Slow</td>
</tr>
<tr>
<td>MOKr</td>
<td>Cream</td>
<td>Small, round, smooth</td>
<td>&lt; 1</td>
<td>Slow</td>
</tr>
<tr>
<td>BDu1</td>
<td>Cream</td>
<td>Very small, round</td>
<td>&lt; 0.5</td>
<td>Slower</td>
</tr>
<tr>
<td>RSFAamb2</td>
<td>Cream</td>
<td>Small, round, smooth, numerous</td>
<td>&lt; 0.5</td>
<td>Slow</td>
</tr>
<tr>
<td>RSFAp1</td>
<td>Clear</td>
<td>Very small round</td>
<td>&lt; 0.5</td>
<td>Slower</td>
</tr>
<tr>
<td>RSFAp2</td>
<td>Cloudy</td>
<td>Very small, round</td>
<td>&lt; 0.5</td>
<td>Slower</td>
</tr>
<tr>
<td>BDu2</td>
<td>Cream</td>
<td>No distinct colonies</td>
<td>EPS</td>
<td>Slow</td>
</tr>
<tr>
<td>MOAsal1</td>
<td>Cream</td>
<td>No distinct colonies</td>
<td>EPS</td>
<td>Slow</td>
</tr>
<tr>
<td>MOGclan3</td>
<td>Cloudy</td>
<td>No distinct colonies</td>
<td>EPS</td>
<td>Slow</td>
</tr>
<tr>
<td>MOHv</td>
<td>Cream</td>
<td>No distinct colonies</td>
<td>EPS</td>
<td>Slower</td>
</tr>
<tr>
<td>MOIa1</td>
<td>Cream</td>
<td>No distinct colonies</td>
<td>EPS</td>
<td>Slow</td>
</tr>
<tr>
<td>MOPspin3</td>
<td>Cream</td>
<td>No distinct colonies</td>
<td>EPS</td>
<td>Slow</td>
</tr>
<tr>
<td>MOTs</td>
<td>Cloudy</td>
<td>No distinct colonies</td>
<td>EPS</td>
<td>Slower</td>
</tr>
<tr>
<td>BAm</td>
<td>Cream</td>
<td>No distinct colonies</td>
<td>EPS</td>
<td>Slow</td>
</tr>
<tr>
<td>BAp1</td>
<td>Cream</td>
<td>No distinct colonies</td>
<td>EPS</td>
<td>Slower</td>
</tr>
<tr>
<td>MOAmp1</td>
<td>Cloudy</td>
<td>No distinct colonies</td>
<td>EPS</td>
<td>Slower</td>
</tr>
<tr>
<td>MOAmp2</td>
<td>Cloudy</td>
<td>No distinct colonies</td>
<td>EPS</td>
<td>Slow</td>
</tr>
<tr>
<td>MOX</td>
<td>Cream</td>
<td>Very few, round, smooth edges, shiny</td>
<td>&lt; 1</td>
<td>Very slow</td>
</tr>
<tr>
<td>MOAdecor</td>
<td>Cloudy</td>
<td>Small, smooth, round, shiny</td>
<td>&lt; 0.5</td>
<td>Very slow</td>
</tr>
<tr>
<td>BDu3</td>
<td>Cream</td>
<td>Large, round, smooth, entire</td>
<td>≤ 3</td>
<td>Fast</td>
</tr>
<tr>
<td>MOAm</td>
<td>Cloudy</td>
<td>Large irregular shape</td>
<td>≤ 3</td>
<td>Fast</td>
</tr>
<tr>
<td>RSFAamb1</td>
<td>Cream</td>
<td>Sparse irregular filaments</td>
<td>Fast</td>
<td></td>
</tr>
<tr>
<td>MOAamb</td>
<td>Cream</td>
<td>Filamentous, loosely curled</td>
<td>Fast</td>
<td></td>
</tr>
<tr>
<td>MOAcu1</td>
<td>Cream</td>
<td>Filamentous, long, loosely curled</td>
<td>Fast</td>
<td></td>
</tr>
<tr>
<td>MODu</td>
<td>Cream</td>
<td>Loose, filamentous</td>
<td>Fast</td>
<td></td>
</tr>
<tr>
<td>MOGclan2</td>
<td>Cream</td>
<td>Filamentous, long sparse filaments</td>
<td>Fast</td>
<td></td>
</tr>
<tr>
<td>MOIa2</td>
<td>Cream</td>
<td>Tight filamentous</td>
<td>Fast</td>
<td></td>
</tr>
<tr>
<td>MOIa3</td>
<td>Cream</td>
<td>Tight filamentous</td>
<td>Fast</td>
<td></td>
</tr>
<tr>
<td>MOPspin1</td>
<td>Cream</td>
<td>Filamentous, straight &amp; curved fils</td>
<td>Fast</td>
<td></td>
</tr>
<tr>
<td>MOPspin2</td>
<td>Cream</td>
<td>Filamentous, long extended fils</td>
<td>Fast</td>
<td></td>
</tr>
</tbody>
</table>
2.3.3 Identification of Pure Cultures by Gram Staining

Gram staining of the various isolates showed that the majority of slow growing isolates, whether those forming discrete colony morphology or the exopolysaccharide producing types, were pure cultures of Gram-negative rods (Table 2.5). Five of the isolates showed mixed growth of either, large and small Gram-negative rods (BAs and RSFAamb2) or small Gram-negative and large Gram-positive rods (MOTs and BAp1, Fig 2.4) indicating bacteria other than rhizobia were present in these cultures. One isolate, MODg showed very small rods or cocci-shaped bacteria.

The majority of the fast growing, filamentous morphology isolates produced mixed cultures of small Gram-negative and large Gram-positive rods, which indicated that bacteria other than rhizobia were present (Fig 2.4). Two of these isolates, MOAamb1 and MOGclan2, were pure cultures of Gram-positive rods, while the other two isolates, MOAcul (medium sized Gram-negative rods) and MODu (a mixed culture of small and large Gram-negative rods) appeared to be at odds with the observed physical appearance of their cultures.
Table 2.5 *In vitro* characteristics of tested isolates showing the results of Gram staining, acid or alkali production and growth at low and high pH.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Gram Stain Results</th>
<th>Acid</th>
<th>Alkali</th>
<th>Growth pH 5.0</th>
<th>Growth pH 9.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAli</td>
<td>- ve rods</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>BAp2</td>
<td>- ve rods</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>BAs</td>
<td>mixed, small -ve &amp; large -ve rods</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>MOAsal2</td>
<td>- ve rods</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>MODg</td>
<td>very small -ve rods or cocci</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>MOKr</td>
<td>- ve rods</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>BDu1</td>
<td>- ve rods</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>RSFAamb2</td>
<td>mixed, small -ve &amp; large -ve rods</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>RSFAp1</td>
<td>- ve rods</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>RSFAp2</td>
<td>- ve rods</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>BDu2</td>
<td>- ve rods</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>MOAsal1</td>
<td>- ve rods</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>MOGclan3</td>
<td>- ve rods</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>MOHv</td>
<td>- ve rods</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>MOIa1</td>
<td>- ve rods</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>MOPspin3</td>
<td>- ve rods</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>MOTs</td>
<td>mixed, small -ve &amp; +ve rods</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>BAM</td>
<td>- ve rods</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>BAp1</td>
<td>mixed, small -ve &amp; large +ve rods</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>MOAimp1</td>
<td>- ve rods</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>MOAimp2</td>
<td>- ve rods</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>MOX</td>
<td>- ve rods</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>MOAdecor</td>
<td>- ve rods</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>BDu3</td>
<td>mixed, small -ve &amp; large +ve rods</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>MOAm</td>
<td>mixed, small -ve &amp; large +ve rods</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>RSFAamb1</td>
<td>mixed, small -ve &amp; large +ve rods</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>MOAamb1</td>
<td>large +ve rods</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>MOAcul</td>
<td>medium +ve rods</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>MODu</td>
<td>mixed, small -ve &amp; large -ve rods</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>MOGclan2</td>
<td>large +ve rods</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>MOIa2</td>
<td>mixed, small -ve &amp; large +ve rods</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>MOIa3</td>
<td>mixed, small -ve &amp; large +ve rods</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>MOPspin1</td>
<td>mixed, small -ve &amp; large +ve rods</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>MOPspin2</td>
<td>mixed, small -ve &amp; large +ve rods</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
</tbody>
</table>
Fig 2.4 Gram stains of representative cultures. A – MOAsal2, Gram negative rods. B – MOAamb1, Gram positive rods. C – BDu3, mixed culture of Gram negative and positive rods.
2.3.4 Antimicrobial Disc Susceptibility Test

The antimicrobial susceptibility test was used to distinguish between duplicate strains of bacteria. It divided isolates into three broad groups, based on an initial response to ampicillin that resulted in 18 resistant isolates, seven intermediate isolates and nine susceptible isolates (Table 2.6).

Table 2.6 Results of the antimicrobial disc susceptibility test, grouping isolates according to sensitivity to the antibiotics trialled. Sus = susceptible, Inter = intermediate and Res = resistant. This nomenclature indicates the size of the zone of inhibition of the bacterial isolates to the antibiotic, measured to the nearest millimetre. Antibiotics are grouped by method of action. Isolates that produced the same pattern are indicated in bold.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Am10</th>
<th>Car100</th>
<th>PnG10</th>
<th>Gn10</th>
<th>K30</th>
<th>St10</th>
<th>Na30</th>
<th>Nv30</th>
<th>Rf5</th>
<th>Te30</th>
</tr>
</thead>
<tbody>
<tr>
<td>MODg</td>
<td>Res</td>
<td>Res</td>
<td>Res</td>
<td>Res</td>
<td>Inter</td>
<td>Inter</td>
<td>Res</td>
<td>Res</td>
<td>Res</td>
<td>Sus</td>
</tr>
<tr>
<td>MOAimp1</td>
<td>Res</td>
<td>Res</td>
<td>Res</td>
<td>Res</td>
<td>Inter</td>
<td>Sus</td>
<td>Res</td>
<td>Sus</td>
<td>Res</td>
<td>Sus</td>
</tr>
<tr>
<td>MODu</td>
<td>Res</td>
<td>Res</td>
<td>Res</td>
<td>Res</td>
<td>Inter</td>
<td>Sus</td>
<td>Inter</td>
<td>Sus</td>
<td>Sus</td>
<td>Res</td>
</tr>
<tr>
<td>MOHv</td>
<td>Res</td>
<td>Res</td>
<td>Res</td>
<td>Res</td>
<td>Inter</td>
<td>Inter</td>
<td>Sus</td>
<td>Res</td>
<td>Res</td>
<td>Sus</td>
</tr>
<tr>
<td>MOla3</td>
<td>Res</td>
<td>Res</td>
<td>Res</td>
<td>Res</td>
<td>Sus</td>
<td>Sus</td>
<td>Inter</td>
<td>Res</td>
<td>Inter</td>
<td>Res</td>
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<td>MOAamb1</td>
<td>Res</td>
<td>Res</td>
<td>Res</td>
<td>Res</td>
<td>Sus</td>
<td>Inter</td>
<td>Sus</td>
<td>Res</td>
<td>Inter</td>
<td>Res</td>
</tr>
<tr>
<td>BAs</td>
<td>Res</td>
<td>Res</td>
<td>Res</td>
<td>Inter</td>
<td>Sus</td>
<td>Res</td>
<td>Inter</td>
<td>Sus</td>
<td>Res</td>
<td>Sus</td>
</tr>
<tr>
<td>BDu1</td>
<td>Res</td>
<td>Res</td>
<td>Res</td>
<td>Res</td>
<td>Inter</td>
<td>Sus</td>
<td>Sus</td>
<td>Sus</td>
<td>Res</td>
<td>Res</td>
</tr>
<tr>
<td>MOAcu1</td>
<td>Res</td>
<td>Res</td>
<td>Res</td>
<td>Sus</td>
<td>Sus</td>
<td>Sus</td>
<td>Res</td>
<td>Inter</td>
<td>Res</td>
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<td>Sus</td>
<td>Sus</td>
<td>Sus</td>
<td>Res</td>
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<td>BAp1</td>
<td>Res</td>
<td>Res</td>
<td>Res</td>
<td>Sus</td>
<td>Sus</td>
<td>Sus</td>
<td>Res</td>
<td>Sus</td>
<td>Res</td>
<td>Sus</td>
</tr>
<tr>
<td>MOPspin2</td>
<td>Res</td>
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<td>Res</td>
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</tr>
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<td>MOGclan3</td>
<td>Res</td>
<td>Res</td>
<td>Inter</td>
<td>Res</td>
<td>Inter</td>
<td>Sus</td>
<td>Sus</td>
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<td>Sus</td>
<td>Sus</td>
</tr>
<tr>
<td>MOGclan2</td>
<td>Inter</td>
<td>Res</td>
<td>Res</td>
<td>Res</td>
<td>Inter</td>
<td>Sus</td>
<td>Sus</td>
<td>Sus</td>
<td>Res</td>
<td>Sus</td>
</tr>
<tr>
<td>RSFAp1</td>
<td>Inter</td>
<td>Res</td>
<td>Res</td>
<td>Res</td>
<td>Inter</td>
<td>Sus</td>
<td>Sus</td>
<td>Sus</td>
<td>Res</td>
<td>Sus</td>
</tr>
<tr>
<td>MOKr</td>
<td>Inter</td>
<td>Res</td>
<td>Res</td>
<td>Res</td>
<td>Sus</td>
<td>Sus</td>
<td>Sus</td>
<td>Sus</td>
<td>Sus</td>
<td>Sus</td>
</tr>
<tr>
<td>MOla2</td>
<td>Inter</td>
<td>Res</td>
<td>Res</td>
<td>Sus</td>
<td>Sus</td>
<td>Sus</td>
<td>Res</td>
<td>Inter</td>
<td>Res</td>
<td>Sus</td>
</tr>
<tr>
<td>BALi</td>
<td>Inter</td>
<td>Res</td>
<td>Res</td>
<td>Sus</td>
<td>Sus</td>
<td>Sus</td>
<td>Res</td>
<td>Sus</td>
<td>Res</td>
<td>Sus</td>
</tr>
<tr>
<td>MOPspin1</td>
<td>Inter</td>
<td>Res</td>
<td>Res</td>
<td>Sus</td>
<td>Sus</td>
<td>Sus</td>
<td>Res</td>
<td>Sus</td>
<td>Res</td>
<td>Sus</td>
</tr>
<tr>
<td>MOX</td>
<td>Inter</td>
<td>Res</td>
<td>Inter</td>
<td>Sus</td>
<td>Sus</td>
<td>Sus</td>
<td>Res</td>
<td>Sus</td>
<td>Sus</td>
<td>Sus</td>
</tr>
<tr>
<td>MOAdcor</td>
<td>Sus</td>
<td>Res</td>
<td>Res</td>
<td>Res</td>
<td>Inter</td>
<td>Sus</td>
<td>Sus</td>
<td>Sus</td>
<td>Res</td>
<td>Sus</td>
</tr>
<tr>
<td>BAm</td>
<td>Sus</td>
<td>Res</td>
<td>Res</td>
<td>Sus</td>
<td>Res</td>
<td>Inter</td>
<td>Sus</td>
<td>Res</td>
<td>Sus</td>
<td>Sus</td>
</tr>
<tr>
<td>MOla1</td>
<td>Sus</td>
<td>Res</td>
<td>Inter</td>
<td>Sus</td>
<td>Sus</td>
<td>Sus</td>
<td>Sus</td>
<td>Sus</td>
<td>Res</td>
<td>Sus</td>
</tr>
<tr>
<td>RSFAmb1</td>
<td>Sus</td>
<td>Res</td>
<td>Res</td>
<td>Sus</td>
<td>Sus</td>
<td>Sus</td>
<td>Res</td>
<td>Inter</td>
<td>Sus</td>
<td>Sus</td>
</tr>
<tr>
<td>MOAsal2</td>
<td>Sus</td>
<td>Inter</td>
<td>Inter</td>
<td>Sus</td>
<td>Sus</td>
<td>Sus</td>
<td>Res</td>
<td>Res</td>
<td>Res</td>
<td>Sus</td>
</tr>
<tr>
<td>BAp2</td>
<td>Sus</td>
<td>Sus</td>
<td>Res</td>
<td>Sus</td>
<td>Sus</td>
<td>Sus</td>
<td>Res</td>
<td>Sus</td>
<td>Res</td>
<td>Sus</td>
</tr>
<tr>
<td>MOTs</td>
<td>Sus</td>
<td>Sus</td>
<td>Inter</td>
<td>Sus</td>
<td>Sus</td>
<td>Sus</td>
<td>Res</td>
<td>Res</td>
<td>Res</td>
<td>Sus</td>
</tr>
<tr>
<td>MOAm</td>
<td>Sus</td>
<td>Sus</td>
<td>Sus</td>
<td>Res</td>
<td>Sus</td>
<td>Res</td>
<td>Sus</td>
<td>Sus</td>
<td>Sus</td>
<td>Res</td>
</tr>
<tr>
<td>BDu3</td>
<td>Sus</td>
<td>Sus</td>
<td>Sus</td>
<td>Sus</td>
<td>Sus</td>
<td>Sus</td>
<td>Inter</td>
<td>Res</td>
<td>Res</td>
<td>Sus</td>
</tr>
</tbody>
</table>
Within these broad responses, and regardless of which of the other antibiotics was used as the primary division, only five isolates returned identical susceptibilities to the 10 antibiotics trialled. RSFAmb2, BAp1 and MOPspin2 grouped together in the ampicillin resistant response. In the smaller ampicillin intermediate response group, two isolates BAli2 and MOPspin1 grouped together. All other isolates returned unique responses taken over the 10 antibiotics. This method indicated that the vast majority of these were different strains of bacteria with very little duplication in collection occurred.

2.3.5 Molecular Identification
Identification of the bacterial isolates using a 16S rDNA fragment provided the results summarised in Table 2.7 (sequences used for identification are presented in Appendix 2). Overall, the 16S rDNA data identified six isolates as Rhizobium and two isolates as Agrobacterium. Three of the Rhizobium isolates were identified as Rhizobium sp. CHNTR53. Two of these three isolates, MOAsal2 and MOTs, had very high homology between the experimental isolate and the sequence held in Genbank (99 and 100% respectively). The remaining isolate sequence, BDu1 had a lower homology at 93%.

Two isolates, BAli and MOPspin3, were identified as strains of R. tropici, while the last isolate, BAp1 was identified as R. miluonense strain CC-BL6. The two isolates that were identified as Agrobacterium were MOIa1 and MOAsal1, a previously uncultured isolate and A. tumefaciens strain H2P4 respectively.

Eight isolates were identified as belonging to the genus Burkholderia. Five of these, BAs, BAp2, BDu1, BAm and MOAm, were identified as uncultured bacterium clone AKIW800, with high homologies of 98 and 99%. Isolate MODu had a lower homology of 97% with Burkholderia species Tat-045. MOPspin2 was identified as an uncultured Eubacterium as the most significant return from GenBank, with all other sequence matches from GenBank and the phylogeny data from Fig 2.5 (see below) grouping this isolate as a member of the Burkholderia complex. The remaining isolate, RSFAp2, was identified as Burkholderia species CC-S-L25.

Three isolates, MOAdcor, MOAimp1 and MOAimp2 were identified as an uncultured bacterium clone that was closely related to the genus Pseudomonas.
Table 2.7 Identification of bacterial isolates using an approximately 500 bp 16S rDNA fragment compared to sequences held in Genbank, showing the name, accession number and percentage homology of the fragment to the bacterial sequence with the greatest similarity. Coloured isolates were identical. Column on the right shows the result of the re-inoculation experiment.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Name</th>
<th>Accession Number</th>
<th>Homology (%)</th>
<th>Re-Inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOAsal1</td>
<td><em>Agrobacterium tumefaciens</em> str H2P4</td>
<td>EU221403.1</td>
<td>100</td>
<td>Y</td>
</tr>
<tr>
<td>MOKr</td>
<td><em>Bacillus cereus</em> str. BFE 5392</td>
<td>GU250443.1</td>
<td>99</td>
<td>Y</td>
</tr>
<tr>
<td>MOX</td>
<td><em>Bacillus</em> sp. Pc2T</td>
<td>GU391510.1</td>
<td>100</td>
<td>Y</td>
</tr>
<tr>
<td>RSFAamb2</td>
<td><em>Bacillus</em> sp. Tp14</td>
<td>EU855759.1</td>
<td>99</td>
<td>Y</td>
</tr>
<tr>
<td>RSFAp2</td>
<td><em>Burkholderia</em> sp. CC-S-L25</td>
<td>DQ830511.1</td>
<td>99</td>
<td>Y</td>
</tr>
<tr>
<td>MOGclan3</td>
<td><em>Chitinophaga</em> sp. CS5-B1</td>
<td>FM865977.1</td>
<td>99</td>
<td>Y</td>
</tr>
<tr>
<td>MOHv</td>
<td><em>Labrys portucalensis</em> str. F11</td>
<td>AY362040.1</td>
<td>99</td>
<td>Y</td>
</tr>
<tr>
<td>BAp1</td>
<td><em>Rhizobium miluonense</em> str. CC-BL6</td>
<td>GU120632.1</td>
<td>99</td>
<td>Y</td>
</tr>
<tr>
<td>MOAsal2</td>
<td><em>Rhizobium</em> sp. CHNTR53</td>
<td>DQ337578.1</td>
<td>99</td>
<td>Y</td>
</tr>
<tr>
<td>MOTs</td>
<td><em>Rhizobium</em> sp. CHNTR53</td>
<td>DQ337578.1</td>
<td>100</td>
<td>Y</td>
</tr>
<tr>
<td>BDu1</td>
<td><em>Rhizobium</em> sp. CHNTR53</td>
<td>DQ337578.1</td>
<td>93</td>
<td>Y</td>
</tr>
<tr>
<td>MOIa3</td>
<td><em>Bacillus</em> sp. Tp14</td>
<td>EU855759.1</td>
<td>99</td>
<td>Y</td>
</tr>
<tr>
<td>RSFAp1</td>
<td>Uncult <em>Acinetobacter</em> sp. clone GI5-007-H09</td>
<td>FJ192831.1</td>
<td>99</td>
<td>Y</td>
</tr>
<tr>
<td>MOIa1</td>
<td>Uncult <em>Agrobacterium</em> sp.</td>
<td>AB451540.1</td>
<td>100</td>
<td>Y</td>
</tr>
<tr>
<td>MOIa2</td>
<td>Uncult <em>Bacillus</em> sp. clone JPL-S3-M01</td>
<td>FJ957645.1</td>
<td>99</td>
<td>Y</td>
</tr>
<tr>
<td>BAs</td>
<td>Uncult bacterium clone AKIW800</td>
<td>DQ129599.1</td>
<td>99</td>
<td>Y</td>
</tr>
<tr>
<td>BAp2</td>
<td>Uncult bacterium clone AKIW800</td>
<td>DQ129599.1</td>
<td>99</td>
<td>Y</td>
</tr>
<tr>
<td>MOAm</td>
<td>Uncult bacterium clone AKIW800</td>
<td>DQ129599.1</td>
<td>98</td>
<td>Y</td>
</tr>
<tr>
<td>BDu2</td>
<td>Uncult bacterium clone AKIW800</td>
<td>DQ129599.1</td>
<td>99</td>
<td>Y</td>
</tr>
<tr>
<td>MOAimp2</td>
<td>Uncult bacterium clone CF7</td>
<td>GU124695.1</td>
<td>99</td>
<td>Y</td>
</tr>
<tr>
<td>MOAdecor</td>
<td>Uncult bacterium clone CF7</td>
<td>GU272304.1</td>
<td>99</td>
<td>Y</td>
</tr>
<tr>
<td>MOAimp1</td>
<td>Uncult bacterium clone CF7</td>
<td>GU124695.1</td>
<td>99</td>
<td>Y</td>
</tr>
<tr>
<td>MODu</td>
<td><em>Burkholderia</em> sp. TAt-045</td>
<td>FJ478405.1</td>
<td>97</td>
<td>Y</td>
</tr>
<tr>
<td>MODg</td>
<td>Bacterium G2</td>
<td>AY34539.1</td>
<td>98</td>
<td>N</td>
</tr>
<tr>
<td>MOPspin1</td>
<td><em>Brevibacillus brevis</em> NBRC 100599</td>
<td>AP008855.1</td>
<td>99</td>
<td>N</td>
</tr>
<tr>
<td>MOAcu</td>
<td><em>Brevibacillus brevis</em> NBRC 100599</td>
<td>AP008855.1</td>
<td>99</td>
<td>N</td>
</tr>
<tr>
<td>RSFAamb1</td>
<td><em>Paenibacillus</em> pabuli</td>
<td>AB045104.1</td>
<td>92</td>
<td>N</td>
</tr>
<tr>
<td>MOAamb</td>
<td><em>Paenibacillus</em> pabuli</td>
<td>AB045104.1</td>
<td>99</td>
<td>N</td>
</tr>
<tr>
<td>MOGclan2</td>
<td><em>Paenibacillus taichungensis</em> str. BK28</td>
<td>EU982882.1</td>
<td>89</td>
<td>N</td>
</tr>
<tr>
<td>BAli</td>
<td><em>Rhizobium tropici</em> strain LNP7</td>
<td>GQ181037.1</td>
<td>99</td>
<td>N</td>
</tr>
<tr>
<td>MOPspin3</td>
<td><em>Rhizobium tropici</em> strain LNW10</td>
<td>GQ181030.1</td>
<td>100</td>
<td>N</td>
</tr>
<tr>
<td>Bam</td>
<td>Uncult bacterium clone AKIW800</td>
<td>DQ129599.1</td>
<td>98</td>
<td>N</td>
</tr>
<tr>
<td>MOPspin2</td>
<td>Uncult eubacterium 16S rRNA (Burkholderia)</td>
<td>AJ233521.1</td>
<td>99</td>
<td>N</td>
</tr>
<tr>
<td>BDu3</td>
<td>Unknown</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
A large group of isolates were identified as belonging to either the genus *Bacillus* (five isolates), *Brevibacillus* (two isolates, both *B. brevis* NBRC 100599) or *Paenibacillus* (three isolates, two of which were identified as *P. pabuli*), all of which are closely related Gram-positive bacteria.

Four of the remaining five isolates, RSFAp1 (an uncultured *Acinetobacter*), MODg (unclassified Bacterium G2), MOGclan3 (*Chitinophaga* sp CS5-B1), MOHv (*Labrys portucalensis*) were identified as belonging to a diverse range of miscellaneous genera. The last isolate, BDu3 was unable to be identified due to unreadable sequencing data.

### 2.3.6 Phylogeny

An rectangular phylogenetic tree was produced from the sequence data used for molecular identification shown in Table 2.7 and includes sequences of a number of type strains (Fig 2.5). Examination of Fig 2.5, starting from the top, shows that the isolates formed four main clusters, with MOGclan3 genetically distinct. The *Burkholderia* isolates, Cluster 1, form two sub-clusters. The five uncultured bacterium clone AKIW800 and type strain *B. cenocepacia* AU1054 cluster together, with the remaining three *Burkholderia* isolates (MOPspin2, RSFAp2 and MODu) forming the second sub-cluster.

The second cluster, Cluster 2 consists of three uncultured *Pseudomonas* group isolates, plus RSFAp1, uncultured *Acinetobacter* and *P. aeruginosa* LESB58, with a more distant sub-cluster of *E. coli* ATCC8739 and MODg, suggesting that this isolate is very closely related to *E. coli*.

The third cluster, Cluster 3 is the rhizobia group and includes several type strains, with three sub-clusters. The three *Rhizobium* sp. CHNTR53 isolates cluster with *R. gallicum*, while the two *R. tropici* strains and *R. miluonense* form another sub-cluster. The two *Agrobacterium* strains cluster together, and separately, while finally, MOHv, is more distantly related to the Rhizobia group.

The fourth cluster, Cluster 4, consists of the *Bacillus*, *Brevibacillus* and *Paenibacillus* species, and group accordingly.

### 2.3.7 Re-inoculation Trial

The re-inoculation trial results are shown in Table 2.7. All 34 isolates were re-inoculated into screw-capped jars and the presence or absence of nodules was observed. 23 isolates produced nodules indicating the presence of root-nodule forming bacteria.
Fig 2.5 Phylogenetetic tree of the bacterial isolates identified and their relationship to selected bacterial type strains. The isolates cluster into four main genera, with one outlier, MOGclan3 not closely related to any other isolate. The four clusters are 1) *Burkholderia* related isolates, 2) *Pseudomonas* (with a related *E. coli* isolate), 3) the Rhizobia cluster, and finally 4) *Bacillus* cluster that includes *Bacillus, Brevibacillus* and *Paenibacillus*. Isolates nodulating in the Re-Inoculation Trial are in red text. MOIa2 is not included in the phylogenetic tree.
2.4 Discussion

The aim of this study was to culture, identify and test the specificity of rhizobial isolates that had been collected from a several native legume hosts. A number of techniques, by no means exhaustive, were used in this chapter to identify and verify if the bacterial isolates in culture were indeed able to nodulate.

Phenotypic characteristics are generally not considered diagnostic to identify and separate bacteria into their different genera and species. Nonetheless it can be seen from Table 2.8 that various characteristics can be used to begin to identify potential rhizobia from other soil bacteria. Note that there are two further genera of “rhizobia” Azorhizobium and Allorhizobium, but as they have not been reported as occurring in Australia, unless these genera are referred to specifically, the term rhizobia will refer to the four genera Rhizobium, Mesorhizobium, Sinorhizobium and Bradyrhizobium.

2.4.1 Colony Morphology and Growth Rate

The *in vitro* characteristics used in this study, such as colony morphology and colour were useful to distinguish between potential rhizobia and other soil bacteria, but are not useful to distinguish between rhizobia strains. Rhizobia strains that do not produce extracellular gum or slime are usually small round colonies that can be white, cloudy or colourless. Rarely do they produce pigmented colonies and most reports of pigmented colonies are for rhizobia isolated from particular hosts (Garrity *et al.*, 2001). From Table 2.4 it can be seen that none of the isolates cultured for this study produced pigmentation. Members of the closely related genera *Bacillus*, Brevibacillus and *Paenibacillus* have a distinctive filamentous colony morphology that readily eliminates them from belonging to rhizobia. Therefore, if colony morphology were a prime criterion used to determine the presence or absence of rhizobia, several of the nodulating cultures that showed this filamentous morphology would have been discarded. As will be discussed later, this would have greatly reduced the number of nodulating isolates used in the specificity trial in Chapter 3. However, given that these genera have been reported to be readily isolated from root nodules, prudence dictated that further tests were conducted to verify nodulation ability.

Growth rate is one characteristic that traditionally has been used to help differentiate rhizobia, both from other bacteria and from each other. Rhizobia are notoriously slow growing in culture.
Table 2.8 Summary of the results from the culture dependent techniques and molecular identification used in this study. Isolates are grouped first according to ability to produce root nodules in the re-inoculation trial, and then second alphabetically from the molecular identification. Isolates with the same colouring were identified from 16S rDNA as being the same isolate.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Colony Morphology</th>
<th>Growth Speed</th>
<th>Gram stain</th>
<th>Re-noc Nods?</th>
<th>Molecular ID</th>
<th>Dendro group</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOAsal1</td>
<td>No distinct colonies</td>
<td>Slow</td>
<td>- ve rods</td>
<td>Y</td>
<td>Agrobacterium tumefaciens str H2P4</td>
<td>Rhiz</td>
</tr>
<tr>
<td>MOKr</td>
<td>Small, round, smooth</td>
<td>Slow</td>
<td>- ve rods</td>
<td>Y</td>
<td>Bacillus cereus strain BFE 5392</td>
<td>Bacillus</td>
</tr>
<tr>
<td>MOX</td>
<td>Few, round, smooth, shiny</td>
<td>V. slow</td>
<td>- ve rods</td>
<td>Y</td>
<td>Bacillus sp. Pc2T</td>
<td>Bacillus</td>
</tr>
<tr>
<td>RSFAamb2</td>
<td>Small, round, smooth, many</td>
<td>Slow</td>
<td>mixed</td>
<td>Y</td>
<td>Bacillus sp. Tp14</td>
<td>Bacillus</td>
</tr>
<tr>
<td>RSFAp2</td>
<td>Very small, round</td>
<td>Slower</td>
<td>- ve rods</td>
<td>Y</td>
<td>Burkholderia sp. CC-S-L25</td>
<td>Burk</td>
</tr>
<tr>
<td>MOGclan3</td>
<td>No distinct colonies</td>
<td>Slow</td>
<td>- ve rods</td>
<td>Y</td>
<td>Chitinophaga sp. CS5-B1</td>
<td>Unique</td>
</tr>
<tr>
<td>MOHv</td>
<td>No distinct colonies</td>
<td>Slower</td>
<td>- ve rods</td>
<td>Y</td>
<td>Labrys portucaleensis strain F11</td>
<td>Rhiz</td>
</tr>
<tr>
<td>BApl</td>
<td>No distinct colonies</td>
<td>Slower</td>
<td>mixed</td>
<td>Y</td>
<td>Rhizobium miluonense strain CC-BL6</td>
<td>Rhiz</td>
</tr>
<tr>
<td>MOAsal2</td>
<td>Small, round, smooth</td>
<td>Slow</td>
<td>- ve rods</td>
<td>Y</td>
<td>Rhizobium sp. CHNTR53</td>
<td>Rhiz</td>
</tr>
<tr>
<td>BDu1</td>
<td>Very small, round</td>
<td>Slower</td>
<td>- ve rods</td>
<td>Y</td>
<td>Rhizobium sp. CHNTR53</td>
<td>Rhiz</td>
</tr>
<tr>
<td>MOTs</td>
<td>No distinct colonies</td>
<td>Slower</td>
<td>mixed</td>
<td>Y</td>
<td>Rhizobium sp. CHNTR53</td>
<td>Rhiz</td>
</tr>
<tr>
<td>BAl</td>
<td>Numerous round colonies</td>
<td>Slow</td>
<td>- ve rods</td>
<td>Y</td>
<td>Rhizobium tropici strain LNP7</td>
<td>Rhiz</td>
</tr>
<tr>
<td>MOla2</td>
<td>Tight filamentous</td>
<td>Fast</td>
<td>mixed</td>
<td>Y</td>
<td>Uncult Bacillus sp. clone JPL-S3 M01</td>
<td>-</td>
</tr>
<tr>
<td>BApl2</td>
<td>Small, round, smooth</td>
<td>Slow</td>
<td>- ve rods</td>
<td>Y</td>
<td>Uncult bacterium clone AKIW800</td>
<td>Burk</td>
</tr>
<tr>
<td>BDu2</td>
<td>No distinct colonies</td>
<td>Slow</td>
<td>- ve rods</td>
<td>Y</td>
<td>Uncult bacterium clone AKIW800</td>
<td>Burk</td>
</tr>
<tr>
<td>BAS</td>
<td>Small, round, smooth</td>
<td>Slow</td>
<td>mixed</td>
<td>Y</td>
<td>Uncult bacterium clone AKIW800</td>
<td>Burk</td>
</tr>
<tr>
<td>MOAm</td>
<td>Large irregular shape</td>
<td>Fast</td>
<td>mixed</td>
<td>Y</td>
<td>Uncult bacterium clone AKIW800</td>
<td>Burk</td>
</tr>
<tr>
<td>MOAimp1</td>
<td>No distinct colonies</td>
<td>Slower</td>
<td>- ve rods</td>
<td>Y</td>
<td>Uncult bacterium clone CF7</td>
<td>Pseudo</td>
</tr>
<tr>
<td>MOAimp2</td>
<td>No distinct colonies</td>
<td>Slow</td>
<td>- ve rods</td>
<td>Y</td>
<td>Uncult bacterium clone CF7</td>
<td>Pseudo</td>
</tr>
<tr>
<td>MOAdecor</td>
<td>Small, smooth, round, shiny</td>
<td>V. slow</td>
<td>- ve rods</td>
<td>Y</td>
<td>Uncult bacterium clone CF7</td>
<td>Pseudo</td>
</tr>
<tr>
<td>RSFAp1</td>
<td>Very small round</td>
<td>Slower</td>
<td>- ve rods</td>
<td>Y</td>
<td>Uncultured Acinetobacter sp. clone G15-007-H09</td>
<td>Pseudo</td>
</tr>
<tr>
<td>MOla1</td>
<td>No distinct colonies</td>
<td>Slow</td>
<td>- ve rods</td>
<td>Y</td>
<td>Uncultured Agrobacterium sp.</td>
<td>Rhiz</td>
</tr>
<tr>
<td>MOla3</td>
<td>Tight filamentous</td>
<td>Fast</td>
<td>mixed</td>
<td>N</td>
<td>Bacillus sp. Tp14</td>
<td>Bacillus</td>
</tr>
<tr>
<td>MODg</td>
<td>Small, smooth, round, shiny</td>
<td>Slow</td>
<td>mixed</td>
<td>N</td>
<td>Bacterium G2 (unclassified bacterium)</td>
<td>E. coli</td>
</tr>
<tr>
<td>MOAcul</td>
<td>Filamentous, loosely curved</td>
<td>Fast</td>
<td>+ve rods</td>
<td>N</td>
<td>Brevibacillus brevis NBRC 100599</td>
<td>Bacillus</td>
</tr>
<tr>
<td>MOPspin1</td>
<td>Filamentous, straight &amp; curved</td>
<td>Fast</td>
<td>mixed</td>
<td>N</td>
<td>Brevibacillus brevis NBRC 100599</td>
<td>Bacillus</td>
</tr>
<tr>
<td>MODu</td>
<td>Loose, filamentous</td>
<td>Fast</td>
<td>mixed</td>
<td>N</td>
<td>Burkholderia sp. TA1-045</td>
<td>Burk</td>
</tr>
<tr>
<td>MOAamb</td>
<td>Filamentous, loosely curled</td>
<td>Fast</td>
<td>+ve rods</td>
<td>N</td>
<td>Paenibacillus pabuli</td>
<td>Bacillus</td>
</tr>
<tr>
<td>RSFAamb1</td>
<td>Sparse irregular filaments</td>
<td>Fast</td>
<td>mixed</td>
<td>N</td>
<td>Paenibacillus pabuli</td>
<td>Bacillus</td>
</tr>
<tr>
<td>MOGclan2</td>
<td>Long sparse filaments</td>
<td>Fast</td>
<td>+ve rods</td>
<td>N</td>
<td>Paenibacillus taichungensis str BK28</td>
<td>Bacillus</td>
</tr>
<tr>
<td>MOPspin3</td>
<td>No distinct colonies</td>
<td>Slow</td>
<td>- ve rods</td>
<td>N</td>
<td>Rhizobium tropici strain LNW10</td>
<td>Rhiz</td>
</tr>
<tr>
<td>BAmp</td>
<td>No distinct colonies</td>
<td>Slow</td>
<td>- ve rods</td>
<td>N</td>
<td>Uncult bacterium clone AKIW800</td>
<td>Burk</td>
</tr>
<tr>
<td>MOPspin2</td>
<td>Filamentous, long extended</td>
<td>Fast</td>
<td>mixed</td>
<td>N</td>
<td>Uncult eubacterium 16S rRNA (Burk)</td>
<td>Burk</td>
</tr>
<tr>
<td>BDu3</td>
<td>Large, round, smooth, entire</td>
<td>Fast</td>
<td>mixed</td>
<td>N</td>
<td>Unknown</td>
<td>-</td>
</tr>
</tbody>
</table>
The “fast” growing genera, *Rhizobium*, *Mesorhizobium* and *Sinorhizobium* (relative to Bradyrhizobium) have a generation time (the time taken to double the population) in culture that is strain dependent, and ranges from 1.5 to five hours, producing colonies on YMA plates two to four days after streaking.

The slow growing genus *Bradyrhizobium* produces colonies seven to ten days after plating, with a generation time of between nine and 18 h (Garrity, *et al*., 2001). In contrast, *Bacillus*, *Brevibacillus*, *Paenibacillus*, *Pseudomonas* and *Burkholderia* are usually much faster growing. *Bacillus subtilis* when cultured at 40°C has a generation time of 26 min (Prescott, *et al*., 1996) whereas *Burkholderia cepacia* has a generation time of 192 min when grown on *p*-hydroxybenzoate (PHB) (Paul *et al*., 2004). The slow growth rate of rhizobia, when compared to common soil dwelling bacteria such as those mentioned above can present difficulties when attempting to isolate rhizobia from contaminating bacteria as they tend to be overgrown or masked by the faster growing bacteria.

The acid or alkali production of the rhizobia was used to differentiate the slow-growing, alkali producing *Bradyrhizobium*, from the fast-growing, acid producing members of the genus *Rhizobium* (Vincent, 1974). From Table 2.5, none of the isolates cultured produced an alkali reaction when grown on acid plates. This indicates that none of the isolates are slow-growing *Bradyrhizobium*, which is unexpected given the prevalence of this genus in other studies of bacteria that have investigated the microsymbionts of Australian native legumes (Lafay and Burdon, 1998; Lafay and Burdon, 2001).

Gram staining proved to be one of the most useful techniques for determining the presence of contaminating or mixed cultures where there was the possibility of more than one bacterial species being present. Rhizobia are Gram-negative rods and as there have been no reports found in the literature, of Gram-positive bacteria as root nodulators, such cultures could therefore be eliminated from the specificity trial, as they would not be expected to form root nodules. Many other common soil dwelling bacteria found in this study such as *Burkholderia*, *Pseudomonas*, that have also been reported to nodulate legumes (Moulin, *et al*., 2001; Shiraishi *et al*., 2010) and *Xanthomonas* are Gram negative rods, and thus Gram staining is not itself diagnostic. Care also must be taken as older cultures of Gram-positive bacteria may not hold the secondary stain, and thus may appear Gram negative (Prescott, *et al*., 1996). This appears to have occurred.
for three mixed cultures, where the bacteria are recorded as small and larger Gram-
negative rods, BAs, RSFAamb2 and MODu.

Nineteen isolates (Table 2.5) were confirmed as being Gram-negative rods, the
correct staining and shape for rhizobia. Another two isolates, MOAamb1 and
MOGclan2 were shown to be pure cultures of Gram-positive rods, clearly not rhizobia
and could therefore possibly be eliminated from the specificity trial in Chapter 3.
Isolates that showed mixed cultures of Gram-negative and positive bacteria, such as
BDu3 and RSFAmb1, could not be eliminated from the specificity trial, as the
presence of the Gram-negative rods could indicate rhizobia bacteria.

### 2.4.2 Intrinsic Antibiotic Resistance or Antimicrobial Disc Susceptibility Test

Intrinsic Antibiotic Resistance (IAR) has been used to differentiate between species and
strains of rhizobia since the early 1960’s (Davis, 1962). This technique was found to be
a quick and easy method to distinguish inoculating strains from indigenous rhizobia,
especially when compared to serological techniques (Beynon and Josey, 1980). The
Antimicrobial Disc Susceptibility Test (AST) is a modified version of IAR based on the
Kirby-Bauer paper disc diffusion method (Bauer et. al., 1966).

The results from this study (Table 2.6) showed that only two small groups of
bacteria had the same pattern of resistance or susceptibility to the antibiotics tested,
indicating that the remaining isolates were unique species or strains. This high degree of
uniqueness is surprising considering the bacterial isolates were located from two sites
only. The likelihood of capturing the same bacteria species is increased if multiple
samples are collected from a limited geographic range (Howieson, et al., 1995).

In past studies, IAR and AST have been used to identify an inoculating strain
retrieved from a field situation (Josey et al., 1979; Beynon and Josey, 1980; Abaidoo,
2002) or as methods of differentiating between large numbers of environmental isolates
(Mueller, et al., 1988; Jenkins and Bottomley, 1985; Date and Hurse, 1991). These
studies examined intra-specific variation, and did not use IAR or AST to investigate
inter-specific or inter-genic variation. Within species variation was noted in one study
where the combination and concentrations of antibiotics used produced consistent
results for *Rhizobium leguminosarum*. However, when these same antibiotic
combinations and concentrations were applied to *R. phaseoli*, which are now recognised
as biovars (bv) of the same species (and hence very closely related), different results
were obtained (Josey, et al., 1979). This highlights the sensitivity of IAR.
The use of AST with mixed cultures may not have made interpretation of the results any clearer and may have lead to erroneous conclusions as to similarities or differences of the isolates. Despite IAR and AST having first been used to differentiate between bacterial species in a clinical setting, it would appear that from an environmental viewpoint, the technique may be too sensitive to discriminate between soil bacteria with the range and concentrations of antibiotics used here. A different range of antibiotics may need to be trialled against reference species and strains, possibly for each division of bacteria that may be encountered, such as proteobacteria, low G+C gram positive bacteria, other Gram positive bacteria.

The antibiotics used across different studies vary considerably. The drugs and concentrations used in this study were based on Labidi, et al., (2002), who used AST with 12 antibiotics to perform intra-specific discrimination between 70 isolates of *R. leguminosarum* bv *viciae*. This indicates that “fine tuning” of the antibiotics being employed may be required for this technique to be successful. Three of the antibiotics used in this study, ampicillin, carbenicillin and penicillin G, are broad-spectrum penicillins and, as stated in Table 2.2, their mode of action inhibits the transpeptidation enzymes involved in cross-linking peptidoglycans, the polysaccharide chains of the bacterial cell wall (Prescott et al., 1996). This renders them effective against almost all Gram-positive and some Gram-negative bacteria, although clinical use of the penicillins is becoming limited due to the increasing rise of bacterial immunity (Prescott et al., 1996).

### 2.4.3 16S rDNA Identification and Phylogeny

Molecular identification showed a high heterogeneity in the 34 isolates used here. In terms of recognised nodulating bacteria, eight isolates were identified as either *Rhizobium* or *Agrobacterium*. *Agrobacterium* have not been considered as nodule forming or nitrogen-fixing bacteria, but members of this species have been recovered from root nodules, both in isolation and as nodule co-inhabitants and have been shown to form both functional and non-functional nodules upon re-infection of legumes (Odee et al., 2002; Bala, et al., 2003; Diouf et al., 2007).

This is reflected in the phylogenetic tree (Fig 2.5) where the rhizobial isolates showed greatest similarities to *Rhizobium gallicum* (MOAsal2, BDu1 and MOTs) and two *R. tropici* isolates, MOPspin3 and BAl1. A third isolate, BAp1, identified as *R. miluonense* str CC-BL6, grouped closely with the two *R. tropici* strains. The two
Agrobacterium species also grouped together. However, some of the other isolates show less stringent matches to rhizobia. For instance, MOHv was identified as Labrys portucalensis but the majority of matches were for members of the Rhizobia, in particular Mesorhizobium, albeit with reduced homology of 92%. Missing from the molecular identification are any members of the genus Bradyrhizobium, which are reported to be the most common native nodulator in most studies that have undertaken investigations into indigenous rhizobia (Marsudi et al., 1999; Lafay and Burdon, 1998; Lafay and Burdon, 2001; Lafay and Burdon, 2007).

MOIa2, which formed nodules in the re-inoculation trial, was not included in the phylogenetic tree because examination of the entire 16S rDNA sequence showed that the sequence was a chimera. The first 500 nucleotides, located at the 5’ end of the 16S gene (from base pair positions 17-585) used for constructing the phylogenetic tree and identification of isolates was shown in Table 2.7. This provided an identification of a previously uncultured Bacillus species clone JPL-S3 M01. However, submission of a 369 bp fragment (from base pair positions 1069 to 1439) to GenBank returned an identification of Rhizobium. The phylogeny program was unable to include this fragment into the analysis.

2.4.4 Re-inoculation Trial
The re-inoculation trial showed that despite some inconsistent results from the other methods used for identification, the most definitive test for the presence or absence of root-nodulating bacteria was re-inoculation onto a suitable host legume (Vincent, 1970) in this case M. atropurpureum. The results in Table 2.7 and 2.8 show that 22 of the 34 isolates produced root-nodules, demonstrating that despite mixed cultures and molecular identification of unlikely nodulating bacteria such as Bacillus species, nodulating rhizobia were still viable within the cultures.

2.4.5 Synthesis
Each of the methods and tests used in this study add to the final goal of culturing and identifying root nodulating bacteria. Where ambiguities exist in identification in the “downstream” techniques of IAR and 16S rDNA, these can be attributed to the inability to obtain pure cultures of the appropriate rhizobia.

The antimicrobial disc susceptibility test was originally envisaged to be a relatively simple and quick method of distinguishing the isolates without necessarily
identifying them. Unfortunately, due to the inability to obtain pure cultures of all isolates and the varied nature of the nodulating species that were identified by 16S rDNA, it is with hindsight unlikely that this method would prove to be useful in the context it was intended for in this study. Where the use of antibiotics may have proved useful was to obtain pure cultures, with the reduction or elimination of Gram-positive bacteria from mixed cultures. As has been mentioned, this was observed in the course of the AST work where lawns of Gram-positive bacterial growth were prevented from growing in the diffusion halo of an antimicrobial disc, leaving small round colonies within the halo, but the significance of the results was not realised at the time.

While the number of rhizobia identified was not as high as was expected given the number of nodulating isolates in the re-inoculation trial, there were some aspects of the molecular identification worth highlighting. There were 22 unique species or strains identified from the 34 isolates cultured (Table 2.7). Of these, six were previously uncultured isolates that had been identified by the use of culture independent methods, such as MODg, not yet officially classified, but closely related to E. coli from the phylogenetic tree in Fig 2.5. Further characterisation of these isolates and investigation of their role in soil ecology is warranted for a number of reasons.

The rhizosphere, that is the zone of soil that is influenced directly by plant roots both physically and chemically, is known to have a much higher population of soil micro-organisms (not just bacteria) than bulk soil (Jones et al., 2004). This is due to plant root exudation of sugars, hormones, amino acids, signalling molecules and the physical sloughing of dead cells and root turnover (Paterson, 2003; Jones et al., 2009). It is highly likely that many of the isolates that were inadvertently cultured were rhizosphere-inhabiting bacteria, are part of this community and may not promote or hinder plant growth. Their characterisation is nevertheless beneficial for expanding the knowledge of soil ecology. Isolates MOHv (L. portucalensis) and MOGclan3 (Chitinophaga, a gliding bacterium) may fit into this category. Both Burkholderia and Pseudomonas species have been known to act as plant growth promoting rhizobacteria (PGPR), as well as being documented nitrogen-fixing symbionts. They benefit plant growth through the production of plant hormones such as indole acetic acid, siderophores and phosphate solubilizing enzymes amongst others (Bloemberg and Lugtenburg, 2001; Chung et al., 2005; Richardson et al., 2009), and, or by acting as suppressors of detrimental micro-organisms (Piex, et al., 2001).
There is also the possibility of new strains of root-nodulators. As raised in the introduction, several new species of bacteria have been documented that form nitrogen-fixing root nodules. Certainly the number of *Burkholderia* species identified in this study is consistent with this possibility. Indeed, it is proposed that isolate RSFAp2 is one such new strain of nitrogen-fixing bacterium. This isolate was re-inoculated onto *M. atropurpureum* and the nodules re-cultured and sequenced on three separate occasions. Each re-inoculation produced nitrogen-fixing nodules (as determined by the survival and growth of the seedling in a nitrogen free medium) and the subsequent sequencing returned near identical results. These results strongly indicate that this isolate is a novel root-nodulating strain of *Burkholderia*. At this stage of analysis, the previously uncultured *Pseudomonas* clone CF7, identified as isolates MOAimp1 and 2 and MOAdcor, could also be a new nodulating strain of this genus. The isolate that was most frequently identified in this study, uncultured bacterium clone AKIW800, which is closely related to *Burkholderia*, appears not to be a novel nodulator given that in one of the five cultures containing this isolate, BAm did not nodulate, indicating that it is more likely to be a nodule co-habiting strain of *Burkholderia* (Table 2.8).

*Rhizobium miluonense* str CC-BL6 also appears to be an indigenous rhizobia, and may be newly reported in Australia. This strain is a relatively new species reported from China by Gu *et al.*, 2008 as a novel nodulator of *Lespedeza* spp.

Another possibility is that some of these isolates may be co-inhabitants of root nodules (Smith, 1989; Theis *et al.*, 2001). As with the relatively recent discovery of novel nodulating species of bacteria, the realisation has come that root nodules may harbour multiple strains of rhizobia, and even multiple species of bacteria (Wang *et al.*, 2006; Liu *et al.*, 2010). It has been hypothesised that in those occasions where more than one strain of rhizobia has been found to occupy a root nodule, only one is actually capable of nitrogen fixation, and the other, or others are endophytic. However, the precise nature of this relationship is yet to be elucidated.

Entry into the root nodule would occur at time of infection, whether by root hair curling or root crack entry. Presumably the superfluous bacteria would “piggyback” with the nitrogen-fixing rhizobia. Thus some of the six mixed cultures that produced root nodules in the re-inoculation trial may have resulted from this scenario. It is also not possible to rule out that many of the cultures that were observed to be entirely Gram-negative bacteria were not in fact mixed cultures.
2.4.6 Recommendations for Specificity Trial

The aim of this work was to collect, isolate, culture and identify rhizobial bacteria for use in a specificity trial to determine their ability to promote the growth of selected native legumes. Twenty-three of the 34 isolates cultured have been shown to form root nodules when re-inoculated onto *M. atropurpureum*. While there are apparently several duplicates of the same bacterial species, each isolate will continue to be treated as unique. If the pattern of nodulation is the same when inoculated onto the same host species, it will add to the conclusion that they are indeed the same species of bacteria.

Of the remaining 12 isolates, nine will be excluded from the specificity trial as they did not re-nodulate *M. atropurpureum*. Three isolates that have been identified as belonging to the genus *Rhizobium* but did not re-nodulate on *M. atropurpureum*, or to a cluster of isolates that otherwise nodulated (uncultured bacterium clone AKIW800, BA$m$) will be included in the specificity trial and may be considered as negative controls.
Chapter 3.

Specificity Between Native Legumes and Rhizobia
3.1 Introduction

The concept of specificity, the range of infection exhibited between the host legume species and symbiotic rhizobium species in the legume-rhizobia symbiosis, started to be investigated at the end of the 19th century in Europe (Breal, 1888; Nobbe et al., 1895 reviewed in Perret et al., 2000). It is now widely recognized that different species of both host legumes and symbiotic nitrogen-fixing bacteria can exhibit variation in their ability to nodulate, or cause nodulation, that is, specificity for their respective symbiotic partners. Thus a spectrum exists from symbiotic partners with a very narrow ability to nodulate with the other partner, to legumes and bacteria that are very promiscuous in their ability to nodulate. For instance, for host legume species, *Lotus corniculatus* has a narrow range, only nodulating with a limited number of *Mesorhizobium loti* strains and *Bradyrhizobium* species (Leiven-Antoniou and Whittam, 1997). In contrast, the tropical pasture species *Macroptilium atropurpureum* is known as a highly promiscuous nodulator, able to nodulate with a wide range of *Bradyrhizobium* and many *Rhizobium* strains. For this reason *Macroptilium atropurpureum* is used widely as an experimental model to test the ability of an uncharacterised rhizobial species to form nodules (Date and Hurse, 1991; Perret, et al., 2000; Parker, 2002; Barrett and Parker, 2005), although recently it has begun to be replaced by re-inoculation onto locally obtained hosts. Examples of specificity between bacteria and their legume hosts include *Sinorhizobium meliloti* that only nodulates with members of the *Medicago, Melilotus* and *Trigonella* genera, while *Rhizobium* sp. NGR234 is very promiscuous, nodulating with species from over 35 different genera of legumes (Mulder et al., 2005).

Historically legume-rhizobia classification was based on specificity and was termed the cross-inoculation concept (Chanway et al., 1991). For instance, tropical legumes nodulated with slow-growing rhizobia, while temperate legumes nodulated with fast-growing rhizobia (Fred et al., 1932 in Dakora, 2000). But, as more and more species from different legume genera, and not just economically and agriculturally important species, are tested for specificity, the cross-inoculation group system has been shown to be an inappropriate system of classification and largely has been abandoned in favour of molecular taxonomy.

In addition to the specificity outlined above between symbiotic partners, when nodulation is successful, there is variation in the effectiveness of nitrogen supply to the host. There is a spectrum of symbioses that ranges from rhizobia that can be highly
effective at promoting growth, to rhizobia that are parasitic and do not provide any benefit to the host plant (Burdon et al., 1999; Deaker et al., 2004; Denison & Kiers, 2004).

With these two aspects of legume-rhizobia symbiosis in mind: infectivity and effectiveness, the provision of suitable nodulating partners for selected host legumes becomes a more complicated process. Potential symbionts must be matched to determine if nodulation is possible, and then evaluated to rank successful combinations for their ability to influence plant growth.

The aim of the experiments described in this Chapter was to select a rhizobial symbiont or isolate as a suitable inoculum to facilitate growth of a selected group of native legume hosts in the field, using appropriately identified isolates collected from legume hosts found in the Hunter Valley (see Chapter 2). The isolates were tested for their ability to form functional nodules based upon the presence or absence of leghaemoglobin and their effectiveness in promoting the growth of their legume hosts by measuring shoot biomass gain. Finally, a measure of the relative efficiency of the host-isolate combination was made informed by estimates of shoot:nodule dry weight ratios.

3.2 Materials and Methods

3.2.1 Host Plant Selection

Within the over-arching requirement of the mining consent to re-construct native forest on the spoil dump, six native legume species were selected for trial based on the following criteria specifically relating to this trial:

- The rehabilitation of Mount Owen open-cut coalmine requires that the species be endemic to the upper Hunter Valley (described in Harden, Flora of NSW, 1991), and that they have been identified from flora surveys as being widespread, or present within Ravensworth State Forest.
- Species should be representative of as many different vegetative strata as possible (Specht and Specht 2002) including the herb layer (plant height <0.5m), tall herbs and sub-shrubs (plant height to 1m), shrubs (plant height to 2m), tall shrubs and small trees (plant height to 10m).
- Seed had to be readily collected from the Ravensworth State Forest and surrounding areas or where that was not possible, from commercial sources.
These criteria resulted in selecting the following host plants:

- *Hardenbergia violacea* (Schneev.) Stearn, a sprawler-climber that also has an erect form to 0.5m in height;
- *Daviesia ulicifolia* Andr., an erect shrub between 0.5m and 1.0m in height;
- *Indigofera australis* Willd., a shrub from 1.0m to 1.5m in height;
- *Acacia amblygona* A. Cunn. ex Benth., a low spreading shrub up to 1.5m in height;
- *Pultenaea retusa* Sm., an erect shrub between 0.5m and 2.0m in height;
- *Acacia parvipinnula* Tindale, a tall shrub or small tree, 2.5m to a reported 10m in height, (although it has not been observed above 4-5m in height in the Ravensworth State Forest) and is the dominant middle storey tree in the Ravensworth State Forest.

### 3.2.2 Seed Preparation

Seeds were obtained from collections made from the Ravensworth State Forest and commercial suppliers (see Appendix 3 for seed sources). All legume species seeds were sterilised by washing in 70% EtOH. All were pre-treated to break dormancy. With the exception of *P. retusa*, seeds were scarified by gently abrading the seed coat with fine grade sand paper, and soaked in hot water of approximately 80 °C. Thereafter, seeds were imbibed until swelling was observable (Langkamp, 1987). For *P. retusa*, seeds were heated in a forced-draught oven at 80°C for three minutes before soaking overnight, until imbibed. Imbibed seeds were germinated on aqueous agar (1.5% w/v) in 90-mm Petri dishes. Once germinated (defined as radicle emergence), seedlings were transferred to growth tubes as described in Section 3.2.3 below.

### 3.2.3 Plant Growth Conditions

A series of experimental trials examining specificity were conducted, over a two year period due to space limitations, in a walk-in growth room manufactured by Thermoline P/L (Sydney). Environmental conditions consisted of a day/night cycle of 14/10 h respectively and a 26/15°C diurnal temperature range. Photosynthetically active radiation was ramped to mimic daily increases and decreases of solar radiation associated with the diurnal rhythm (Fig. 3.1). Maximum photosynthetically active radiation of approximately 675 µmol m⁻² s⁻¹ was incident on the leaf surfaces for seven hours per cycle. Lighting was provided by 14 Venture® 1000 w mercury halide globes
arranged in four banks and by 20 Compton 100 w incandescent globes arranged in 2 banks.

Seedlings were initially grown in square 50 mm x 50 mm x 160 mm tall PVC growth tubes in a 3:1:1 washed river sand : perlite : coconut fibre mix. While this mix proved satisfactory for plant growth, coconut fibre made harvesting and identification of root nodules extremely difficult. Subsequent rounds of the trials were conducted omitting coconut fibre from the soil mix.

PVC growth tubes were placed in 700 mL rectangular plastic containers, with a maximum of three growth tubes per container (Fig 3.2A). All seedlings within each container were inoculated with a single bacterial isolate.

All seedling/bacterial isolate combinations were organised into a randomised block design with 3 replicates per combination. The blocks were rotated within the growth room and the position of individual seedling containers randomised within each block on a weekly basis (Fig 3.2 A & C). One uninoculated, -N control of each species was included within each block (Fig 3.2 B). Due to the space limitations mentioned previously, the specificity trials were conducted in series. The six hosts species were divided into two groups of three species *A. parvipinnula*, *H. violacea* and *P. retusa* in group 1 (Fig 3.2A) and the remaining three hosts *A. amblygona*, *D. ulicifolia* and

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**Fig 3.1** Stepping of the photosynthetically-active radiation used in the growth room for the specificity trials.
I. australis in the second group. Isolates were randomly selected and inoculated onto hosts, resulting in five isolates per series per group of host species.

During the initial trial, growth tubes were open exposing seedlings to growth room conditions, which, due to room design, had strong air currents blowing whenever lights were operating. Incorporating uninoculated seedlings proved that cross-contamination did not occur under these growth conditions, but seedling desiccation proved to be a problem. Therefore, in subsequent trials, growth tubes were positioned inside plastic cups covered with 90 mm Petri dish lids (Fig 3.2 D). This arrangement reduced seedling mortality, and further reduced any likelihood of cross-contamination from airborne particles and water droplets.

Seedlings were watered twice weekly with 30 mL of distilled water and weekly with 30 mL of –N modified Hoagland’s nutrient solution (Appendix 2).
3.2.4 Bacterial Inoculation of Seedlings
Rhizobial isolates were grown in yeast-mannitol liquid culture, pH 7, for 96 h at 28°C on an orbital shaker at 120rpm (see Chapter 2). No attempt was undertaken to maintain the same bacterial concentration between the different isolates. However, each individual isolate (e.g. RSFAp2) was subject to the same growth conditions and the same volume (1 mL) of liquid culture was used for all inoculations of host legumes. This ensured a similar number of bacteria for each of the individual isolates was applied as inoculum for each round in the specificity trials.
Seedlings were acclimatised to growth room conditions for 1 week before inoculation. Each growth tube was inoculated by syringing 1 mL of rhizobial solution at the base of the hypocotyl.

3.2.5 Plant and Nodule Harvest
Seedlings were grown for 8 to 12 weeks post inoculation before harvesting, with duration of growth varying by block. At harvest, plants were removed from growth tubes and the sand-perlite mix washed from their roots. The plants were separated into root, shoot and nodule components, dried in a forced-draught oven at 80 °C for 48 h and thereafter weighed using a Mettler A340 four place electronic balance. Nodule numbers were not recorded.

Plants were scored for presence/absence of nodules, no nodules were found on uninoculated controls. If present, nodules were classified as functional or non-functional based on visual inspection. Here nodules were cut in half and examined for presence of leghaemoglobin, as indicated by a dark red to brown colouration (Fig 3.3). The presence of a single functional nodule was used to classify that isolate as functional.
3.2.6 Determination of Isolate Effectiveness

Effectiveness of growth promotion was determined by comparing shoot dry weight growth rates of the host plants, modified from Burdon et al., (1999). \(-N\) uninoculated controls were not used as growth references to compare to inoculated plants as the uninoculated controls became moribund within 4 - 6 weeks of growth. \(+N\) plants were not used as growth references due to the uncertainty of fertilizer application rates needed to obtain optimum growth for these native species. Plants were harvested, dried in a force draught oven at 80\(^\circ\)C for 48 hours and dried weight of stems recorded. The isolate’s level of effectiveness was determined by comparing growth attained by the best performing isolate in the trial for each host species. Highly effective isolates produced growth rates greater than 70% of the best performing isolate, moderately effective isolates achieved growth rates between 70% and 40% and ineffective isolates produced growth rates below 40%.

![Fig 3.3 Photograph of three nodules harvested from D. ulicifolia each cut in half to display the red-brown colour characteristic of the presence of leghaemoglobin. Large grid is 5 mm square.](image)

3.2.7 Statistical Analysis

Data were analysed using two-factor ANOVA with partitioning of variance. If sums of squares contributed by blocks were low, c.a. 10%, they were pooled as replicates and the data re-analysed using one factor ANOVA. In all analyses, a Cochran’s test was conducted to assess data for homogeneity. Non-homogenous data were then transformed using log, square root, arcsine or reciprocal methods. With block analysis, Cochran’s test is not possible on the two factor ANOVA, as essentially \(n = 1\).
The data are illustrated using least significance difference (LSD) bars to indicate the difference between means required to produce a $p<0.05$, $p<0.01$ or $p<0.001$ level of significance. Where there were no significant differences at the $p<0.05$ level, the remaining LSD bars have not been shown.

Letters above the data columns indicate whether mean values were statistically significant from one another at the $p<0.05$ level. Where data have been transformed due to heterogeneity, the data are displayed in the raw form and LSD’s are not indicated.

3.3 Results

3.3.1 Nodulation Ability – Bacterial Isolates

The 23 isolates that formed nodules when inoculated onto *M. atropurpureum* in the re-inoculation trial in Chapter 2 were inoculated onto six host legumes. Additionally, three isolates that did not nodulate *M. atropurpureum* were included in the trial to verify that they would not nodulate the target hosts, effectively acting as negative controls. These were BAli and MOPspin3 identified as *R. tropici* str. LNP7 and LNW10 respectively, and BAm identified as one of the five uncultured Bacteria AKIW800 isolates, the other four of which nodulated. These isolates are given special mention here as they were either identified as Rhizobium, (BAli and MOSpin3) or were part of a group of isolates that did nodulate (BAm). They did not nodulate in the specificity trial, and their data are not shown.

Of the nodulating isolates, 39% (9) isolates, termed generalist nodulators, nodulated all six hosts, while 61% (14) were capable of nodulating at least one host species. The generalist nodulators were divided into two smaller groups; six were able to form functional nodules on all host species, and have been labelled “Functional Generalists”. Another three isolates formed functional nodules on five host species and non-functional nodules on the remaining host species *P. retusa*. These have been termed “Semi-functional Generalists” (Table 3.1).
Table 3.1 Summary of the specificity trials showing the relative abilities of 23 bacterial isolates to form nodules with each host plant species. ++ = functional nodules formed (red), + = non-functional nodules formed (green), - = no nodules formed. Isolates are grouped by heavy outline boxes according to ability to nodulate. First six isolates are functional generalist nodulators. Following three isolates are semi-functional generalist nodulators, followed by remaining nodulating isolates. See footnote for a key explaining isolate designations.

<table>
<thead>
<tr>
<th>Bacterial Isolates &amp; Source Hosts</th>
<th>Target Host Plant Species</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A. parvipinnula</td>
</tr>
<tr>
<td>Host Name</td>
<td></td>
</tr>
<tr>
<td>MOAdcor</td>
<td>Uncult. bact clone CF7</td>
</tr>
<tr>
<td>MOAmp2</td>
<td>Uncult. bact clone CF7</td>
</tr>
<tr>
<td>MOAm</td>
<td>Uncult bact AKIW800</td>
</tr>
<tr>
<td>MOAsal2</td>
<td>Rhizobium sp. CHNTR53</td>
</tr>
<tr>
<td>RSFAmb2</td>
<td>Bacillus sp. Tp14</td>
</tr>
<tr>
<td>RSFAp2</td>
<td>Burkholderia sp. CC-S-L25</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>BAp2</td>
<td>Uncult bact AKIW800</td>
</tr>
<tr>
<td>MOAsal1</td>
<td>A. tumefaciens str H2P4</td>
</tr>
<tr>
<td>MOKr</td>
<td>Bacillus cereus str BFE 5392</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>BAp1</td>
<td>R. miluonense str CC-BL6</td>
</tr>
<tr>
<td>BAa</td>
<td>Uncult bact AKIW800</td>
</tr>
<tr>
<td>RSFAp1</td>
<td>Uncult Acinetobacter sp.</td>
</tr>
<tr>
<td>MOAmp1</td>
<td>Uncult bact clone CF7</td>
</tr>
<tr>
<td>MOAa3</td>
<td>Bacillus sp. Tp14</td>
</tr>
<tr>
<td>MOK</td>
<td>Bacillus sp. Pe2T</td>
</tr>
<tr>
<td>BDu1</td>
<td>Rhizobium sp. CHNTR53</td>
</tr>
<tr>
<td>MOGclan3</td>
<td>Chitinophaga sp. CS5-B1</td>
</tr>
<tr>
<td>MOHv</td>
<td>Labrys portocalensis</td>
</tr>
<tr>
<td>MOTs</td>
<td>Rhizobium sp. CHNTR53</td>
</tr>
<tr>
<td>MODu</td>
<td>Burkholderia sp. TAt-045</td>
</tr>
</tbody>
</table>

Total Isolates Nodulated (%)  
96  87  83  78  52  87

% Functional Nodules of Nodulating Isolates  
100  90  89  100  92  40

Bacterial isolates are denoted by collection site B = Beresfield (Donaldson Coal Mine), RSF = Ravensworth State Forest and MO = Mount Owen coal mine rehabilitated site. Following letters indicate host plant species that nodules were collected from, with the final number identifying multiple collections from a host plant species. See Chapter 2 Section 2.2.1 listing plant species from which bacterial isolates were collected.
3.3.2 Nodulation Ability – Host Plants

Host plant species also showed considerable variation in their nodulation capacity and their functionality with the isolates tested (Table 3.1).

No single host species nodulated with all 23 isolates. *A. parvipinnula*, the most promiscuous host, nodulated with 96% (22 from 23) of the nodulating strains. The isolate that did not nodulate *A. parvipinnula* (of the subset of nodulating isolates) was MODu1, a *Burkholderia* species that only formed non-functional nodules with *A. amblygona*. Four of the remaining host species *P. retusa*, *H. violacea*, *A. amblygona* and *I. australis* were marginally less promiscuous in their capacity to nodulate with all isolates, nodulating 87%, 87%, 83% and 78% respectively (Table 3.1). The host *D. ulicifolia* was the exception, nodulating with only 52% (12 from 23) of isolates.

Nodule functionality also varied with host species (Table 3.1). Two hosts, *A. parvipinnula* and *I. australis* formed 100% functional nodules. Three hosts, *A. amblygona*, *H. violacea* and *D. ulicifolia* formed functional nodules with 89% to 91% of the nodulating isolates. Only *P. retusa* showed considerable variation from this pattern nodulating with a large number of isolates, but only 40% (8 from 20) of these formed functional nodules.

3.3.3 Nodulation Effectiveness on Host Growth

Bacterial isolates produced a range of growth rates on the host legumes (for primary data see Appendix 5 which divided the isolates into highly effective, moderately effective and least effective at growth promotion (Table 3.2).

With the exception of *D. ulicifolia*, the number of highly effective isolates varied only slightly between the hosts. The remaining hosts had between 15 and 22% (ie. 3-4) isolates that were evaluated as highly effective.

The largest variation between the hosts was in the proportion of moderately effective isolates, where *A. parvipinnula* and *H. violacea* had <40% (9/22 and 9/20 respectively) of the nodulating isolates promoting moderate growth, while at the other extreme, *A. amblygona* and *P. retusa* had only 5% (1/19 and 1/20 respectively) moderately effective isolates.
The composition of the bacterial isolates that were classified as highly effective varied between the legume hosts (Table 3.3). Eleven different isolates accounted for the 19 possible combinations (the sum of the highly effective isolates for the six hosts, e.g. *A. parvipinnula* had four isolates plus *H. violacea* with three and so on) further indicating that there was only a low level of duplication across the hosts. Six isolates were recorded as highly effective on a single host, leaving five isolates occurring on multiple hosts. MOKr and MOAsal1 were the most commonly occurring highly effective isolates, with four and three hosts respectively.

Table 3.3 Identity of the highly effective bacterial isolates and the shoot biomass produced, for each of the host legumes. Bacterial isolates are ranked highest to lowest, left to right, in order of production of shoot dry weight.
3.3.4 Correlation Between Shoot Dry Weight and Nodule Dry Weight

Plotting the shoot dry weight against the nodule dry weight (standardised for variations in growth time) produced a series of graphs examining the correlation between the two parameters (Appendix 5). Therefore higher shoot:nodule ratios indicate the symbioses are utilizing the nitrogenase-dependent process, whereas lower shoot:nodule ratios indicate the biomass-dependent process.

The mean shoot:nodule dry weight ratios for each of the host legumes are shown in Table 3.4. Examination of the mean shoot:nodule dry weight ratios showed that host species produced varying shoot biomass for given nodule mass, indicating the overall efficiency of the host legume and rhizobia symbionts at producing biomass. Based upon these shoot:nodule ratios *A. parvipinnula* was the least efficient host with a mean ratio of 7.48, while *A. amblygona* was the most efficient with a mean ratio of 20.60.

**Table 3.4** Mean shoot:nodule dry weights for each of the host legumes and correlations of the bacterial isolates shown in Fig 3.4.

<table>
<thead>
<tr>
<th>Host Species</th>
<th>Mean Shoot:Nodule Ratio</th>
<th>Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. parvipinnula</em></td>
<td>7.48</td>
<td>0.8438</td>
</tr>
<tr>
<td><em>P. retusa</em></td>
<td>7.67</td>
<td>0.9939</td>
</tr>
<tr>
<td><em>H. violacea</em></td>
<td>9.17</td>
<td>0.8505</td>
</tr>
<tr>
<td><em>I. australis</em></td>
<td>11.34</td>
<td>0.6909</td>
</tr>
<tr>
<td><em>D. ulicifolia</em></td>
<td>11.75</td>
<td>0.7058</td>
</tr>
<tr>
<td><em>A. amblygona</em></td>
<td>20.60</td>
<td>0.9350</td>
</tr>
</tbody>
</table>

Individual bacterial isolate shoot dry weights and nodule dry weights were plotted and regressions calculated (Fig 3.4) and an analysis of covariance performed. The highly effective isolates and the trial inoculum isolate are labelled in the figures. Comparison of the slopes of the regressions produced in Fig 3.5 using an analysis of covariance showed that there were significant differences between each of the host legumes (Table 3.5).

**Table 3.5** ANCOVA table for the comparison of the regressions from for the shoot:nodule dry weight ratios in Fig 3.4.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>Fs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adjusted means (among species)</td>
<td>5</td>
<td>0.0578</td>
<td>0.0116</td>
<td>1.7136</td>
</tr>
<tr>
<td>Adjusted means (common slopes)</td>
<td>100</td>
<td>0.6749</td>
<td>0.0067</td>
<td></td>
</tr>
</tbody>
</table>
The highly effective isolates were chosen based on their ability to promote shoot growth; however, the nodule biomass required to produce the shoot biomass varied depending upon species of host legume and isolates. For instance, isolate MOKr was a highly effective isolate for four of the hosts and the most effective for two of these (Fig 3.4). Where it was the most effective, *A. amblygona* and *D. ulicifolia* it also produced the greatest mass of root nodules, with shoot:nodule ratios of 17.47 and 12.22 respectively. It should be noted that this isolate also produced the greatest nodule biomass for *H. violacea*.
Host legumes can be divided into two groups based on the relative performance of the most effective isolate. For three hosts, *A. parvipinnula, H. violacea* and *I. australis* the most effective isolate did not produce the greatest nodule biomass. For the remaining three hosts, *A. amblygona, D. ulicifolia* and *P. retusa* the most effective isolate also produced the greatest nodule biomass.

### 3.3.5 Inoculum Selection

Given the concurrent nature of the experimental work performed, an early decision was made on the selection of an inoculating strain for shadehouse and field trials. RSFAp2 was chosen, as it was the first of the functional generalist nodulating isolates identified. In terms of growth promotion, this isolate was highly effective on one host, *A. parvipinnula*, moderately effective on four hosts, *H. violacea, A. amblygona, D. ulicifolia* and *P. retusa* and fell into the least effective category for the remaining host, *I. australis*.

### 3.4 Discussion

#### 3.4.1 Specificity and Effectiveness

Host promiscuity for nodulation with a range of rhizobia is reported for both Mimosaceae and Fabaceae suggesting that promiscuity is common and is not confined to any particular plant taxonomic group or growth habit (Perret et al., 2000). Similarly, many studies investigating relationships between native Australian legumes and rhizobia have found limited instances of specificity in terms of nodulation ability, for example, *Acacia* species (Barnet and Catt, 1991; Marsudi et al., 1998; Burdon et al., 1999) and Fabaceae (Lafay and Burdon, 1998). In this study examples of host legumes that were relatively promiscuous such as *A. parvipinnula* and *H. violacea* contrast with hosts that were more specific with regards to nodulation, for example, *I. australis* and *D. ulicifolia* (Table 3.1). This indicates that at least on the local scale specificity for nodulation can vary widely between native legumes.

Differences in rhizobial promiscuity/specificity with regards to nodulating hosts has also been reported, and in this study rhizobial isolates displayed a high degree of difference. One isolate, MODu (*Burkholderia* sp.) only nodulated one host, while several isolates, the functional generalists nodulated all hosts (Table 3.1). Thus, the
ability to form symbioses is not controlled by one party, but results from the interaction of the macro- and micro-symbiont. The ability to form symbioses with a wide range of symbionts may confer adaptive advantages to the promiscuous hosts or rhizobia (Murray et al., 2001). Promiscuous legume hosts able to form symbioses with different species of rhizobia may have the opportunity to become more widespread, increasing their chances of survival when spread over large geographic ranges, although there are conflicting reports where this hypothesis appears to have been confirmed (Thrall et al., 2000) and discounted (Doignon-Bourcier et al., 2000).

Rhizobia are reported to be facultative symbionts and indeed there are many strains that do not nodulate at all, having lost or not acquired the ability to nodulate (O’Hara, 2001; Thrall et al., 2001). However, their ability to survive and persist in the soil without engaging a host is highly species and strain variable and ranges from a season or two, to many years (Carter et al., 1995; Howieson, 1995). Therefore, promiscuity by rhizobia in their selection of hosts is one method of ensuring survival. With competition in the rhizosphere for nodulation sites on legume roots reportedly intense, specificity can impart survival advantages for rhizobia by ensuring “its place at the table”. The trade off being that the rhizobia must be saprophytically competent to survive in the soil environment if the host is not present. Conversely, for legumes, specificity can ensure that rhizobia of lower effectiveness will be excluded from forming symbioses, and thus increasing its, the legume’s, competitive advantage. However promiscuity of nodulation does not necessarily lead to indiscriminate nodulation, with at least one promiscuous host, Phaseolus vulgaris (common bean) able to exert specificity in mixed rhizobial populations (Aquillar et al., 2001; Martinez-Romero, 2003).

Ability to nodulate does not guarantee effectiveness of nitrogen supply. Variation in effectiveness is well documented in the literature, both in agricultural systems (Slattery and Pearce, 2002; Deaker et al., 2004; Herridge et al., 2008) and in Australian non-agricultural systems (Burdon et al., 1999; Thrall et al., 2000). The former forms the basis of the commercial inoculant industry.

This range of effectiveness can also lead to symbiotic “partners” that have moved beyond any actual mutualism and have become parasitic in their association, thus benefiting only one member, usually the microbe (Thrall et al., 2001; Thrall et al., 2007). Thus some rhizobia, such as those that have lost the ability to fix nitrogen
mentioned above, may be perpetual “cheaters”, able to infect hosts without providing nitrogen in return, or the relationship may change depending upon internal or external factors (Neuhauser and Fargione, 2004). These may include increasing soil fertility, drought or other stressors (Thrall et al., 2007). As an ecosystem becomes more complex through increasing resources, or maturity with increased structure and interactions between hosts, there is an increase in the likelihood that cheating will increase. The ability for hosts to enact sanctions or exert influence on non-performing partners becomes more important.

Thus recruiting potential symbionts from nodules in old growth or mature systems may lead to the increased possibility of culturing isolates that will be of no benefit in promoting plant growth and hence not useful for revegetation projects. This increases the need for specificity trials as performed here.

Effectiveness of legume-rhizobia combinations ranged from totally ineffective, where only non-functional nodules were formed to what were considered to be highly effective combinations. For example, with *A. amblygona*, isolate MODu (a *Burkholderia* sp. TAt-045) and MOTs (a *Rhizobium* CHNTR53) formed non-functional nodules, whereas isolate MOKr (*Bacillus cereus* str. BFE 5392) was the most effective (See Appendix 4 for primary data).

The actual number of highly effective isolates was consistent across five of the host species, with three or four isolates each, *D. ulicifolia* being the exception (Table 3.2). What was not as consistent was the composition of these isolates (Table 3.3). No single isolate was highly effective for all hosts. The two most commonly effective isolates, MOKr and MOAsal1, were highly effective on four and three hosts respectively. A total of eleven different isolates, (almost half of the total) were highly effective for at least one host, but interestingly seven of these were sourced from *Acacia* hosts, suggesting a higher degree of specificity by the Fabaceae hosts from which the isolates were collected. Thus the performance of one isolate on a particular host could not be used to predict its performance on another.

To date this study has separated specificity from effectiveness, but the two could in fact be linked, where an effective host-isolate combination can be considered to have displayed a greater degree of specificity than a less effective combination.
3.4.2 Determination of Effectiveness

In this study the determination of effectiveness was by production of shoot biomass of a host-isolate combination when compared to the isolate that promoted the greatest growth for that host (Table 3.2). This produces a measure of relative effectiveness, rather than an absolute measure of the effectiveness of the host-legume combination.

Most commercially available crop species have been grown for many decades, if not longer. Legume crops have been evaluated for their performance with appropriate rhizobial symbionts and these have in many cases been formulated into commercially available inoculants and introduced around the world (Howieson et al., 2000). Thus any new rhizobia that may be used as an inoculum can be trialled against these known benchmarks and the resulting harvest of commercial product evaluated (Collins et al., 2002; Fening and Danso, 2002; Slattery and Pearce, 2002). Mineral supplied nitrogen can also be used as a control (Fening and Danso, 2002; Slattery and Pearce, 2002; Musiyiwa et al., 2005).

However, unless a species of native plant is of particular economic importance, particularly rare or iconic from a cultural or ecological viewpoint, it is unlikely to have been subjected to the same level of scrutiny as commercial species. Without this level of research comparisons to inoculating strains or applications of mineral nutrients cannot be made. It would be necessary to perform dose-response experiments to assess optimum nitrogen levels, and given the reported adaptations of most Australian natives to low levels of phosphorus (Handreck, 1997) and the coupling of nitrogen fixation to levels of available phosphorus in other non-native species (Stamford et al., 1997; Almeida et al., 2000; Reed et al., 2007) and Australian native species (Adams et al., 2002) suggests double dose-response experiments (matching levels of nitrogen and of phosphorous to produce optimum growth) may be required on a species by species basis. Therefore another method of determining effectiveness is required.

The measure of relative effectiveness used here was adapted from Burdon et al. 1999, where relative performance is compared to the isolate-host combination that produced the highest shoot dry weight for that species. Without a statement as to why particular levels of effectiveness were chosen, it was assumed that ineffective isolates would not have produced successfully reproducing plants under field conditions (see Chapter 4).

Relative effectiveness may also be made by comparison to isolates that produced non-functional nodules, such as MOGlan3 (Chitinophaga sp.) with H. violacea, D.
ulicifolia and P. retusa, or MOTs (Rhizobium sp.) and MODu (Burkholderia sp.) for A. amblygona (Table 3.1). These isolates produced nodules and presumably have a carbon draw down upon the host, without any nitrogen return. However, not all hosts had non-functional isolates so that this method was not uniformly applicable.

3.4.3 Correlation of Shoot:Nodule Ratios and Implications for Nitrogen
The original purpose of the inclusion of analysis of shoot:nodule ratios (Fig 3.4) was to provide a measure of nitrogen supply (by the root nodules) or use (by the host legumes) efficiency as an aid to selection of an inoculum.

Legumes and rhizobia can increase their ability to fix nitrogen by employing two strategies that form the end points of a continuum. At one end, they can increase the biomass of nodules where nitrogen is fixed by producing more nodules or larger nodules or both. At the other end, they can increase the specificity of nitrogenase, the enzyme responsible for nitrogen fixation, that is, improve the efficiency of nitrogen fixation per unit of nodule mass. These two processes can be synergistic or antagonistic (Schortemeyer et al., 2002).

Legume-rhizobia combinations where increases in nodule biomass were responsible for increases in shoot biomass occur for three of the legume species trialled in this study (Fig 3.4). The most effective rhizobial isolate for A. amblygona, D. ulicifolia and P. retusa produced the greatest nodule biomass (and shoot biomass). This was not the case for the other three legume species where the most effective rhizobial isolate did not result from the greatest nodule biomass. This suggests that for A. parvipinnula, H. violacea and I. australis an increase in nitrogenase specificity is responsible for the increase in shoot biomass, at least for the most effective isolate. Both processes, and the continuum between, would appear to operate in the majority of legumes. Legume-rhizobia combinations favouring the high nodule biomass end of the spectrum return lower than the average shoot:nodule ratio for that host and the rhizobia in question, whereas combinations that favour the nitrogenase process would have higher than average shoot:nodule ratios. H. violacea (Fig 3.4) provides an example of this with the three most effective isolates. This host had an average shoot:nodule ratio of 9.17 (Table 3.4), with isolate MOAsal2 and MOX both higher than the average with 16.89 and 12.53 respectively, while MOKr was below the average at 7.52. Thus MOAsal2 represents the nitrogenase favouring process, MOKr the nodule biomass process and MOX roughly between the two.
It is hypothesised that shoot:nodule ratios could be used to indicate which process is prevalent in a given set of rhizobial isolates, but this would need to be confirmed with further research. At the very least, inclusion of the roots in the plant biomass data could change the ratios and alter the slopes of the regression lines in Fig 3.4. Root biomass data is extremely difficult if not impossible to collect from field data (Chapter 4), so that in an attempt to maintain consistency of data throughout the study, only shoot biomass data was collected from the specificity trial here.

Shoot:nodule ratios did provide an indication of the nitrogen use efficiency of each of the legumes. ANCOVA performed on the regressions of these ratios (Table 3.5) showed that there were highly significant differences between the legumes. Given that all other conditions were the same throughout the trial, and nitrogen was only supplied by nitrogen fixation, this would indicate real differences in nitrogen utilization between the hosts. While not the primary focus of this study, this preliminary finding does indicate other areas of research that could be pursued in the future. For instance, comparison of relative nitrogen fixation under conditions of abiotic stress (drought or nutrient deficiency) could determine whether the purported different mechanisms of increasing nitrogen supply and nitrogen use efficiency have any real ecological significance.

3.4.4 Inoculum Selection

There are three broad strategies that can be followed when selecting strains of rhizobia for the inoculation of legumes. Each has advantages and disadvantages (Odee et al., 2002).

1. Select a broad-spectrum isolate that is highly or at least moderately effective on the target legumes.
2. Select individual strains for individual legumes.
3. Select multiple strains that are highly effective for as many of the target legumes as possible.

Using a single broad-spectrum isolate is the easiest option from a practical point of view. Ascertaining suitable culture, growth and application conditions of a single organism will be easier than with many, and no doubt less expensive, if the latter is a consideration. However, the discovery of a single strain that is highly or moderately effective on all target legumes would appear to be more difficult as the number of target legume species is increased.
Selecting individual strains for individual legumes optimises performance for each legume, but increases the associated costs and procedures for handling different rhizobial strains. This strategy does introduce a wider number of rhizobia to the soil, which may be as many as there are target legumes.

A multi-strain rhizobial inoculant introduces direct competition issues for rhizobia in culture and for nodulation sites on legumes. The performance of multi-strain inoculants compared to single strain inoculation is still open to debate (Somasegaran, 1990; Stephens and Rask, 2000), and appears to depend largely on a combination of strain selection and the proportion of nodules occupied by each strain, and legume tolerance for multiple strains (Sutherland et al., 2000). Multi-strain inocula usually consist of three or four highly effective strains and can greatly increase the costs of production of the inocula. This method does allow for rapid introduction of rhizobia to a soil, one important consideration for increasing soil bacterial biodiversity, but may not introduce as many as the previous strategy.

The inoculum selected from this trial was driven by time constraints; that is the full specificity trials were unable to be completed before an inoculating strain was required for use in the shadehouse trials performed in Chapter 4. Therefore isolate RSFAp2, (*Burkholderia* sp.) was chosen for its ability to form functional nodules on all of the host legumes. Its effectiveness was not optimal for most of the legumes (Table 3.4), and its performance in shadehouse and field trials will be presented in Chapter 4.

Utilising the three different strategies outlined above, could a better selection have been made?

**Strategy 1**
A single broad-spectrum isolate could be selected only from the six isolates that formed functional nodules on all six hosts. Further, if any isolate is in the least effective category, it should be ruled out. Essentially this eliminates this strategy, as all of the 23-nodulating isolates were ineffective on at least one host. Two isolates, MOKr (*Bacillus*) and MOAsal1 (*Agrobacterium*) would have better suited as an inoculant as they were highly effective, moderately effective and ineffective on 4, 1 and 1 (MOKr) and 3, 2 and 1 (MOAsal1) of the host legumes respectively. Both were ineffective on *P. retusa*.

**Strategy 2**
Selecting specific rhizobia for specific legumes would appear to offer a simple set of four rhizobia, as there was duplication on two hosts.
Strategy 3
Selecting a multi-strain inoculum suggests three isolates could be used. Strains selected for inclusion should be as effective as possible on as many hosts as possible, as less effective isolates may compromise the survival of the host legume (Thrall, et al., 2000; Murray et al., 2001). Whichever isolates are selected for this strategy a compromise must be reached. With all of the most suitable isolates (those that are highly effective on the most legumes) also ineffective on P. retusa, or effective on P. retusa but ineffective on two or more legumes, the complexities of inoculum selection using this strategy become apparent.

Thus strategy 2 would appear to have been the most suitable of the options put forward. This discussion of inoculum selection was conducted from the viewpoint of legume growth only, and did not take into account the (re-) establishment of soil biodiversity. When biodiversity is given equal weighting to optimum growth, then a fourth strategy, multiple multi-strain inocula, consisting of the highly effective isolates for each legume would be one method of introducing as many rhizobia to a soil as possible.

It is acknowledged that for this discussion based as it is on a small collection of isolates, a more systematic approach to rhizobial sampling using multiple trap hosts would ensure that a more complete comparison could be achieved.
Investigating the Success of Rhizobial Inoculation
4.1 Introduction

In the Australian open cut mining industry best practice for the rehabilitation of mined lands requires the restoration of land to a native vegetation ecosystem which may include native pasture, woodland or forest depending upon pre-existing vegetation types and, or mining consent conditions. The method that has produced the best results for areas previously covered with forest or woodland involves removal of the topsoil layer from the area to be mined, direct transfer of this topsoil to spoil areas, and re-spreading it over the area to be rehabilitated. This practice preserves whatever seed bank, soil microbial community and nutritional characteristics that the topsoil may contain, provided it has not been stockpiled for an extended period (Newman, 1996; Bell, 1996). Where the stated goal of mine site rehabilitation is to re-establish native vegetation, topsoil spreading may not be the most suitable or may not be possible. Previous land use practice such as grazing, logging for timber or firewood may have resulted in loss of the native soil seed bank accompanied by a build up of exotic grasses and weeds either intentionally or through neglect. Use of this topsoil material would result in undesirable plant species outcompeting native tree and shrub species (Cole et al., 2006). Additionally, these past practices have often resulted in the degradation or loss of the topsoil layer, (with a consequent loss of the native seed bank as mentioned above), soil microbial community and organic matter, leaving only the underlying strata, which may or may not be suitable material for native plant growth.

At the Mount Owen Mine, mining operations pass through Ravensworth State Forest, destroying approximately 50% of one of the largest forest remnants in the upper Hunter Valley. The mine lease consent conditions require that the area of native forest destroyed by the mining operations, approximately 220 ha, be reconstructed to a native forest ecosystem on the spoil dump. The mine owners have determined to return the entire disturbance area of approximately 940 ha to native forest or woodland.

As much of the area occupied by the mine spoil dump was pasture prior to mining (Fig 4.1), the mine operators must rehabilitate an area that is considerably larger than the area of forest that was destroyed. Hence there is not enough forest topsoil to cover the entire dump area. This has led to investigations into the efficacy of alternative soil replacement media as substrates for a forest ecosystem in a project funded by the Australian Coal Association Research Program (ACARP), ACARP C12033 “Topsoil Substitutes and the Sustainability of Native Forest Ecosystem in the Hunter Valley”.
It was specified that any such soil replacement medium must be readily available in bulk, and if not available on site, able to be transported economically to site. Consequently mine spoil, pasture subsoil, chitter and biosolids and combinations thereof were trialled, giving a number of treatments (see Table 4.1) plus forest topsoil as a reference.

Spoil, which is overburden or crushed rock, acts as the underlying substrate for all of the experimental plots.
Pasture subsoil, the material contained in a layer 10cm to 20cm under the pasture topsoil, was used to remove the competitive effect of pasture plant species found in the topsoil or upper 10cm of soil.

Chitter is a waste product of the coal washing process whereby substandard material is removed from the coal. It consists of crushed rock strata immediately above, below and imbedded within the coal seams as well as coal of inferior quality that has been industrially washed and is readily available at all coal mines. It was used here to prevent the formation of a hard crust that occurs on the spoil and subsoil after wetting.

Biosolids, or sewage waste, is another readily available waste product sourced from the nearby Branxton plant of the Hunter Water Corporation that provides a rich source of organic nutrients and has been used as a soil ameliorant in both mine rehabilitation and agriculture in the past (Brown et al., 2003; Cooper, 2005; Halofsky and McCormick, 2005; Paschke et al., 2005).

With the exception of the pasture subsoil, these materials are not soils and have not been subjected to soil forming processes such as weathering and the accumulation of organic matter. As a consequence there are abiotic factors that include but are not restricted to pH, structure and mineral content that may influence how these materials support revegetation efforts. Additionally, there are biotic factors that will impact plant growth. Without suitable soil microbes, many of the biogeochemical cycles responsible for releasing or acquiring the mineral elements from the soil, or in this case soil replacement media, necessary for sustainable plant growth will not occur at all, or will only be partially present.

Plant acquisition of nitrogen, the major essential plant nutrient that limits plant growth by its absence, is especially dependent on soil dwelling microbes. Spoil and chitter are essentially devoid of plant available nitrogen. Biosolids will contain relatively high levels of nitrogen, but its application is restricted due to fears of heavy metal contamination, which can accumulate in biosolids and be detrimental to plant and bacterial growth. Pasture subsoil contains relatively more plant available nitrogen when compared to spoil or chitter and is postulated to contain some rhizobia bacteria necessary for symbiotic nitrogen fixation. It is however unlikely that these are native rhizobia, and they may or may not nodulate native legumes.

Introducing suitable native rhizobia to the soil replacement media as an inoculum, along with native legumes is one method of ensuring sustainable nitrogen cycles are established.
In two shadehouse trials, which were undertaken concurrently with a field trial, plant species of different strata typically found in the dry sclerophyll forest of Ravensworth State Forest and representative of the plant community (see Chapter 3) were planted into pots. The shadehouse trials were conducted as part of the ACARP C12033 “Topsoil Substitutes and the Sustainability of Native Forest Ecosystem in the Hunter Valley” study referred to earlier. The first shadehouse trial tested a total of 14 materials for suitability as topsoil substitutes under controlled conditions. The second shadehouse trial involved the 14 materials and a series of secondary treatments including gypsum, zeolite and flyash. Inoculation with rhizobia was included as a tertiary treatment for the ACARP study. From these two trials, suitable soil replacement materials were then selected for testing the effect of inoculation under field conditions. Throughout this chapter, the various soil materials, that is the spoil, pasture subsoil, chitter and biosolids are referred to as “materials”, “soil replacement media” or “SRM”, or as “ameliorants”, as in ameliorating the spoil. Forest topsoil is generally referred to as “forest topsoil reference”.

The first experiment, the uninoculated pot trial, investigated the suitability of spoil either singly or in combination with the three ameliorants, that is chitter, biosolids and pasture subsoil to support plant growth. It also investigated whether resident rhizobia were present and if they were capable of nodulating with native legumes when compared to forest topsoil.

The second experiment, the inoculated pot trial, examined the effect of inoculation with rhizobia on plant growth when grown in a subset of the soil replacement media. The inoculant was selected from the specificity trials conducted in Chapter 3, and its effect on plant growth was tested on a subset of the media used in the first pot trial.

A field trial was then undertaken where six legume species from the Mount Owen/Ravensworth State Forest area, as used in Chapter 3, were inoculated with the same rhizobial isolate as the shadehouse pot trials and grown on selected soil replacement media.
4.2 Materials and Methods

4.2.1 Shadehouse Pot Trial Design

4.2.1.1 Soil Replacement Media Collection

All media were collected from stockpiles of material used in establishing the field site and, with the exception of the biosolids, were sourced from the Mount Owen Mine site or immediate surrounds (Fig 4.1). Spoil was collected from the spoil dump approximately 100 m from the field site for this experiment. Forest topsoil, a yellow podzolic vegetated by open forest with a grassy herb layer, was collected from a section of the northern remnant of the Ravensworth State Forest and was used as the reference medium. Pasture subsoil, a non-calcic brown podzolic, was obtained from nearby pasture at the base of the spoil dump. Chitter was sourced from stockpiles on top of the mine spoil dump. Biosolids were trucked from the nearby Branxton plant of the Hunter Water Corporation and placed in short-term stockpiles on top of the spoil hill above the experiment. All media were transported to the glasshouse complex at the University of Newcastle and stored on, and covered by, plastic sheeting in the case of the spoil or in sealed plastic drums for all other media prior to potting.

4.2.1.2 Potting

All soil replacement media were potted into 250 mm polyurethane plastic pots. These were filled to within 12 cm of the top with a 1:1 mixture of spoil and perlite. Perlite was applied to “open up” the media, prevent water logging and aid root penetration. Previous experimental work (Newman, 1996; Nussbaumer 2005) had shown that when used alone in pot trials, spoil became waterlogged and anaerobic. This was further verified by including spoil control pots within each trial (data not shown). These pots had the least germinations, reduced growth and increased death of test plants.

The top 10 cm of each pot was made up of each of the soil replacement media, either singly or in combination. Each pot, overlying the base spoil + perlite mix, consisted of either spoil, pasture subsoil, pasture subsoil + chitter, pasture subsoil + biosolids, chitter, chitter + biosolids, biosolids or forest topsoil, which except for the biosolids were applied in a 1:1 ratio (Table 4.1). Biosolids was applied at the rate equivalent to 89 dry solid tonnes per hectare, as per Nussbaumer (2005). The soil ripping process used in the field was simulated in the pot trial by pouring the contents of one pot into a bucket and then back. The surface of the media was then turned over by four scoops with the trowel around the circumference of the pot. Space limitations
prevented the inclusion of a ninth media combination, pasture subsoil + chitter + biosolids.

**Table 4.1** List of soil replacement media trialled in the shadehouse pot trials. All eight SRM were used in the uninoculated trial. The four SRM also used in the inoculated trial are indicated in bold.

<table>
<thead>
<tr>
<th>Spoil</th>
<th>Spoil + forest topsoil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spoil + biosolids</td>
<td>Spoil + pasture subsoil</td>
</tr>
<tr>
<td>Spoil + chitter</td>
<td>Spoil + pasture subsoil + biosolids</td>
</tr>
<tr>
<td>Spoil + chitter + biosolids</td>
<td>Spoil + pasture subsoil + chitter</td>
</tr>
</tbody>
</table>

**4.2.1.3 Seeding**

Seed numbers of each species per pot are outlined in Table 4.2. Multiple seeds were placed at each position and four positions were used within each pot to adjust for variation in rates of germination, seedling and plant mortality. Seeding took place according to the plan detailed in Fig 4.2. The Mimosaceae and Fabaceae were pre-treated either by boiling for 1 min or being covered by boiling water until imbibed. The seeding pattern was determined randomly and repeated for each pot using a cut out cardboard template (Fig. 4.2). Each substrate had a designated template, pipette and dowel, which had all been sterilized by autoclaving and thorough washing in 70% EtOH respectively prior to commencement of seeding. A hole was made in the surface of the substrates with the dowel and seeds of a specified species were placed in each according to the template pattern. For each of the two pot trials, pots were then immediately watered and placed in four randomised blocks on benches (Fig 4.3). Pots were randomised on a weekly basis, both as blocks, and within blocks. Pots were watered every second day or as required throughout the experiment.

In addition to my legume host species identified from findings reported in Chapter 3, several other species were included in the shadehouse trials. These were three understorey legume species, and two canopy Eucalyptus species (Table 4.2). Inclusion of the Eucalypt species was a compromise to accommodate the large scale experimental objectives of a concurrently run Australian Coal Association Research Programme funded experiment.
Table 4.2 Number of seeds used in both the inoculated and uninoculated shadehouse trials.

<table>
<thead>
<tr>
<th>Species</th>
<th>Number of seed in each position</th>
<th>Total seeds per pot</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Corymbia maculata</em></td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td><em>Eucalyptus crebra</em></td>
<td>Scoop approx. 0.04g</td>
<td>0.16 g</td>
</tr>
<tr>
<td><em>Acacia amblygona</em></td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td><em>Acacia filicifolia</em></td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td><em>Acacia parvifina</em></td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td><em>Hardenbergia violacea</em></td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td><em>Indigofera australis</em></td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td><em>Jacksonia scoparia</em></td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td><em>Kennedia prostrata</em></td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td><em>Pultenaea retusa</em></td>
<td>3</td>
<td>12</td>
</tr>
</tbody>
</table>

![Seeding pattern used for both the inoculated and uninoculated shadehouse trials.](image)

**Fig 4.2** Seeding pattern used for both the inoculated and uninoculated shadehouse trials.
4.2.1.4 Inoculation

Pots were inoculated with the generalist nodulator, RSFAp2, *Burkholderia* sp. CC-S-L25 (Chapter 3 Table 3.1), when plants were four months of age. Ten ml of liquid culture was applied with a 20 mL syringe over the surface of each pot, priority given to the nodulating species and then spreading the rest evenly across the pot. This rate provided $1 \times 10^{10}$ rhizobia per pot.

4.2.1.5 Measurements of Plant Growth and Root Nodule Weights

Plant growth was determined by destructive harvest of the pots at the termination of the shadehouse trial. Stems were combined on a community or pot basis, bagged and dried in a force draught oven at $80^\circ$C for 48 h before weighing.

Root nodules were collected from the pots, combined after washing out from the soil replacement media, and the oven drying procedure above followed.
4.2.2 Field Trial Design

4.2.2.1 Soil Replacement Media

The soil replacement media were set out as a randomly blocked design as part of the ACARP Project C12033 (Cole et al., 2006) using machinery and techniques that would typically be used by mine operators for rehabilitation of mine spoil dumps (Fig 4.4 A-C). Each treatment material, with the exception of the biosolids, was brought onto the site by bottom dumping scraper and deposited onto the experimental blocks in layers approximately 100 mm thick (Fig 4.5 A & B).

![Fig 4.5 Machinery used during construction of the field site. A) Bottom dumper spreading pasture subsoil. B) Excavator used to spread biosolids onto the plots. C) Deep contour ripping was undertaken by bulldozer, here an example of a spoil + pasture subsoil plot being ripped.](image)
The biosolids were applied at 50 dry solid tonnes per hectare and spread by a small excavator as a thin layer only a few millimetres thick over the selected experimental blocks (Fig 4.4 B). This lower rate was used based on data provided by the Hunter Water Board indicating that no further improvement in plant growth would be achieved with higher application rates. Each material was layered over the previously spread material in various combinations to produce eight experimental treatments, plus a reference forest topsoil treatment (Fig 4.4 A & B).

After all materials were spread, the entire site was subjected to deep contour ripping (300-400 mm depth) by dozer to loosen the spoil after compaction by the heavy machinery, allowing for water penetration, establishment of plant roots and slowing of erosion (Fig 4.4 C). Plate 1 shows the ACARP field site A) pre-seeding and B) three years post seeding.

Six blocks, consisting of eight experimental plots of 20m x 20m, were constructed on a northwest-facing slope of mine spoil. This area was contoured and graded to approximate the surrounding landscape, giving an average final slope of approximately 8° and an elevation of 240 m above the surrounding pasture. The forest topsoil plots used as control or reference treatments were constructed in two sections. Two plots were located on the extreme northeast of the site, and were assigned to blocks one and two, with the remaining reference plots constructed in a strip between blocks three and four and assigned to blocks three to six (Fig 4.6).
Five of these six blocks were selected for the inoculation field trial. Block 4 was not used due to loss of a drainage bank leading to excessive erosion in some treatments prior to seeding.

The inoculation trial was conducted in a series of 4 m x 4 m experimental subplots. Two of these were established at the lowermost down slope 4 m strip of each of the 20 m x 20 m experimental plots used in the ACARP experiment (Fig 4.6 and Fig 4.7). One of these subplots acted as an uninoculated control, while the second was treated with the rhizobial inoculate. The subplots were located to minimise the chance of inoculum spreading to the rest of the ACARP experimental plots. The inoculated subplot was chosen randomly (left or right by coin toss), except when the inoculated plot would have drained towards the uninoculated plot, leading to potential contamination through water runoff.
Each 4 m x 4 m subplot was then further subdivided into 16 1 m x 1 m squares, the centre of which was staked to establish an exact seeding location for the plant species (Fig 4.7B). The position of each plant species was randomly allocated. Thus each of the six species trialled had two positions (pseudoreplicates) within each subplot, with four of the squares not seeded.

![Plan view of the layout of the experimental subplots. A) Example location of the inoculated and uninoculated subplots within the 20 m x 20 m treatment plots. B) Example layout of an experimental subplot showing the position of the host plant species. Aa = *A. amblygona*, Ap = *A. parvipinnula*, Du = *D. ulicifolia*, Hv = *H. violacea*, Ia = *I. australis*, Pr = *P. retusa*.](image)

**4.2.2.2 Seeding**

Six pre-treated seeds of each of the host species were seeded into a small pre-dug hole containing a “plug” of washed river sand and perlite (3:1) mix, 2 cm down slope from the centre stake in each 1 m x 1 m subplot. Seeding took place over a two-week period between April 4 and April 18, 2005.

**4.2.2.3 Inoculation**

Initial inoculation by rhizobial isolates was conducted on August 24, 2005, with a second inoculation conducted on December 13, 2005. One rhizobial isolate, RSFAp2 *Burkholderia* sp. CC-S-L25 selected for its ability as a generalist nodulator (Chapter 2) was used for the field trials. The isolate was grown in yeast mannitol liquid (see Appendix 1) in conical flasks for 96 h at 26°C on a rotating shaker at 120 rpm. Culture
Plate 1 The ACARP field site at Mount Owen Mine. Top September 2004 prior to seeding. Bottom, field site after three years taken in November 2007, prior to final harvest of the inoculation field trials. The effect of the different soil replacement media upon growth is clearly visible, with forest topsoil and pasture subsoil plots having the best cover.
flasks were then combined, kept on ice in an insulated box and transported to the field site. Direct surface application of 10 mL of inoculant culture was applied to each of the seeding sites in the inoculated subplots, with 10 mL of yeast mannitol liquid (without rhizobial culture) applied to the uninoculated subplots. Cutting using a hand held saw shortened a 50 mL Falcon tube so that it held exactly 10 mL. This was immersed into the container containing the inoculant solution and then applied at the seeding location, as per Section 4.2.2.2 above. A sub-sample of the inoculant culture was collected and used to calculate the cell density as measured using a Thermo Spectronic Biomate 3 spectrophotometer at OD600. Cell density was calculated using the formula:

$$\text{Bacterial number (x } 10^8/\text{mL}) = \frac{(\text{OD600}-0.083)}{0.073}$$

This resulted in $3.51 \times 10^8/\text{mL}$ bacteria being applied to the seedlings.

4.2.2.4 Measurements of Plant Growth and Survival

Measurements of plant height, combined with a destructive harvest of the plant stems were conducted to determine the efficacy of the treatments. This was performed in February and March 2008, thirty-six months after the start of the field trial. The shoots were cut off at the soil surface and transported to the laboratory, dried in an 80°C forced-draught oven for 48 h and weighed. Data are presented for the entire “Community” as well as of each individual species. Community data refers to the combined data of the surviving trialled plants, and does not include native or weed species germinated from seed banks contained in the soil ameliorants.

Surveys of plant height and survival were conducted on a six monthly basis on which the time course data is based. Plant height data are presented at final harvest only, as evidence of herbivory compromised plant height particularly during the establishment phase.

Survival was monitored on weekly basis from sowing, and highest seedling emergence determined. Survival was calculated from this point in late July/early August 2005.

4.2.3 Statistical Analysis

Data were analysed using two- or three-factor ANOVA with partitioning of variance. If the sum of squares contributed by blocks was low, ca 10%, they were pooled as replicated to simplify the design and data presentation. In all analyses, a Cochran’s test was conducted to assess data for homogeneity. Non-homogenous data were then arcsine
transformed. With block analysis, Cochran’s test is not possible on the two factor ANOVA, as essentially n = 1.

The data are illustrated using least significance difference (LSD) bars to indicate the difference between means required to produce a p<0.05, p<0.01 or p<0.001 level of significance. Where there were no significant differences greater than the p<0.05 level, the remaining LSD bars have not been shown.

Letters above the data columns indicate whether mean values were statistically different from each other at the p<0.05 level. Where data have been transformed due to heterogeneity, the data are displayed in the raw form and LSD’s are not indicated.

4.3 Results

4.3.1 Shadehouse Trials

The successful establishment of native legume species and their rhizobial symbionts is essential for re-establishing the nitrogen cycle upon the spoil dump. One of the reported obstacles to establishing populations of rhizobial inoculants is the presence of a resident or indigenous population (Stephens and Rask, 2000; Lopez-Garcia et al. 2002). To determine the efficacy of topsoil replacement media three factors were considered. Does the replacement media adequately support plant growth? Will the replacement media support growth of the inoculating culture? Is there a resident population of rhizobia that will nodulate the native legume species? In addition, will the rhizobia used as an inoculant produce a discernable improvement in community plant growth?

4.3.1.1 Support of Plant Growth of Uninoculated Soil Replacement Media

When compared to bare spoil, plant growth in the uninoculated shadehouse trial was significantly increased by addition of biosolids with or without pasture subsoil, and forest topsoil (Fig 4.8). Addition of chitter did not significantly affect growth.

Comparison of individual soil replacement media shows that addition of biosolids significantly increased community shoot dry weight (Fig 4.8). For instance, spoil + biosolids significantly (p<0.001) increased growth over bare spoil. When chitter was incorporated, whether alone or in combination with biosolids, growth was not significantly improved. Spoil + pasture subsoil produced improved community growth although not to a significant degree over bare spoil medium. However, spoil + pasture subsoil + biosolids media improved growth significantly (p<0.001) over all other media.
When effects of the media are pooled (data not shown), addition of pasture subsoil significantly \((p<0.01)\) increased community growth over those media that did not include pasture subsoil. Likewise, media with biosolids produced significantly \((p<0.001)\) increased growth when compared to media without biosolids. It should be noted that for these pooled data, spoil + chitter + biosolids medium was omitted from the analysis. This was done to balance the numbers of soil replacement media (there was no spoil + pasture subsoil + chitter + biosolids equivalent) to allow valid comparison.

4.3.1.2 Resident Rhizobial Population

The success of an introduced rhizobial inoculant can be affected by the presence of a resident rhizobial population, and therefore the presence or absence of such needs to be determined.

Root nodules were not recovered from spoil + chitter soil replacement media (Fig 4.9) whereas one pot in each of the other three spoil-based media produced

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**Fig 4.8** Shoot dry weight from the uninoculated shadehouse pot trial. Data show total plant community shoot dry weight per pot for each of the seven soil replacement media and the forest topsoil reference. Columns with the same letters are not statistically different at the 5% level. Bars at top left indicate least significant differences of the means for the individual soil replacement treatments at the probability levels indicated above them.
extremely low nodulation (<0.25 g of nodules each). The functional ability of nodules collected from these pots to fix nitrogen was verified by these nodules containing leghaemoglobin.

When collecting soil material for this experiment, every effort was made to only use pure spoil. However, forest subsoil may inadvertently have been included which contained some rhizobia (data not shown). Media containing pasture subsoil consistently produced root nodules. Spoil + pasture subsoil + biosolids had the greatest nodule dry weight. Spoil + pasture subsoil and spoil + pasture subsoil + chitter were not significantly different to the forest topsoil reference.

Partitioning of variance showed that nodule dry weight was significantly (p<0.001) greater in media with pasture subsoil than spoil alone and that addition of biosolids significantly increased (p<0.01) nodule dry weight over all soil replacement media.

**Fig 4.9** Nodule dry weight produced on plants grown in specified soil media without additional inoculum. Total nodule dry weight per pot for each of the seven soil replacement media and the forest topsoil reference. Columns with the same letters are not statistically different at the 5% level. Bars at top left indicate least significant differences of the means for the individual soil replacement treatments at the probability levels indicated above them.
Ratio of shoot to nodule dry weight indicates the relative efficiencies of unit root nodule biomass to support plant community biomass (Fig 4.10). After partitioning spoil-based media due to their lack of nodulation from the other media, spoil + forest topsoil shoot to nodule ratio was significantly ($p<0.05$) higher than either the spoil + pasture subsoil or the pasture subsoil + chitter media. Spoil + pasture subsoil + biosolids media ratio was not significantly different from ratios on any of the three other media.

### 4.3.1.3 Effect of Inoculation Upon Plant Growth

Inoculation produced no statistically significant changes to community shoot dry weights between the different media (Fig 4.11). Spoil and spoil + pasture subsoil media increased slightly, while spoil + biosolids and spoil + forest topsoil media decreased slightly. A two-way ANOVA (data not shown) with spoil + biosolids shoot dry weight partitioned from the other media, showed it was significantly higher ($p<0.001$), with no significant differences between the remaining three media.

Nodule dry weight (Fig 4.12) increased in all the soil replacement media after inoculation, including spoil + forest topsoil reference. Both spoil + pasture subsoil and spoil + biosolids nodule weights were statistically significant ($p<0.01$ and $p<0.05$ respectively) although the $p$-value for the spoil + biosolids media was $p=0.049$, indicating that inoculation produced highly variable results across blocks.

![Figure 4.10](image)

**Fig 4.10** Ratio of shoot to nodule dry weights of plants raised in the uninoculated shadehouse trial. Ratios are from four media that had indigenous rhizobial populations. Columns with the same letters are not statistically different at the 5% level. Bar at top left indicates least significant difference of the means at the level indicated.
Fig 4.11 Comparison of the total community shoot dry weight of plants raised in uninoculated and inoculated shadehouse trials for the common media. Columns with the same letters are not significantly different at the 5% level within each soil replacement media only.

Fig 4.12 Nodule dry weight comparison between plants raised in uninoculated and inoculated shade house trial for common media. Columns with the same letters are not statistically different at the 5% level within media.
The shoot:nodule dry weight ratio is not significantly different between media in the inoculated pot trial (Fig 4.13). Only spoil + forest topsoil and spoil + pasture subsoil shoot/root ratios can be compared directly between the two trials (Fig. 4.10). The media have a reduction in their shoot/nodule DW ratios by about half in both spoil + forest topsoil and spoil + pasture topsoil.

Presenting data on a community or per pot basis does not show if changes in species composition, of those plants that germinated and survived, occurred between the two trials. Photographs of representative pots from the four common media show that in at least one medium, spoil + biosolids, inoculation appeared to affect legume species more than the canopy species (Fig 4.14 A-D and Fig 4.15 A-D). Examination of Fig 4.14 shows almost no qualitative difference between uninoculated and inoculated pots in Fig 4.15 A & B, the spoil medium, and the same is also true of Fig 4.15 A & B, spoil + pasture subsoil medium, or Fig 4.15 C & D spoil + forest topsoil medium. However, in spoil + biosolids medium, the change from shoot dry weight composed almost entirely of canopy species (Fig 4.14 C) to one of legume dominated shoot dry weight (Fig 4.14 D) is quite evident.

![Fig 4.13 Ratio of shoot to nodule dry weights from plants in the inoculated shade house trial. Columns with the same letters are not significantly different at the 5% level.](image)
Fig 4.14 Photographs of representative treatments common to the two shade house trials. Spoil - A) Uninoculated trial, B) Inoculated trial. Spoil + biosolids - C) Uninoculated trial, D) Inoculated trial. Red scale bars indicate 250mm. Plants are labelled Cm = Corymbia maculata, Aa = Acacia amblygona, Ap = A. parvipinnula, Hv = Hardenbergia violacea.
Fig 4.15 Photographs of representative treatments common to the two shadehouse trials. Spoil + pasture subsoil - A) Uninoculated trial, B) Inoculated trial. Spoil + forest topsoil - C) Uninoculated trial, D) Inoculated trial. Red scale bars indicate 250mm. Plants are labelled Cm = Corymbia maculata, Aa = Acacia amblygona, Ap = A. parvipinnula, Hv = Hardenbergia violacea, Pr = Pultenaea retusa
4.3.2 Field Trial

4.3.2.1 Plant Survival

One measure of success of vegetation community reconstruction is survival of target species. In these trials, the effect of rhizobial inoculation upon target legume species was tested in different soil media, and therefore effects of inoculation and media were ascertained. Survival data were standardised as a percentage of the highest emergence of seedlings. This was done to remove possible effects of seed viability and germination between target species. However, data demonstrate that soil replacement media and individual species had an effect upon rates of seedling emergence, which in turn had an effect on the final numbers of plants that survived.

When the mean highest seedling emergence expressed as a percentage of sown seed was pooled for all species for each of the soil replacement media (Fig 4.16), it was evident that pasture subsoil ameliorated media had significantly reduced emergence when compared to spoil + forest topsoil reference. Indeed, emergence on spoil + pasture subsoil + biosolids and spoil + pasture subsoil + chitter + biosolids was significantly reduced when compared to all other media, with the exception of spoil + chitter + biosolids.

Partitioning of variance (data not shown) showed that the inclusion of biosolids had a significant effect (p<0.01), reducing emergence, while incorporation of chitter had no effect on emergence. Inclusion of pasture subsoil reduced emergence when compared to media without pasture subsoil. The effect was not statistically significant, but the p value of p<0.056 approached significance.
Fig 4.16 The highest seedling emergence as a percentage of total seed sown for the combined host species prior to inoculation for each soil replacement media. Data have been arcsine transformed prior to analysis by two-way ANOVA, and are presented here transformed. Columns with the same letters are not significantly different at the 5% level.

Fig 4.17 Seedling emergence for each host species as a percentage of planted seed pooled across the soil replacement media. Data have been arcsine transformed prior to analysis by two-way ANOVA, and are presented here transformed. Columns with the same letter are not significantly different at the 5% level.
Rainfall data were available from the Mount Owen mine from October 1996. From that date to the beginning of the field experiment in April 2005, average monthly rainfall was 59 mm, with a minimum of 0.6 mm (April 2003) to a maximum of 213 mm (March 2000). Rainfall over the course of the experiment also influenced plant survival, a not unexpected consequence of field trials (Fig 4.18). Rainfall was sporadic and only one-third of the months recorded above average rainfall (average for the duration of the field trial of 47 mm). Monthly totals varied from a minimum of 2.6 mm recorded in October 2006, to a maximum of 279 mm recorded in June 2007. Average rainfall for the year of 2006 was the lowest recorded for this period with 25 mm.

![Rainfall Graph]

**Fig 4.18** The monthly rainfall at the Mount Owen mine for the period of the field trials presented as days post sowing of seedlings in April 2005 until the final harvest in March 2008. The horizontal line is the average rainfall for the period, 47 mm per month.

Time course data for each soil replacement media and each plant species show community survival over the life of the experiment. Statistical significance is provided in the analyses that follow in sections 4.3.2.5, 4.2.3.6 and 4.2.3.7. In the four soil replacement media without pasture subsoil (Fig 4.19 A to D), seedling survival declined markedly in the period immediately post inoculation before a “base level” was achieved. Inoculation did not affect the trajectory of community survival, with both uninoculated and inoculated plots following the same trend of sharp declines until 200 days post inoculation.

Survival of plant communities grown in media containing pasture subsoil and the spoil + forest topsoil reference did not exhibit such a marked decline in survival,
with inoculated plants generally having improved survival compared to uninoculated plants (Fig 4.19 E to I). Additionally, the spoil + forest topsoil reference and spoil + pasture subsoil media appeared to buffer plant communities against the rapid declines observed in the remaining pasture subsoil containing media and media without pasture subsoil.

Survival of individual plant species pooled for all soil replacement media exhibited a marked decline during 200 - 250 days post inoculation. However, the severity and extent of this trend was species dependent (Fig 4.20 A-F). *P. retusa, D. ulicifolia*, and *I. australis* (Fig 4.20 A, B & C respectively) exhibited decreased survival when compared to the remaining three species *H. violacea, A. amblygona* and *A. parvipinnula* (Fig 4.20 D, E & F respectively). Inoculation improved survival for all species with the exception of *A. amblygona* (Fig 4.20 F), and for *P. retusa* only inoculated plants survived. While all other species appear to have stabilised to a certain degree, *H. violacea* and *A. parvipinnula* numbers were still continuing to decrease at the conclusion of the experiment (Fig 4.20 D & E respectively).
Fig 4.19 Time course for survival of the combined plant species for each soil replacement medium of both uninoculated and inoculated treatments. Survival is shown as a proportion of seedling emergence.
Fig 4.20 Time course of survival of each individual plant species, both uninoculated and inoculated, for the combined soil replacement media treatments. Survival data presented as a proportion of emergence.
4.3.2.2 Community Survival

When community survival data at harvest were pooled across soil replacement media and analysed for effects of inoculation using 2-way ANOVA (Fig 4.21 A), overall seedling survival was not significantly improved by inoculation, although there is a trend of improved survival with inoculation.

Analysis of each soil replacement media for effects of inoculation upon seedling survival (Fig 4.21 B) using three-way ANOVA, shows there was a clear trend for inoculation increasing plant survival. Six of the nine media produced significant results. Inoculation with rhizobia improved survival in spoil and spoil + pasture subsoil (p<0.01) and in spoil + biosolids, spoil + chitter + biosolids and spoil + forest topsoil (p<0.05).

Combining data from uninoculated and inoculated plots showed the effects soil replacement media had on community survival (Fig 4.21 C). Plant survival was generally higher in spoil + forest topsoil and spoil + pasture subsoil media when compared to media without pasture subsoil. Spoil + pasture subsoil and spoil + forest topsoil supported significantly higher plant survival than spoil + chitter (p<0.05) and the remaining spoil media (p<0.001).

Internal partitioning of variance to determine effects of soil replacement media (data not shown) demonstrated that addition of pasture subsoil significantly improved survival of both uninoculated and inoculated plants (p<0.001 respectively). Addition of biosolids impacted negatively upon survival of both uninoculated and inoculated plants (p<0.001), while chitter had no impact on survival of uninoculated plants or inoculated plants.

Fig 4.21 Community plant survival. (Following page)
A) Survival data for inoculated and uninoculated plots of combined plant species and soil replacement media.
B) Survival data for inoculated and uninoculated plots within soil replacement media.
C) Survival data for the combined inoculated and uninoculated soil replacement media.
For all graphs:-
Columns with the same letters are not significantly different at the 5% level.
Bars at top left indicate least significant differences between the mean values at the levels indicated.
Data have been arcsine transformed before analysis by two-way ANOVA for graph A, and three-way ANOVA for graphs B & C.
4.3.2.3 Community Height

Plant height was used as a measure of plant health, indicating vigour and ability to produce new growth.

Pooling plant height data across soil replacement media and analysing for effects of inoculation using 2-way ANOVA (Fig 4.22 A) showed that inoculation produced a significant (p<0.01) increase in plant community height.

Inoculation increased community height over uninoculated plant communities in eight of the nine soil replacement media (Fig 4.22 B). Community height was significantly increased by inoculation in spoil + forest topsoil and spoil + pasture subsoil + biosolids (p<0.001), in the remaining media containing pasture subsoil and in spoil + chitter (p<0.01). A positive trend towards greater height was observed in spoil and spoil + chitter + biosolids media. The only exception was spoil + biosolids medium where uninoculated plants were significantly taller (p<0.05).

Plant height data of the combined inoculated and uninoculated plots produced significant differences between communities grown in the different soil replacement media (Fig 4.22 C). The spoil + forest topsoil reference plant community was significantly greater in height than the next ranked soil replacement media, spoil + pasture subsoil (p<0.05), and significantly greater (p<0.001) than the lowest ranked media, spoil. Spoil-grown community heights were significantly shorter than all other media except spoil + biosolids.

Almost without exception, media with pasture subsoil produced taller communities than spoil based media. The tallest spoil based media, spoil + chitter + biosolids was significantly taller than the shortest pasture subsoil plus media, spoil + pasture subsoil + chitter + biosolids.

Fig 4.22 Community plant height. (Following page)
A) Plant height data for inoculated and uninoculated plots of combined plant species and soil replacement media.
B) Plant height data for inoculated and uninoculated plots within soil replacement media.
C) Plant height data for the combined inoculated and uninoculated soil replacement media.
For all graphs:-
Columns with the same letters are not significantly different at the 5% level.
Bars at top left indicate least significant differences between the mean values at the levels indicated.
4.3.2.4 Community Shoot Dry Weight

Plant biomass, as measured by dry weight, is a more accurate measure of plant performance compared to plant height. Plant height can be subject to changes in plant architecture or form due to herbivory or plant growth habit. Given the extremely difficult task of recovering entire root systems of field-grown plants, shoot dry weight was used as indicator of plant performance, with the comparison between inoculated and uninoculated plants being used as a correlation of nitrogen supply.

Comparing effects of inoculation on shoot dry weight across all plant species and soil replacement media showed no significant difference between inoculated and uninoculated plots (Fig 4.2 A). However, a strong trend toward increased shoot dry weight with inoculation was apparent.

Inoculation had a statistically significant positive effect upon community shoot dry weight for two of the nine soil replacement media (Fig 4.2 B), with a trend toward increased shoot dry weight for plants raised on another four media. The two exceptions were spoil + chitter + biosolids and spoil + biosolids.

Community shoot dry weights across the combined inoculated and uninoculated plots of soil replacement media produced relatively clear differences between spoil and pasture subsoil based media (Fig 4.2 C). Internal partitioning of variance for uninoculated plots showed no significant difference in shoot dry weight when media with pasture subsoil was compared to media without. The same was true of media with and without biosolids and with and without chitter. When inoculated, media with pasture subsoil significantly ($p<0.001$) increased shoot dry weight when compared to those media without. But as with uninoculated plants, incorporation of chitter or biosolids had no significant effect upon plant growth.

**Fig 4.23** Community shoot dry weight. (Following page)
A) Shoot dry weight for inoculated and uninoculated plots of combined plant species and soil replacement media.
B) Shoot dry weight for inoculated and uninoculated plots within soil replacement media.
C) Shoot dry weight for the combined inoculated and uninoculated soil replacement media.
For all graphs:-
Columns with the same letters are not significantly different at the 5% level.
Bars at top left indicate least significant differences between the mean values at the levels indicated.
4.3.2.5 *Individual Species Survival-Low Survival Species*

*P. retusa* survival was extremely low, and no plants survived in uninoculated plots across any soil replacement media (Fig 4.24). Only inoculated *spoil + forest topsoil* medium showed any success. Lack of survival of this species was highly unexpected given its common occurrence in the Ravensworth State Forest and success in the shadehouse trials. Because of this low survival, no further data analyses are presented for this species.

*I. australis* survival was limited to media containing pasture subsoil and the *spoil + forest topsoil* reference. Inoculation had no statistical effect upon survival when data were pooled across soil replacement media and analysed by two-way ANOVA (Fig 4.25 A). However, a strong trend towards increased survival was observed.

Comparing effects of inoculation upon seedling survival within soil replacement media showed that there were significant increased survival (Fig 4.25 B) in *spoil + pasture subsoil + biosolids* ($p<0.01$) and the *spoil + forest topsoil* reference ($p<0.05$). A trend for increased seedling survival was apparent in *spoil + pasture subsoil* and *spoil + pasture subsoil + chitter*.

The effect that different soil replacement media had upon *I. australis* survival can clearly be seen in Fig 4.25 C. Three soil replacement media, *spoil + pasture subsoil*,...
spoil + pasture subsoil + biosolids and the spoil + forest topsoil reference supported significantly higher survival compared to the remaining two spoil + pasture subsoil media (p<0.05 and p<0.01). It should also be noted that addition of chitter appeared to have a deleterious effect upon this species survival. While not as pronounced as *P. retusa* overall, survival was very low for *I. australis* and no further analyses were undertaken.

**Fig 4.25** *I. australis* survival. (Following page)
A) Survival data for inoculated and uninoculated plots of combined plant species and soil replacement media.
B) Survival data for inoculated and uninoculated plots within soil replacement media.
C) Survival data for the combined inoculated and uninoculated soil replacement media.
For all graphs:
Columns with the same letters are not significantly different at the 5% level.
Bars at top left indicate least significant differences of the means at the levels indicated.
Data have been arcsine transformed before analysis by two-way ANOVA for graph A, and three-way ANOVA for graphs B & C.
D. ulicifolia survival was shown not to be significantly affected by inoculation when results were pooled across soil replacement media (Fig 4.26 A).

When seedling survival was analysed within soil replacement media (Fig 4.26B) only spoil + forest topsoil produced a significant result (p<0.05). Despite the reverse trend for spoil + pasture subsoil and spoil + pasture subsoil + chitter, overall there was a trend of inoculation increasing survival of this species.

Pooling results from uninoculated and inoculated plots showed that soil replacement media had a significant effect on survival of D. ulicifolia (Fig 4.26C). Spoil + forest topsoil reference and spoil + pasture subsoil had significantly increased survival (p<0.01) compared to other media. Other media producing statistically similar, low survival. As with the previous two species, overall plant survival was very low precluding any further analyses.

Fig 4.26 D. ulicifolia survival. (Following page)
A) Survival data for inoculated and uninoculated plots of combined plant species and soil replacement media.
B) Survival data for inoculated and uninoculated plots within soil replacement media.
C) Survival data for the combined inoculated and uninoculated soil replacement media.
For all graphs:-
Columns with the same letters are not significantly different at the 5% level.
Bars at top left indicate least significant differences of the means at the levels indicated.
Data have been arcsine transformed before analysis by two-way ANOVA for graph A, and three-way ANOVA for graphs B & C.
4.2.3.6 Individual Species Survival-Intermediate Survival Species

*H. violacea* exhibited no influence by inoculation when results were pooled across soil replacement media (Fig 4.27A).

However, effects of inoculation within each soil replacement medium showed considerable variation (Fig 4.27B), and put this species into an intermediate group of survival. Inoculation enhanced survival in five media, while survival decreased in the remaining four media. Spoil + chitter, spoil + pasture + chitter and spoil + forest topsoil each supported significantly (p<0.05) lower seedling survival in inoculated plots. Spoil + pasture subsoil and spoil + pasture subsoil + biosolids supported a significant (p<0.05) increased survival in inoculated plots.

Pooling results from inoculated and uninoculated plots showed some variation, although an overall trend for increased seedling survival in spoil + pasture subsoil media was apparent (Fig 4.27C). Internal partitioning of variance confirmed this trend (data not shown). Media that contained pasture subsoil had significantly enhanced seedling survival with inoculation (p<0.01) and without inoculation (p<0.05) when compared to media without pasture subsoil. Inclusion of biosolids significantly reduced survival of both inoculated (p<0.01) and uninoculated (p<0.001) plants. Chitter did not have any effect on survival with or without inoculation.

**Fig 4.27** *H. violacea* survival. (Following page)
A) Survival data for inoculated and uninoculated plots of combined plant species and soil replacement media.
B) Survival data for inoculated and uninoculated plots within soil replacement media.
C) Survival data for the combined inoculated and uninoculated soil replacement media.
For all graphs:-
Columns with the same letters are not significantly different at the 5% level.
Bars at top left indicate least significant differences of the means at the levels indicated.
Data have been arcsine transformed before analysis by two-way ANOVA for graph A, and three-way ANOVA for graphs B & C.
*A. amblygona* survival was shown to be negatively impacted by inoculation when results are pooled for soil replacement media (Fig 4.28A).

Examination of individual soil replacement media showed that inoculation produced a mixed survival response. Survival was improved in three media (Fig 4.28B). These were spoil alone, spoil + pasture subsoil (significant at p<0.05) and spoil + pasture subsoil + biosolids. But survival in the remaining six media including the spoil + forest topsoil reference, spoil + chitter + biosolids and spoil + chitter (all significant at p<0.05) were all negatively impacted by inoculation, with no survival in the inoculated spoil + biosolids and spoil + chitter + biosolids media.

The soil replacement media had a significant effect upon seedling survival (Fig 4.28C). Survival was greatest in spoil + pasture subsoil and spoil + forest topsoil media, and was significantly decreased with added ameliorants. Partitioning of variance for effects of media showed that survival of uninoculated plants was not significantly increased by incorporation of pasture subsoil or chitter. Incorporation of biosolids significantly (p<0.05) decreased this species survival. Survival of inoculated plants was significantly increased by inclusion of pasture subsoil and with the absence of biosolids (p<0.05 for both). Chitter had no significant effect on seedling survival.

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**Fig 4.28** *A. amblygona* survival. (Following page)
A) Survival data for inoculated and uninoculated plots of combined plant species and soil replacement media.
B) Survival data for inoculated and uninoculated plots within soil replacement media.
C) Survival data for the combined inoculated and uninoculated soil replacement media.
For all graphs:-
Columns with same the same letters are not significantly different at the 5% level.
Bars at top left indicate least significant differences of the means at the levels indicated.
Data have been arcsine transformed before analysis by two-way ANOVA for graph A, and three-way ANOVA for graphs B & C.
4.2.3.7 Individual Species Survival—High Survival Species

*A. parvipinnula* survival was significantly enhanced (p<0.01) by inoculation when results were pooled across the soil replacement media (Fig 4.29A).

Inoculation of *A. parvipinnula* significantly increased seedling survival in seven media, with a trend increase in an eighth medium (Fig 4.29B). Seedling survival was increased to a very highly significant level (p<0.001) in spoil + chitter and the spoil + pasture subsoil + chitter media ranging to the spoil + forest topsoil and spoil media, with significance of p<0.05.

Pooling uninoculated and inoculated data to determine effects of soil replacement media showed relatively uniform seedling survival (Fig 4.29C). When compared to the spoil + forest topsoil reference, two media had significantly (p<0.05) reduced survival, with the remaining media not significantly different from these two extremes. Internal partitioning of variance showed that soil ameliorants did not significantly affect survival of this species, regardless of inoculation. In uninoculated media, there was a trend for increased seedling survival in media containing pasture subsoil compared to media without. Survival also tended to be increased for media without biosolids and without chitter in comparison to those with these ameliorants. Inoculation did not alter these trends.

**Fig 4.29** *A. parvipinnula* survival. (Following page)

A) Survival data for inoculated and uninoculated plots of combined plant species and soil replacement media.
B) Survival data for inoculated and uninoculated plots within soil replacement media.
C) Survival data for the combined inoculated and uninoculated soil replacement media.

For all graphs:-
Columns with the same letters are not significantly different at the 5% level.
Bars at top left indicate least significant differences of the means at the levels indicated.
Data have been arcsine transformed before analysis by two-way ANOVA for graph A, and three-way ANOVA for graphs B & C.
4.3.2.8 Individual Species Plant Height  As argued in the introduction to the field trials, only data for three plant species will be presented. In order, these are *H. violacea*, *A. parvipinnula* and *A. amblygona*. Height data are included to ascertain whether inoculation or soil replacement media had an effect upon plant form, providing an improved competitive ability, especially in the spoil + forest topsoil reference where a significant seed soil bank was evident.

*H. violacea* height (or length as it can be a climber/twiner), although not statistically significant, tended to be greater with inoculation (Fig 4.30A).

Analysis of plant height within the soil replacement media showed a strong positive trend with inoculation across almost all media (Fig 4.30B). However, only spoil + pasture subsoil + chitter + biosolids (p<0.05) and spoil + forest topsoil (p<0.01) showed a significant increase. Spoil + biosolids and spoil + chitter + biosolids could not be internally analysed as there were no surviving plants in uninoculated plots. Spoil + chitter + biosolids produced a very large plant; a surprising result against the trend recorded for other spoil based media. Apart from this one example, inoculated plots in media containing pasture subsoil and forest topsoil produced taller plants than spoil-based media.

**Fig 4.30** *H. violacea* plant height. (Following page)
A) Plant height for inoculated and uninoculated plots of combined plant species and soil replacement media.
B) Plant height for inoculated and uninoculated plots within soil replacement media.
C) Plant height for the combined inoculated and uninoculated soil replacement media.
For all graphs:-
Columns with same the same letters are not significantly different at the 5% level.
Bars at top left indicate least significant differences between the mean values at the levels indicated.
Data have been arcsine transformed before analysis by two-way ANOVA for graph A, and three-way ANOVA for graphs B & C.
A. parvipinnula showed a trend of increased plant height with inoculation across soil replacement media (Fig 4.3A).

When effects of inoculation were analysed within soil replacement media, plant height was increased in seven of the media (Fig 4.3B). Spoil + chitter (p<0.0.5), spoil + pasture subsoil + chitter and spoil + pasture subsoil + chitter + biosolids (p<0.01 each) supported significant increases in plant height with inoculation. A reverse trend was evident in plants raised on spoil + biosolids and spoil + chitter + biosolids. This was counter to responses for other media, especially given the positive effect of chitter on plant height.

Plant heights appear to be rather homogeneous across media for this species (Fig 4.3C), suggesting that media may have had less of an effect than for other species, as was the case for survival data. Pure spoil is the exception to this generalisation as it produced significantly (p<0.01) smaller plants compared to most other media. Fig 4.3C shows that the two media with the greatest plant height, spoil + chitter and spoil + pasture subsoil + chitter + biosolids, also exhibit the most significant response to inoculation.

**Fig 4.31** A. parvipinnula plant height. (Following page)
A) Plant height for inoculated and uninoculated plots of combined plant species and soil replacement media.
B) Plant height for inoculated and uninoculated plots within soil replacement media.
C) Plant height for the combined inoculated and uninoculated soil replacement media.
For all graphs:-
Columns with same the same letters are not significantly different at the 5% level.
Bars at top left indicate least significant differences between the mean values at the levels indicated.
Data have been arcsine transformed before analysis by two-way ANOVA for graph A, and three-way ANOVA for graphs B & C.
A. amblygona showed an almost negligible difference in plant height between uninoculated and inoculated plots across soil replacement media (Fig 4.32A).

Within soil replacement media (Fig 4.32B), inoculation significantly increased plant height in only spoil + chitter (p<0.05). Overall there was no clear trend with regard to plant height. As two of the spoil-based media, spoil +biosolids and spoil + chitter + biosolids, had no survivors in inoculated plots, no comparisons can be made and thus any apparent trends are based on scant data. This also negatively impacts the results presented in Fig 4.32C. Inoculation made little, if any difference to plant height with regards to pasture subsoil inclusive media or the spoil + forest topsoil medium.

Plants raised on soil replacement media displayed some interesting trends (Fig 4.33C). The spoil only medium produced plants as tall as spoil + forest topsoil reference plants and significantly taller than the remaining spoil-based media (p<0.05 for the spoil + biosolids and spoil + chitter, and p<0.01 for spoil + chitter + biosolids medium).

Fig 4.32 A. amblygona plant height. (Following page)
A) Plant height for inoculated and uninoculated plots of combined plant species and soil replacement media.
B) Plant height for inoculated and uninoculated plots within soil replacement media.
C) Plant height for combined inoculated and uninoculated soil replacement media.
For all graphs:-
Columns with same the same letters are not significantly different at the 5% level.
Bars at top left indicate least significant differences between the mean values at the levels indicated.
4.3.2.9 Individual Species Shoot Dry Weight

Shoot dry weight provides a more accurate assessment of success of the plant under investigation when subjected to differing conditions. Total plant biomass would be the ideal as it allows comparison between shoots and roots reflecting any partitioning of plant resources that may have occurred.

Although not statistically significant, *H. violacea* showed a very strong trend towards increased shoot dry weight with inoculation (Fig 4.33A). The lack of a statistically significant result was surprising given the response of shoot dry weight within each soil replacement medium (Fig 4.33B). Here, shoot dry weight of inoculated plants was significantly increased in six of the nine media and one medium, spoil + chitter, showed a similar trend. Responses on spoil + biosolids and spoil + chitter + biosolids, were unable to be internally compared as no uninoculated plants survived, a fact that has implications for statistical bias towards inoculation. This consideration aside, levels of statistical significance were very high. Spoil was significant (p<0.05), spoil + pasture subsoil + chitter + biosolids medium was very significant (p<0.01) and the remaining four media were very highly significant (p<0.001). Together these data indicate that inoculation had a strong positive effect on shoot dry weight.

Of the soil replacement media, spoil + forest topsoil supported the greatest shoot dry weight, although spoil + pasture subsoil with or without biosolids also produced relatively high shoot dry weights (Fig 4.33C). Internal partitioning of variation showed that even though there are very strong trends, none of the partitioned factors elicited a significant response. Therefore, for both inoculated and uninoculated plants, there were trends indicating that inclusion of pasture subsoil increased, while biosolids and chitter decreased shoot dry weight.

**Fig 4.33 H. violacea** shoot dry weight. (Following page)
A) Shoot dry weight for inoculated and uninoculated plots of combined plant species and soil replacement media.
B) Shoot dry weight for inoculated and uninoculated plots within soil replacement media.
C) Shoot dry weight for the combined inoculated and uninoculated soil replacement media.
For all graphs:-
Columns with same the same letters are not significantly different at the 5% level.
Bars at top left indicate least significant differences between the mean values at the levels indicated.
Pooled data for soil replacement media of inoculated versus uninoculated *A. parvipinnula* showed a statistically non-significant trend of increased shoot dry weight with inoculation (Fig 4.34A).

As with plant height, this is unexpected when comparisons are made between uninoculated and inoculated plots within each soil replacement media (Fig 4.34B). Inoculated plots produced plants with significantly greater shoot dry weights in all pasture subsoil containing media (p<0.05, except spoil + pasture subsoil + chitter + biosolids, p<0.01). Spoil + forest topsoil reference, while not significant, supported an increase in shoot dry weight of inoculated plants. An interesting observation is the reverse trend for spoil-based media. In these cases, except in spoil only medium, inoculated plants trended to have lower shoot dry weights. Indeed, shoot dry weights generated in the spoil + chitter + biosolids medium were very close to being significantly different.

When inoculation treatments are pooled and growth responses to soil replacement media examined, this species appeared to respond very well to additions of chitter and biosolids with or without inoculation (Fig 4.34C). This trend was not always statistically significant, but became more evident with internal partitioning of variance. When uninoculated, there was a statistically non-significant trend for increased shoot dry weight in media without pasture subsoil. A similar trend was apparent for media with chitter with the p value of p<0.055 approaching significance. Indeed, shoot dry weights of plants raised on media with biosolids were significantly (p<0.05) larger. When inoculated, plants were significantly (p<0.001) larger in media with pasture subsoil, and significantly larger (p<0.05) when media contained biosolids or chitter.

**Fig 4.34** *A. parvipinnula* shoot dry weight. (Following page)

A) Shoot dry weight for inoculated and uninoculated plots of combined plant species and soil replacement media.
B) Shoot dry weight for inoculated and uninoculated plots within soil replacement media.
C) Shoot dry weight for the combined inoculated and uninoculated soil replacement media.

For all graphs:-

Columns with same the same letters are not significantly different at the 5% level.
Bars at top left indicate least significant differences between the mean values at the levels indicated.
Inoculation had no discernable effect on *A. amblygona* shoot dry weights when data were pooled across the soil replacement media (Fig 4.35A). In contrast, when analysed within each soil replacement medium, inoculation produced mixed results (Fig 4.35B). As noted previously, inoculated plots within two of the spoil-based media had no survivors, and thus any observed trends in spoil-based media are of limited value. However, inoculation increased shoot dry weight slightly in spoil and spoil + chitter media, but not to a significant level. Plant growth responses were very mixed within pasture subsoil containing media. Spoil + pasture subsoil (not significant) and spoil + pasture subsoil + biosolids \((p<0.05)\) increased shoot dry weight. The remaining two media generated smaller plants in inoculated plots. The spoil + forest topsoil reference produced significantly \((p<0.05)\) smaller plants when inoculated, an outcome further confounding evaluating the success of inoculation with this combination of host plant and inoculant.

Effects of soil replacement media were analysed using pooled inoculation and non-inoculation data sets. These analyses showed that *A. amblygona* did not respond well to addition of biosolids or chitter (Fig 4.35C). Internal partitioning of variance did not detect any statistically significant results, but did confirm the observed trend. Whether plants were inoculated or not, increased shoot dry weights occurred in media with pasture subsoil but without biosolids or chitter.

The surprise result with this species was the apparent success of plants grown in spoil, with shoot dry weights not significantly different from either spoil + pasture subsoil or spoil + forest topsoil.

**Fig 4.35** *A. amblygona* shoot dry weight. (Following page)
A) Shoot dry weight for inoculated and uninoculated plots of combined plant species and soil replacement media.
B) Shoot dry weight for inoculated and uninoculated plots within soil replacement media.
C) Shoot dry weight for the combined inoculated and uninoculated soil replacement media.
For all graphs:-
Columns with same the same letters are not significantly different at the 5% level.
Bars at top left indicate least significant differences between the mean values at the levels indicated.
A) 

Shoot dry weight (g) 

Uninoculated vs. Inoculated 

B) 

Shoot dry weight (g) 

0.05 0.01 

Spoil 
Spoil + chitter 
Spoil + biosolids 
Spoil + pasture + biosolids 
Spoil + pasture subsoil 
Spoil + pasture subsoil + chitter 
Spoil + pasture subsoil + biosolids 
Spoil + pasture subsoil + chitter + biosolids 
Spoil + forest topsoil 

Uninoculated 
Inoculated 

C) 

Shoot dry weight (g) 

0.05 0.01 

Spoil 
Spoil + chitter 
Spoil + biosolids 
Spoil + pasture subsoil 
Spoil + pasture subsoil + chitter 
Spoil + pasture subsoil + biosolids 
Spoil + pasture subsoil + chitter + biosolids 
Spoil + forest topsoil 

Abc
4.4 Discussion

Eight soil replacement media were selected to test their ability to support the growth of a community of native vegetation used in the rehabilitation of an open cut coalmine. The first shadehouse trial examined the various soil replacement media for the presence or absence of an indigenous rhizobia population. The second shadehouse trial determined whether inoculation with a native rhizobium would enhance community plant growth in a reduced number of the soil replacement media.

4.4.1 Plant Growth, Resident Rhizobia and Soil Replacement Media in Shadehouse Trials

The soil replacement media used in the shadehouse trial can be divided into two groups; media without or with pasture subsoil amelioration. Both were compared to the spoil + forest topsoil reference.

The shadehouse results can be summarised as follows. The first shadehouse trial tested the effect of the of the soil replacement media on plant community growth and tested for the presence of a resident population of rhizobia by destructive harvest and measuring shot dry weight (Fig 4.8). When compared to the spoil + forest topsoil reference, community shoot dry weight was significantly lower in spoil, and spoil + chitter treatments, whilst significantly higher in those soil replacement media that included biosolids. Spoil + pasture subsoil + biosolids produced the greatest community shoot dry weight, significantly higher than the remaining media.

The presence of a resident rhizobia population was deduced by collection and determination of dry weight of root nodules from the native legumes in each of the soil replacement media (Fig 4.9). Small populations were recovered from some individual pots within the blocks of the experiment from the four soil replacement media that did not include pasture subsoil or forest topsoil. Larger nodule dry weights were recovered from the three media that included pasture subsoil and the spoil + forest topsoil reference, with spoil + pasture subsoil + biosolids having significantly higher nodule dry weights than all other media.

The second shadehouse trial compared the results of inoculation with a single rhizobial isolate, RSFAp2, of 4 media common to the two trials. Shoot dry weights were not significantly different between the media when directly compared (Fig 4.11). However, nodule dry weights were increased in the four soil replacement media. This
increase was significant in two, spoil + biosolids and soil + pasture subsoil. Observational data (Fig 4.14 and 4.15) showed that the native legumes became more competitive with the canopy species upon inoculation.

4.4.1.1 Soil Replacement Media Without Subsoil

In the uninoculated trial neither spoil, nor spoil + chitter produced good plant growth (Fig 4.8), with both significantly less than spoil + forest topsoil. Spoil at this mine is crushed rock that has been buried for approximately 230 million years (Herbert and Helby, 1980). Chitter is a waste product that results from industrial washing of mined coal, and is therefore of the same age. Both of these materials are essentially sterile due to the length of time and depth of burial (Charnock and Grant, 2005; Maiti, 2006). Neither material has been subjected to any soil forming processes. Soils form as result of several processes interacting over time including climate, organisms and human activity, relief or topography and the original parent material (Young and Young, 2001). When used as a growth medium for plants, they lack many of the attributes that a “good” soil would have evolved. These attributes include structure, organic material and an ecosystem of micro- and macro-flora and fauna (Briggs and Smithson, 1995), all of which allow for water retention and provision of essential mineral nutrients, especially nitrogen.

Chitter can be used as a mulch preventing surface crusting thereby increasing water penetration and reducing evaporation (Hannan, 1995). Chitter can also contain relatively high levels of organic carbon, up to 6.9%, that can be utilised by plants and microorganisms (Masters, 1996). However this geogenic carbon is not as readily available as soil organic carbon (Waschkies and Hüt tl, 1999). Additionally, chitter is reported to have significant levels of nitrogen although this was measured as total nitrogen tightly bonded to the carbonaceous material and not readily plant available (Waschkies and Hüttl, 1999; Charnock and Grant, 2005). Therefore, while chitter is reported to have some potentially useful properties, when used as the only ameliorant of spoil, it is not surprising that no discernible effect was measured (Fig 4.8).

When biosolids were used to amend the spoil, significant increases in shoot biomass were obtained when compared to spoil only and the forest topsoil reference (Fig 4.8). Biosolids are a high nutrient media containing high levels of essential plant nutrients including nitrogen and phosphorus (Sarooshi et al., 2002; Krogstad et al., 2005), and analysis for plant available nitrogen of the biosolids used in this study
showed 4, 511 mg/kg (Nussbaumer, 2005; Cole et al., 2006). Biosolids, dried sewage sludge and liquid slurries have been studied and used extensively in many parts of the world to dispose of the waste, as an aid to plant growth and as soil ameliorants to improve the physical characteristics of soil (Whatmuff, 2002). There are many studies that examine the effect of biosolids application on the survival, composition and nitrogen fixation of rhizobia in different soils (e.g. Giller et al., 1998; Munn et al., 2001; Chaudri et al., 2008), but to the knowledge of this author, none report biosolids containing rhizobia. Examination of the resident microbial community of biosolids was not undertaken as part of this study, but from Fig 4.9, no rhizobia were recovered from the roots of legumes growing in spoil, chitter or biosolids.

### 4.4.1.2 Soil Replacement With Subsoil

Ameliorating spoil with pasture subsoil increased shoot biomass substantially in comparison to spoil only. The physical effects of pasture subsoil + chitter produced a significant increase over and above pasture subsoil and was equivalent to the forest topsoil reference (Fig 4.8). Pasture subsoil had a resident rhizobial population as evidenced by the presence of root nodules from the uninoculated trial, as opposed to the media that was not ameliorated with pasture subsoil (Fig 4.9). The presence of nitrogen fixing bacteria and a history of fertilizer addition to the pasture topsoil (G. Marschke, Thiess P/L Environmental Officer, Mount Owen Mine) produced higher levels of nitrogen than the spoil itself. Analysis of plant available nitrogen showed 15.6 mg/kg for pasture subsoil, 0.2 mg/kg for spoil and 5.4 mg/kg for forest topsoil (Nussbaumer, 2005; Cole et al., 2006).

The greatest increase resulted from an apparently synergistic pairing of pasture subsoil and biosolids, due largely to the combined nutrient levels. Apart from the direct effects of increased nutrients, biosolids may also have an indirect effect on plant growth. In the presence of a resident microbial population (it is safe to presume that the pasture subsoil would possess both rhizobia and other soil bacteria and microorganisms) the increase in organic matter will stimulate the growth of other soil dwelling organisms (Emmerling et al., 2000) including free-living rhizobia. This will increase nutrient cycling, making further nutrient resources available, and increase the numbers of rhizobia in the soil.
4.4.1.3 Effects of Inoculation in the Shadehouse

Inoculation with the isolate selected from Chapter 3 (RSFAp2, *Burkholderia* sp., hereafter referred to as the inoculum) doubled the mean shoot biomass in spoil, although this was not statistically significant (Fig 4.1). The increased biomass that resulted from inoculation in spoil was not equivalent to the biomass produced by any of the other uninoculated soil replacement media (spoil + chitter excepted). This indicates that abiotic or biotic factors, other than a lack of nitrogen, retarded, or were not available, to promote plant growth in spoil.

Inoculation did not produce a statistically significant increase in community plant growth in any soil replacement media (Fig 4.11). However, inoculation did increase nodule biomass across the four media, with spoil + pasture subsoil and spoil + biosolids increasing significantly (Fig 4.12). Interpretation of these two figures plus photographic evidence from Fig 4.14 and 4.15 indicates that there was a change in community species composition responsible for the shoot dry weight responses. Uninoculated spoil + biosolids media (Fig 4.14 C) supported larger canopy plants, in this case *C. maculata*, with little growth of the legume species. Inoculation changed the distribution of shoot dry weight of the community to be dominated by growth of the legume species (Fig 4.14 D), with a concomitant increase in nodule dry weight (Fig 4.12). Spoil + pasture subsoil showed a less pronounced change and probably needs to be interpreted more cautiously. However, the response may indicate a slight preference by one or more of the legumes for the inoculum rhizobia over the resident rhizobia.

The relative shift in species dry weights before and after inoculation may indicate a limited ability of the legumes to utilize soil nitrogen, in the form that is present in the biosolids, or better adaptation of *C. maculata* to soil nitrogen acquisition over the legumes. As a consequence, the legumes were not competitive until inoculated.

This trial showed that both forest topsoil and pasture subsoil support a resident rhizobial population (Fig 4.9). This was to be expected given that a number of species of legumes are found in forest and pasture flora communities. Flora surveys conducted in the Ravensworth State Forest have identified a total of 23 native and 1 exotic Fabaceae species and 12 native *Acacia* species (Cole *et al.*, 2004).

Surveys in surrounding pastures from which the pasture subsoil was sourced have identified 9 species of exotic legumes belonging to *Trifolium*, *Melilotus* and *Medicago* genera (C. Castor, pers. com.). These exotic pasture species have very specific rhizobial symbiont requirements. For instance, the medics are only nodulated
by *Sinorhizobium meliloti* or *S. medicae* (Eardley *et al*., 1992; Bailly *et al*., 2006) while *Trifolium* species are only nodulated by various strains of *Rhizobium leguminosarum* biovar *trifolii* (Pryor and Lowther, 2002). This would suggest that these legumes would be unable to nodulate and maintain populations of native rhizobia in the pasture soils.

Without native legumes growing in the pasture soils for this length of time, survival of native rhizobia would not be expected (Thrall *et al*., 2001 and 2005). Results from Chapter 2 (Table 2.7) strongly suggest that at least some native legumes were able to nodulate with non-native rhizobia, thus forming potential competitors for any re-introduced native rhizobia. Observations of the surrounding pastures indicate that some native species of legume such as *Daviesia genistifolia*, *Swainsonia galegifolia* and *Acacia implexa* re-invade and nodulate with these rhizobia.

While some very minor nodulation was observed in spoil-based media, this can be attributed to the open-cut mining process where forest subsoil is included in what is called the pre-strip. This may include soil immediately under the topsoil used for rehabilitating the unweathered bedrock above the coal seams. This forest subsoil, a 10 – 20 cm layer below the forest topsoil, similar to the pasture subsoil included in the present study, also supported nodulation (data not shown, Cole *et al*., 2006). These findings were included in the ACARP report to demonstrate the value of all subsoils in rehabilitating spoil dumps. It was not used in this study, as it is not regularly conserved in current mining practice.

Establishing inoculant species and strains into soil situations where a resident rhizobia population exists can be difficult to achieve. Resident rhizobia enjoy a positional advantage, whereby they occupy the soil void through which plant roots grow, while inoculant rhizobia maybe concentrated around the seed (Lopez-Garcia *et al*., 2002). Moreover, the resident population may have a physiological advantage in having a greater specificity for the host than the inoculating strain (Thies *et al*., 1991) or be present in sufficient numbers to nullify any inoculant effect (Singleton and Tavares 1986).

Some of these advantages of residency may be overcome by using inoculating strains sourced from the same soils. This should ensure that the inoculum is adapted to the soil conditions, although in the case of spoil dumps and severe disturbance this may not be especially important. Positional advantage may be overcome to some extent by the use of a liquid carrier that facilitates spread of the inoculum through a larger portion of the immediate soil profile. In addition, “flooding” the soil with very high numbers of
bacteria will give the inoculum every opportunity to nodulate the host legume. If the host legume that is being re/introduced is novel or has been absent for a significant amount of time, for instance when revegetating abandoned agricultural land, then the inoculum may have a greater specificity for the host, and thus outperform any resident rhizobia.

Taking the results from the shadehouse trials, it was possible to make some predictions as to outcomes for the field trials. Firstly, spoil would not make a suitable media for plant growth. Plant growth was considerably reduced in pure spoil when compared to the other soil replacement media, and inoculation alone was not enough to produce growth comparable to the other media. Secondly, amelioration of the spoil with any of the other media, except chitter, will improve plant growth, although chitter may have a positive physical effect when included with other ameliorants, such as pasture subsoil. The best combination of media for promoting plant growth without inoculation was spoil + pasture subsoil + biosolids (Fig 4.9). Inoculation will generally improve plant growth, although the presence of resident rhizobia that are native (forest topsoil) or introduced (pasture subsoil), may reduce the benefit of inoculation.

### 4.4.2 Survival and Growth of Selected Legumes Under Field Conditions

The field trials investigated specific responses of six native legume species to inoculation and soil replacement media under field conditions. Species were selected for their common occurrence in nearby native forest, their vegetative structure and seed availability. Final survival of host legume species was presented as a proportion of highest seedling emergence to reduce any fecundity or germination effects that may be inherent in species used.

The field trials showed that responses to inoculation with the same single rhizobial isolate, RSFAp2, as used in the shadehouse trials, were dependent upon both soil replacement media and host plant species. The results were presented comparing pooled (that is, combined) uninoculated to pooled inoculated plant data across all soil replacement media, between uninoculated and inoculated plants within soil replacement media, and finally uninoculated and inoculated plants pooled within each of the soil replacement media. Three parameters were measured; survival, plant height and shoot dry weight. These parameters were presented for the entire “community” of legumes, and then for each of the legume species individually.
On a community basis, survival was not significantly increased by inoculation, (Fig 4.21A) although significant differences were observed within soil replacement media (Fig 4.21B) with survival increased by soil replacement media with pasture subsoil, and inoculation significantly increasing survival in three of the four media without pasture subsoil. Combined plant height was significantly increased with inoculation (Fig 4.22A) and not surprisingly this was reflected in the individual soil replacement media (Fig 4.22B), with all spoil + pasture subsoil and spoil + forest topsoil media having significantly increased height with inoculation. Shoot dry weight was higher for the inoculated community, but was not significant for the pooled data (Fig 4.23A). However within the individual soil replacement media (Fig 4.23B) that included pasture subsoil or forest topsoil, there were substantial, and in two media, significant increases in shoot dry weight with inoculation.

Examination of the individual species data showed that pooling data on a community basis had masked some important trends. Individually the six species could be divided into three groups based on survival. Three species were deemed to be low survival species. These were *P. retusa* (Fig 4.24), *I. australis* (Fig 4.25) and *D. ulicifolia* (Fig 4.26). Inoculation greatly increased survival of these three species. Survival was also increased by inclusion of pasture subsoil or forest topsoil. Indeed, *P. retusa* only produced survivors when inoculated in spoil + pasture subsoil and spoil + forest topsoil. *I. australis* only survived in pasture subsoil included media, with inoculation increasing survival further. *D. ulicifolia* survival was greater than previous species, but the same trend of enhanced survival with inoculation and inclusion of pasture subsoil was exhibited. The second group consisted of *H. violacea* (Fig 4.27) and *A. amblygona* (Fig 4.28) and were termed intermediate survival species. Inoculation did not enhance *H. violacea* survival, and this was reflected in the individual soil replacement media, where inoculation produced mixed results. Pasture subsoil containing media generally increased survival. *A. amblygona* showed a slight trend of negative response to inoculation, but did show increased survival when grown in pasture subsoil or forest topsoil containing media, with a surprising result of uninoculated plants only surviving in two spoil based media. The final species, *A. parvipinnula* had higher survival overall, but also significantly enhanced survival (Fig 4.29) when inoculated and a trend for increased survival when grown in pasture subsoil and forest topsoil media.

The poor survival of the first three species precluded any discussion with regards to height or shoot dry weight.
Both *H. violacea* (Fig 4.30) and *A. parvipinnula* (Fig 4.31) showed increased height with inoculation, although neither was significant when pooled. However, individual soil replacement media were often significantly so, especially for the pasture subsoil and forest topsoil media. *A. amblygona* (Fig 4.32) showed almost no difference between inoculated and uninoculated plants, with the spoil only media producing heights equal to the spoil + forest topsoil media.

Shoot dry weights for *H. violacea* (Fig 4.33) and *A. parvipinnula* (Fig 4.34) both increased overall in response to inoculation. *H. violacea* was significant, surprisingly given that many of the individual soil replacement media supported significantly higher shoot dry weights. Indeed all pasture subsoil and forest topsoil media were significantly increased. *A. parvipinnula* displayed significantly increased shoot dry weights across pasture subsoil media, and surprisingly reversed results in the pasture absent media. *A. amblygona* (Fig 4.35) did not increase shoot dry weight when inoculated with this rhizobial isolate, and showed a negative response to with biosolids or chitter present, in some cases significantly so.

Survival of plants at the final harvest was affected by two factors other than inoculation. These were:-

1. Soil replacement media, physical and biotic characteristics
2. Individual species fecundity and ability to survive.

Extreme soil conditions, such as are found in rehabilitating coalmines, can have adverse effects on germination, emergence and survival of plants (Monterroso et al., 1998; Dutta and Agrawal, 2003). While germination was not measured, seedling emergence was recorded to ascertain whether the soil replacement media affected this parameter of plant survival, perhaps by the chemical composition of the soil replacement media.

The inclusion of biosolids in the pasture subsoil amended media produced a significant decrease in seedling emergence when compared to the forest topsoil reference or the spoil + pasture subsoil media (Fig 4.16). The biosolids used in this experiment had been stockpiled and were extensively colonised by exotic grass and weed species that germinated and competed with the native species (Cole et al., 2006).

Recruitment of exotic weeds after a lengthy period of stockpiling is a management issue rather than any inherent property of this media. But it does highlight a difficulty of using this material as an ameliorant. Recruitment of rapid life-cycle weed species into high nutrient amendments such as sewage sludge is of common occurrence (Halofsky & McCormick, 2005). There is substantial evidence that survival and
recruitment of tree species as well as overall species diversity has been adversely affected by biosolids during coal mine reclamation, setting back forest development by an estimated 10 - 20 years (Nord, 1998 quoted in Halofsky & McCormick, 2005), and up to 24 years for other community types (Paschke et al., 2005).

The individual legumes also displayed significant differences in emergence (Fig 4.1). *I. australis* and *P. retusa* had significantly reduced emergence compared to the *A. parvipinnula* and *H. violacea*, with the remaining two species, *A. amblygona* and *D. ulicifolia* giving an intermediate response. These differences may be explained by individual tolerances to the edaphic characteristics of the soil replacement media.

The replacement media that did not incorporate biosolids or chitter, exhibited pH values of 4.7 for pasture subsoil, 5.6 for forest topsoil and 9.2 for spoil, (pH for chitter was not determined) which has implications for nutrient availability. The mechanics of the experimental design, amelioration and mixing of media by ripping, would result in a change in pH of the combined media, especially in the zone where ripping occurred, i.e. about the top 400mm. However the considerable differences in the chemical make up of the media would have exerted a strong influence on emergence rates and ultimately survival.

The physical characteristics of the soil replacement media appeared to have a pronounced effect on survival and rates of decline in the proportion of surviving plants (Fig 4.19). This was largely attributed to the relative water retention abilities of the soil replacement media, in particular forest topsoil and pasture subsoil. Both have a higher clay and organic matter content than the spoil or chitter that would have contributed to a greater water holding capacity (Newman, 1996). While biosolids generally have high organic matter contents (Chennon et al., 2003; Chaudri et al., 2008) it would be unlikely that the rate of application was sufficiently high to affect water retention, physically resulting in a layer of biosolids only a few millimetres in thickness.

Rainfall distribution was sporadic, and in the first nine months only four months had near or above average rainfall (Fig 4.18). Time course survival profiles in soil replacement media showed that inclusion of pasture subsoil had a positive effect on plant survival, reducing the rate of decline. For instance, spoil + pasture subsoil media, final proportional survival for all species was 0.49 for uninoculated plants and 0.72 for inoculated plants, which compares well to forest topsoil with 0.50 and 0.63 survival respectively. In comparison, survival rates for spoil + chitter of 0.03 and 0.10 for uninoculated and inoculated plants respectively were the lowest recorded.
Lastly, while this issue was not investigated, it may be possible that the presence of ecto- or endo- mycorrhizal fungi present in pasture subsoil and forest topsoil (Newman, 1996; Nussbaumer, 2005) may have increased drought resistance and, or accessed nutrients not otherwise available to plants growing in the remaining, non-pasture subsoil amended media.

The effect of these different factors acting or interacting to affect survival was highlighted by *P. retusa, I. australis* and *D. ulicifolia*. Survival data for these species (Fig 4.24, 4.25 and 4.26 respectively) shows varying responses in order of severity. *P. retusa* only survived on spoil + pasture subsoil and forest topsoil, indicating that spoil itself, chitter and biosolids all impacted negatively, on this species’ survival. *I. australis* only survived in pasture subsoil amended media and forest topsoil, while *D. ulicifolia* survival was adversely affected by biosolids. Two of the three remaining species, *H. violacea* and *A. amblygona* were also significantly affected by interactions with the soil replacement media (Figs 4.27C and 4.28C respectively). *A. parvipinnula* was not significantly affected, but showed a trend of increased survival in pasture subsoil and without chitter (Fig 4.29C).

These issues were not the main focus of the study here, but certainly the initial emergence and ability of the plants to survive when grown in the varying soil conditions presented by the media, must be taken into account when the effects of inoculation are taken into consideration. Additionally they point to further areas of research where the suitability of species selected for use in restoration and reconstruction projects needs to be determined.

4.4.3 Inoculation Improves Plant Survival and Growth

Australian legume species generally have been shown to form functional nodules with a wide range of rhizobia from across the four genera reported as occurring in Australia (Barnett & Catt, 1991; Burdon *et al*., 1999; Lafay & Burdon, 1998 and 2001; Marsudi *et al*., 1999; Thrall *et al*., 2000). The effectiveness of these legume-rhizobia combinations has been shown to vary widely (Burdon *et al*., 1999; Thrall *et al*., 2000), with inoculation of native legumes by highly effective isolates increasing survival in the field significantly (Thrall *et al*., 2000).

The inoculum, RSFAp2 was collected from root nodules of *A. parvipinnula* growing in the Ravensworth State Forest. This isolate was chosen for this field trial because it was a generalist nodulator that formed functional nitrogen fixing nodules on
all six species (Chapter 3, Table 3.1). It was a highly effective nodulator (as defined in Chapter 3) on one of the target legumes, *A. parvipinnula*, moderately effective on four (*H. violacea, A. amblygona, D. ulicifolia* and *P. retusa*) and least effective on *I. australis*. This difference in nodulation effectiveness maybe one explanation for the varying success of the legumes in the field trial, in that different effectiveness of nitrogen supply to the host may have effected plant growth, and hence ability to access other soil nutrients or water through an expanded root area.

There is evidence (Murray *et al.*, 2001; Thrall *et al.*, 2005) that inoculation with suitable nitrogen-fixing symbiotic bacteria will improve survival and growth of target legumes, especially when planted into soils or materials of low fertility and with little if any resident rhizobial population. If there is a resident rhizobial population, obtaining a positive inoculation result will be achieved if the inoculant is more effective and more competitive for the target legumes, and, or the resident rhizobia only sparsely populate the soil. If inoculation does not produce a positive effect, preferably for both increased survival and growth, it must be seen as a failure requiring some form of remedial action such as changes to inoculant or inoculation strategy.

Combined survival and plant growth, both height and biomass was improved by inoculation (Fig 4.21, 4.22 and 4.23 respectively). The breakdown of the individual species highlights the variable and species-specific effects of inoculation.

Three species, *P. retusa, I. australis* and *D. ulicifolia* performed poorly in terms of survival and growth, had survival enhanced by inoculation. Only inoculated plants for *P. retusa* survived (Fig 4.24) while the majority of surviving *D. ulicifolia* plants were inoculated (Fig 4.26B). *I. australis* had increased survival overall when inoculated (Fig 4.25A) and significantly so for two soil replacement media, spoil + forest topsoil and spoil + pasture subsoil + biosolids (Fig 4.25B). As noted above, the inoculating isolate was not highly effective for any of these species, which suggests the use of a highly effective isolate would further improve survival and plant biomass.

The benefits of using a highly effective isolate versus a moderately effective isolate can be seen with the three remaining species, *A. parvipinnula, H. violacea* and *A. amblygona*. Overall survival was significantly better for these species when compared to the previous three. Not surprisingly, inoculation produced differing outcomes.

The inoculum was evaluated as highly effective for *A. parvipinnula* (Chapter 3 Table 3.3) and this was reflected in significantly improved survival compared to uninoculated plants (Fig 4.29A). In addition, this inoculum effectiveness was also
reflected in the almost uniform increase across the soil replacement media (Fig 4.29B). This may reflect an inherent ability of this species to be successful in a range of edaphic soil conditions given that it was the only species to survive in all media, regardless of inoculation. Biomass production was more equivocal and showed a strong media influence (Fig 4.34B). Nonetheless, inoculation consistently and significantly increased biomass in the pasture subsoil amended media. The reversal in the non-pasture subsoil media was perplexing and requires further investigation. The allocation of biomass as reflected by comparing the plant height data (Fig 4.31) to the shoot dry weight data, indicates that competition from older plants in the seed bank of the forest topsoil, causing shading, and may have been responsible for the reduced biomass of plants in the forest topsoil plots.

*H. violacea* presents a mid-way point in successful inoculation with a moderately effective isolate. Survival was not significantly enhanced by inoculation (Fig 4.27A), and demonstrated inconsistent responses to inoculation when grown in different media. However, inoculation did substantially increase shoot dry weights across all media, and in this respect must be seen as successful. This species would appear to be able to form at least moderately effective combinations with a wide range of rhizobia (Thrall *et al.*, 2000) and see Chapter 3 Table 3.2.

The last species, *A. amblygona* did not perform well in the field with the inoculum. Survival was actually slightly reduced with inoculation (Fig 4.28A), with uninoculated experimental plants significantly outperforming inoculated plants in several media (Fig 4.28B). Biomass data was not improved by inoculation (Fig 4.35A), and when analysed by individual soil replacement media (Fig 4.35B) presented mixed results. The non-significant differences between uninoculated and inoculated plants in spoil alone and spoil + pasture subsoil media suggests that for this species an equally effective symbiont is resident in these media. The result observed in the forest topsoil media requires further investigation, but may involve physiological aspects of the symbiosis not observed for the other species such as carbon draw down and the ability of the host legume to up-regulate photosynthesis to accommodate this requirement, or simply indicate that this species does not respond as well to inoculation generally, or to the inoculum used here.

As stated above, successful inoculation requires the inoculum to be more competitive for nodulation and more effective at nitrogen fixation and, or outnumber the resident rhizobia where present (Brockwell and Bottomley, 1995; Deaker *et al.*, 2004).
Enumeration of the resident rhizobial population was not performed for either the shadehouse or field trials, but resident rhizobia were recovered from pasture subsoil and forest topsoils by the collection of root nodules. Biosolids, chitter and spoil did not possess resident rhizobia, although incidental inclusion of forest subsoil in some of the spoil both in the shadehouse trial and in the field demonstrated that rhizobia could be present. Under favourable conditions, rhizobia are reported to number as high as $1 \times 10^5$ g$^{-1}$ of soil (Brockwell and Bottomley, 1995), but can be detected using the Most Probable Number (MPN) method when there are as few as 4 rhizobia g$^{-1}$ of soil (Thrall et al., 2005), and this low number is reported to be sufficient to induce inoculation, although this is open to further clarification. Rhizobial numbers would be expected to be lower than those for less disturbed soils. The poor state of the forest topsoil has been mentioned already, and dilution of this soil by ripping into spoil would serve to further reduce rhizobial numbers throughout the medium. Rhizobial numbers are known to decrease with soil depth (Brockwell et al., 1995), thus the main source of rhizobia, the subsoils, would also have reduced numbers of rhizobia, and again, would be further reduced once mixed into spoil by machinery. Realistically, any inclusion of soil containing rhizobia could be expected to induce inoculation in legumes, given satisfactory specificity and effectiveness. Therefore, equal or improved growth and, or survival in uninoculated plants suggests the presence of rhizobia more effective in terms of nitrogen fixation than the inoculant.

4.4.4 Conclusion

In conclusion, it can be seen that amelioration was required for the successful establishment of native vegetation on spoil dumps and that inoculation with native locally sourced rhizobia alone is not enough to overcome the shortcomings in several of the media used here. Once those detrimental abiotic factors are accounted for, inoculation can significantly improve survival and growth of the plants used in these trials, thus greatly improving the quality and overall success of revegetation efforts.

The caveat is that the inoculum used was not successful for every species and demonstrated again that the rhizobia used must be matched to the host plant species. For this trial the host species used represented some of the most widespread species found locally at the Ravensworth State Forest Vegetation Complex, but were only a few of the total legume species. Less commonly found species, or species more difficult to obtain seed for, may have more specific symbiotic requirements yet may be important for
native forest reconstruction from a biodiversity viewpoint. This would seemingly preclude the use of limited numbers of inoculating rhizobia species or strains for rehabilitation of native vegetation.
Chapter 5.

General Discussion
5.1 Introduction

In many regions from other parts of the world with younger more fertile soils, legumes are primarily early succession plants that are replaced by other plants in later successional stages. In Australian terrestrial ecosystems legumes are often the dominant vegetation, whether measured by species or biomass (Groves, 1998). Thus the introduction of legumes early in the revegetation of a landscape not only increases the nitrogen capital of a soil, but also reflects the vegetation of the mature ecosystem.

Restoration of Ravensworth State Forest under the Mount Owen Mine “Plan of Management” has as its stated principal objective the re-establishment and enhancement of the ecological values of the forest and specified surrounding areas. This is to be achieved through effective management that:

- Maintains the diversity and genetic resource of the flora currently existing within the locality, and
- Provides the basis for an expanded (that is geographically extended, this author’s emphasis) forest-woodland ecosystem that is self-sustaining in the long term.

This phrase, “self-sustaining” provides the key to the approach undertaken in this study. Self-sustaining is defined here as a minimal, if any, on-going intervention into the natural processes of vegetation establishment and succession of the restored ecosystem. This requires not just revegetation, but re-establishment of the entire ecosystem including nutrient acquisition and restoration of biogeochemical cycling allowing for a self-sustaining ecosystem. Where suitable soils exist, such as forest topsoil, little intervention may be required to achieve this objective. However, in many cases even with the best available suitable soils many of the shrubs, sub-shrubs, herbs and more cryptic plant species may have disappeared and knowledge of what was present pre-European settlement has been lost. Suitable soils can be defined as those that have a functioning ecosystem of soil flora and fauna, such that all biogeochemical cycling processes can be performed. However, where soils have been degraded by mining or other means for example, grazing or intense cropping, efforts to enhance the biodiversity or physically extend the area of the forest and woodland ecosystems require more intervention to achieve the stated goals. These interventions may include seeding of locally sourced provenance plants and, or inoculation of suitable soil microbes such
as rhizobia or mycorrhizal fungi and their hosts plants, to (re-) establish nitrogen and phosphorus cycles respectively.

While the field site was the Mount Owen mine and the adjoining Ravensworth State Forest, the issues outlined here are not restricted to this site. The issues of how to extend the reserve so as to improve its conservation value and how this can be achieved in a self-sustaining manner are relevant to any mining province, or indeed any land area where the restoration, or reconstruction, of native vegetation is being attempted. This is especially so where the soils have been subjected to alternate land uses, leading to losses of topsoil and the resident soil flora and fauna that perform key ecological functions.

Key Problems for Ecosystem Restoration include:-

1. Availability of suitable topsoil
2. Introduced non-native legumes and bacterial symbionts both in pastures and in the Ravensworth State Forest

Topsoil transfer is a technique that has been applied to mine site rehabilitation for a considerable period of time (Bradshaw, 1997). This process provides a layer of material containing organic matter, microbes and a seed bank spread over the spoil to varying depths (Hannan, 1995; Huxtable et al., 2005; Nussbaumer, 2005). When forest topsoil is available, this method is the most successful for the reintroduction of native seed, soil fauna and flora and microbes, including nitrogen-fixing rhizobia. Topsoil may need to be stockpiled before spreading, especially early in the life of a mine, and this can result in changes in physical soil characteristics, loss of seed viability and a reduced soil microbial community (Newman, 1996; Kundu and Ghose, 1997; Huxtable et al., 2005). One of the key microbial groups that may be lost is the rhizobial bacteria. These are obligate aerobes when free-living in the soil, and stockpiling can lead to anaerobic conditions, dependent upon the shape and depth of the stockpile. That is soil that has been piled into mounds with low surface area to volume ratios will lead to anaerobic conditions within the stockpile away from the surface. With extended stockpiling, that is, over six months (Harris and Rengasamy, 2004), there may even be a requirement to inoculate topsoil with rhizobia to ensure nodulation potential.

At the Mount Owen mine, topsoil spreading is the preferred method of re-establishing forest vegetation. However, the supply of forest topsoil will not be sufficient to allow topsoil respreading over the entire disturbed area. Further, there are no other forest topsoils available as the surrounding areas are either native grasslands or
introduced pasture. The native grasslands are areas worthy of preservation in their own right, while the pastures have been under cultivation for almost 125 years (Chapter 4 Fig 4.1). This long period of cultivation has two detrimental impacts on the suitability of these soils for use as a topsoil replacement. While there may still be many species of native herbs in these pasture areas, they have become colonised by many weeds and exotic grasses including *Senecio madagascariensis, Galenia pubescens, Hypericum perforatum, Chloris gayana* and *Panicum maximum* rendering them unsuitable for reconstructing a native forest ecosystem. In addition, the pasture areas have been sown with exotic legume species that in the areas immediately surrounding Mount Owen include three *Medicago* species, six *Trifolium* species and *Melilotus indicus* (Cole et al., 2009), all legumes that have specific non-native rhizobial symbionts (Garau et al., 2005; Bailly et al., 2006).

With over 99% of native forest cleared from the central and lower Hunter Valley floor since European settlement, mainly for agriculture (Peake, 2006), as a remnant of native biota the importance of the Ravensworth State Forest is very high. But as with most relatively isolated remnants it is not in pristine condition. Vegetation surveys (Cole et al., 2004, 2005, 2006, 2007, 2008, 2009) have identified many introduced plants, including some non-native legumes such as *Trifolium repens*. Soil microbial activity in the Ravensworth State Forest has also been found to be as low as 10% of normal soil microbial activity when compared to topsoil from old growth forest (Newman, 1996; Cole et al., 2008). This has resulted from the combination of past land use including logging, fire wood extraction and grazing and the extended drought in the early part of the decade. The consequence is that while the forest topsoil is the best resource available, an argument could be mounted that this remnant would itself benefit from a program of inoculation with native rhizobia and suitable host legumes. For instance current research and monitoring of the rehabilitated areas of the spoil dump being conducted by the Centre for Sustainable Ecosystem Restoration at the Mount Owen mine where forest topsoil has been used (Chapter 4, Fig 4.1) indicates that excavation, respreading and the subsequent de-compaction of the topsoil increases the root infection of native legumes by mycorrhizal fungi (M. Cole pers comm.) The rehabilitation process has led to a flush of middle- and understorey legume species (including at least one species, *Acacia ulicifolia* not identified from the vegetation surveys in the RSF (C. Castor pers. com.), and which also indicates that increased nodulation of legumes may result.
5.2 Re-establishing Native Rhizobia

The identification of rhizobial nodulators in Chapter 2 (Table 2.7, isolate *Rhizobium CHNTR53*) showed that non-native rhizobia have successfully naturalised in remnant vegetation. Combined with the colonization of pasture areas by several species of native legume (see below) this shows that some non-specificity between native legumes and non-native rhizobia is possible. This result highlights one difficulty in any revegetation or reconstruction project; the conundrum of what can be practically done to remove or prevent the spread of introduced species.

If the decision is made that nothing can be done, then what these new combinations of rhizobia-legume produce is a hybrid or novel ecosystem (Seastedt, *et al.*, 2008; Hobbs *et al.*, 2009). These are ecosystems that are not historically intact, containing organisms that were not part of the original environment. However, given the expense and, or inability to fully eradicate these introduced organisms, their presence creates a new ecosystem, unlike the original. As long as these organisms are not detrimental to the overall ecology and co-exist or only in part replace native organisms, they can be acceptable given that the larger goals of vegetation restoration, animal habitat and ecosystem functionality have been met (Hobbs *et al.*, 2009).

In terms of native legumes and introduced rhizobia, this may be acceptable if the new combinations are as effective at supplying nitrogen to their native legume hosts and there are no alternatives for re-introduction of native rhizobia. Certainly the homogenisation and reduction of rhizobial biodiversity is an issue in itself. High biodiversity provides an ecosystem with resistance to (ability to withstand), and resilience after (ability to return to its original state) disturbance (Bengtsson *et al.*, 2000). Therefore the presence of non-indigenous rhizobia does not preclude the need to attempt to identify and introduce native rhizobia and other soil biota where possible. In the absence of native rhizobia, the presence of the non-natives rhizobia then ensures that functionality of the ecosystem can be maintained, at least until such time as native rhizobia can be introduced.

Given the stated difficulties of establishing rhizobia into a soil with resident rhizobia (Chapter 4, Section 4.4.3), preventing the spread of introduced rhizobia would appear to be the best strategy. On a bare substrate such as spoil, revegetation with native legumes heavily inoculated with native rhizobia as soon as possible should give the native rhizobia a positional and numerical advantage, monopolizing nodulation sites on
plant roots. This is purely a physical mechanism and does not take into account any physiological or specificity issues that allow naturalised or resident rhizobia to outcompete any inoculating strains, as apparently occurred with *A. amblygona* (Chapter 4, Fig 4.27B, forest topsoil).

This study confirmed that when isolates rated as highly effective were used as an inoculum increased survival and growth resulted as on *A. parvipinnula* (Chapter 4, Fig 4.28 and Fig 4.33 respectively; Thrall *et al.*, 2005). Use of a moderately effective isolate as an inoculum can produce mixed results, as was apparent from the results for *H. violacea*. This species had statistically significant increased biomass (Chapter 4, Fig 4.32), but inconsistent survival across the soil replacement media (Chapter 4, Fig 4.26). Even for those species with low overall survival, *P. retusa, I. australis* and *D. ulicifolia* survival was enhanced by inoculation with a rhizobial isolate that was only moderately effective in its performance with these species (Chapter 4 Figs 4.23, 4.24 and 4.25 respectively). The negative *A. amblygona* results in the forest topsoil (Chapter 4, Fig 4.27B) highlight one of the drawbacks of the inoculum approach used in this study, where one generalist strain was used for all legumes.

Although the results obtained using a single inoculum were variable, all surviving plants of five species of legumes were observed to flower within the first year of the study, with *A. parvipinnula* flowering and seeding the next and following years, indicating the beginning of successful life cycling of the plants.

### 5.3 Competition and Effect on Inoculation

After determining that a rhizobial strain effectively nodulates a target legume species, its success will ultimately depend on field performance. Selecting inoculating strains on the basis of effectiveness under controlled conditions does not guarantee success in the field (Mrabet *et al.*, 2005). Under natural soil conditions, many different rhizobia and indeed a whole ecosystem of soil microbes are present, and the effects on the growth and competitiveness of the isolates and the host may be altered. Large resident populations of rhizobia provide a competitive barrier to inoculation success (Thies *et al.*, 1991). Resident rhizobia may not only possess an adaptive advantage, but a positional advantage may also exist (Lopez-Garcia *et al.*, 2002). That is, resident rhizobia already physically occupy the soil space the roots will travel through. These factors were not a major consideration in the spoil based soil replacement media trialled
in Chapter 4, (except where forest subsoil had been incorporated by machinery prior to the commencement of mining) but were certainly a potential barrier to success with the forest topsoil and pasture subsoil based treatments. Inoculating seeds and seedlings with a liquid inoculum, as opposed to sticking agents that allow rhizobia to adhere to the seed, will help alleviate the physical positional advantage to some extent. Applying a liquid allowed the rhizobia to permeate through the soil, and establish in a larger volume of soil, thus being able to come into contact with roots of the legume as they penetrate the soil. It is speculated that this allowed a high concentration of viable rhizobia to be delivered to the root zone, outcompeting resident rhizobia.

By using a strain sourced from the Ravensworth State Forest, the disadvantage of non-adaptiveness was removed in the forest topsoil reference, especially as the inoculating strain was isolated from one of the species used in the trial. This advantage only applies to the forest topsoil; given the highly disturbed and artificial nature of the spoil dump, any inoculating rhizobia, regardless of its origin will be introduced into a soil medium that is inherently unnatural, that is, a novel system.

5.4 Rhizobial Occurrence and Implications

5.4.1 Rhizobial Identification

Members of the rhizobial genus *Bradyrhizobium* are reported to be the most common and widespread indigenous nodulators across Australia. This has been found for studies undertaken in other states such as south-west Western Australia (Lange, 1961; Marsudi *et al.*, 1998), Queensland (Bowen, 1956), Victoria (Lawrie, 1983), and widely spread areas of New South Wales (Barnet, *et al.*, 1985; Barnet and Catt, 1991). Many of these older studies were only able to divide the rhizobia isolated into fast- and slow-growing rhizobia, corresponding presumably to *Rhizobium* and *Bradyrhizobium* species respectively. In two studies covering large areas of south-east New South Wales and south-east Australia, Lafay and Burdon, (1998, 2001) identified over 88% and 96% respectively, of isolates collected as *Bradyrhizobium*, all previously undescribed, with minor percentages of isolates showing a high affinity to *Rhizobium tropici* and previously undescribed *Mesorhizobium*. None of the 863 isolates tested in the two Lafay and Burdon studies, which were sourced from 12 sites in New South Wales (Lafay and Burdon, 1998) and 44 sites from South East Queensland to Tasmania, (Lafay and Burdon, 2001) were identified as *Sinorhizobium*. In tropical Australia the diversity is
reportedly greater, but *Bradyrhizobium* still dominates (Lafay and Burdon, 2007). Another recent study conducted across western New South Wales has found many novel nodulating genera of bacteria including *Mesorhizobium*, *Burkholderia*, *Phyllobacterium* and *Devosia*, in addition to many *Bradyrhizobium* and novel endophytic root-nodule bacteria (Hoque et al., 2010). In north-west Western Australia fast growing isolates were identified as *Sinorhizobium meliloti*, and made up more than half of a relatively small sample of some 31 rhizobial isolates (Yates et al., 2004), which may indicate that these are naturalised rhizobia.

In the study undertaken here, four of the 22 nodulating isolates were identified as belonging to *Rhizobium*, with another two *Agrobacterium* strains, one previously uncultured, and one a putatively novel *Burkholderia*. There were no isolates identified conclusively as *Bradyrhizobium* either indirectly by culture dependent methods or directly through rDNA (Chapter 2, Tables 2.4 and 2.7 respectively). Two strains of *Rhizobium* that were identified as *Rhizobium tropici* may be considered as indigenous rhizobia, (Lafay and Burdon, 1998; 2001) although neither of these nodulated in the re-inoculation trial (Chapter 2, Table 2.7). Of the remaining 16 isolates, molecular analysis identified various genera of soil dwelling bacteria, many of which have not been reported as nodule forming or nitrogen fixing bacteria. These include *Acinetobacter* (RSFAp1), *Chitinophaga* (MOGclan3), *Labrys portucalensis* (MOHv) and *Bacillus* spp. With one *Burkholderia* identified as a nitrogen fixing nodulator, it is possible that three of the other isolates identified as *Burkholderia*, Uncultured bacterium clone AKIW800, *Burkholderia* sp. CC-S-L25, and *Burkholderia* sp. TAt-045 may likewise be novel nodulators capable of fixing nitrogen.

Taking the lack of *Bradyrhizobia* at face value is an indication that there may be a population of naturalized rhizobia in the two reference systems from which the bacterial isolates used in this study have been sourced, although the lack of definitive identification of the bacterial isolates makes this problematical. That these two areas of native forest may harbour naturalised rhizobia is not surprising. Given their locations either adjacent to encroaching suburban developments in the case of the Beresfield site, or the surrounding pasture and grazing lands of the Ravensworth State Forest which was also extensively slashed and grazed, the reference system for the reconstruction, is in itself a hybrid ecosystem.

The establishment of non-native rhizobia and a lack of the most common native rhizobia may have implications for the ability of both native legume species and
introduced legume species to spread throughout the rehabilitated areas of the mine and into and from the adjacent pasture areas that have been set aside as biodiversity offsets. There are several examples from around the world where the invasiveness of weed or introduced species, (plant and animal) has been facilitated by the introduction of a symbiont or mutualist (Richardson et al., 2000). Alternatively, generalist plants have been successful in their spread without the need for the co-introduction of their symbiont due to their ability to form an association with the resident population. In a restoration situation this can be both a hindrance, as unwanted plants invade, and a benefit as desirable plants spread outwards. At Mount Owen, instances of both have been observed. *Acacia saligna*, a species native to West Australia, has become a severe pest in part due to its non-specificity with regards to rhizobia, and is fast becoming more widespread throughout the Hunter Valley (Marsudi, et al., 1998; Targett 2001). Conversely, three species of native locally occurring legumes, *Acacia implexa*, *Daviesia genistifolia* and *Swainsonia galegifolia* have been observed colonising surrounding pasture areas from the adjacent forest remnants.

In Chapter 2 it was postulated that those isolates not identified as known nodule forming nitrogen fixers were rhizosphere dwelling bacteria that may have been plant growth promoting or hindering in their effects. However there is a second possible explanation. Members of several genera identified here have been found to be endophytic inhabitants of root nodules, including *Acinetobacter*, *Bacillus*, *Burkholderia*, *Paenibacillus*, *Pseudomonas* (Zakhia et al., 2006; Li, et al., 2008; Hoque et al., 2010; Palaniappan et al., 2010). Palaniappan and co-workers investigated the plant growth promoting ability of the endophytes finding that through the production of indole acetic acid and siderophores, growth was promoted in their own right and as co-inoculants with *Rhizobium* and *Bradyrhizobium*. The presence of these potential endophytic bacteria has two major implications for this study.

Firstly, as the endophytic bacteria were included in the Specificity Trial in Chapter 3, the relative performance rankings of the isolates are compromised. The reported plant growth promoting properties of these endophytes would then have an additive effect upon plant growth skewing the performance of those isolates. Thus comparison of isolates where culture-dependent and independent techniques provided an identification of “rhizobia” (see Chapter 2, Table 2.4 and 2.7) for instance MOAsal2 (identified as *Rhizobium* sp. CHNTR53) compared to MOKr (identified as *Bacillus cereus*) may have resulted in MOKr outperforming pure isolate cultures, where it was
ranked as the best performing isolate for two of the legume species (Chapter 3, Table 3.3). When the best performing isolates for each of the legume hosts are examined from this perspective, there were four isolates not identified as rhizobia MOAsal1 (A. parvipinnula and I. australis), MOAdecorr (P. retusa), MOKr (A. amblygona and D. ulicifolia) and MOX (H. violacea). This suggests that endophytic bacteria may have a positive influence on plant growth.

Secondly, their presence opens another avenue of investigation into the formulation of inocula to be used for establishing plant growth. Somewhat serendipitously, a small collection of endophytic bacteria is now available for further research on how they influence plant growth (See Section 5.6 below for further investigations).

5.4.2 Further Investigations of Rhizobial Identity

Targeted isolation of the nodule forming bacteria contained in the cultures and verified by re-inoculation onto M. atropurpureum, or the host species that the isolates was originally sourced from, should be re-performed and definitive identifications made. Attempts have been made to formulate selective media for the isolation of rhizobia. These are either semi-selective in nature by virtue of reducing, rather than eliminating, the growth of other non-target bacteria (Louvrier et al., 1995), or they are selective for only one genus of rhizobia, for example Bradyrhizobium (Tong and Sadowsky, 1994). Therefore obtaining pure cultures of rhizobia from root nodules is still the preferred method, and with correct microbiological techniques (for example, surface sterilization of root nodules and repeated streaking on suitable growth medium agar plates) past studies have been successful in isolating and culturing rhizobia.

Culture-independent methods have been at the forefront of microbial identification since the advent of the polymerase chain reaction (PCR), with the 16S rRNA gene still the most widely used gene for molecular identification. But it’s very attractiveness as a highly conserved gene, and hence use of “universal” primers means that only small differences in base pair composition can lead to quite different identities being established. Additionally, PCR is a competitive enzymatic process and rRNA templates are amplified according to relative abundance in a sample (Forney et al., 2004). Therefore in these samples, cultures containing more than one bacterial strain, such as endophytic bacteria or rhizosphere bacteria not eliminated from the root nodule.
during sterilization, may outnumber rhizobia due to their superior generation time and rhizobial rDNA would not be amplified when the PCR is performed.

Therefore identification of rhizobia from mixed DNA samples should be performed using genes that can reasonably be assumed to belong exclusively to rhizobia, or show enough variability to allow for rhizobia-specific primers to be designed. The obvious candidates are the nitrogen fixation (*nif*) genes and the nodulation (*nod*) genes, although much work has been performed on the 16S-23S intergenic spacer-region showing significant inter- and intra-specific differentiation between *Bradyrhizobium* and *Rhizobium* species, but especially in the former (Laguerre *et al*., 1996; Vinuesa *et al*., 1998; Tan *et al*., 2001; Parker, 2003).

Nitrogen fixation is, of course, not unique to rhizobia, and is performed by a range of saprophytic bacteria from the α-, β- and γ-proteobacteria, symbiotic and saprophytic cyanobacteria and symbiotic actinomycetes. Most studies undertaken that use culture-independent methods to investigate diazotrophic nitrogen fixation target the *nifH* gene which encodes for the iron-protein subunit of the nitrogenase enzyme (Roesch *et al*., 2008). Additionally, nitrogenase genes have become the largest collection of gene sequences held in databases for non-ribosomal genes for non-cultivable organisms, at about 1500 sequences (Zehr, *et al*., 2003). This suggests that appropriate primers could be used to investigate for the presence and identity of nitrogen fixing microbes in a sample or mixed culture, especially if it can be reasonably assumed that only one or a very limited number of such nitrogen fixers are present.

Genes encoding for nodulation would appear to be ideal for the identification of rhizobia, however *nod* genes are reported to be rhizobia species specific, making them unsuitable for broad investigative studies or identification of unknown or suspected rhizobia (Laguerre, *et al*., 1996, Sánchez-Contreras *et al*., 2001).

Judicious targeting of several different genes during the same identification procedure, would allow elucidation of the identity of nodule forming nitrogen-fixing bacteria regardless of the complexity of the culture being investigated.

### 5.5 Recommendations for Ravensworth State Forest Vegetation Complex (RSFVC)

This study investigated the specificity of six of the native legumes found in the RSFVC. With a total of 26 native Fabaceae and 16 Mimosaceae species found from vegetation
surveys (Cole et al., 2009) only a modest proportion of the total native legume community has been investigated for rhizobial specificity and effectiveness. Many of the species were unable to be considered for the trial due to unavailability of seed. Seeds are not collected for a variety of reasons. Plants may produce too few seeds to be economic for commercial seed collectors. For instance Daviesia genistifolia and Pultenaea spinosa were two species that were only available from commercial sources sporadically. Seed collection from within the RSFVC was also limited by seed production. Other species such as Zornia dyctiocarpa are small sub-shrubs, rather cryptic and again produce few seeds. Such issues make investigation of any symbiotic associations difficult, but with the prospect of limited supply of forest topsoil these species will require further investigation of their specificity if they are to be used for reconstruction within the RSFVC boundary in the future.

In conjunction with the above studies, a more systematic collection of rhizobia should be undertaken. This would determine if more effective inoculating strains can be found, whether they be more efficient nitrogen suppliers or more competitive with established rhizobial populations, or whether there are specificity issues between host legumes and rhizobia. This was apparent from the A. amblygona results from the Field Trial (Chapter 4, Fig 4.27 Survival, Fig 4.31 Height and Fig 4.34 Shoot Dry Weight) where the inoculating strain did not perform as well or better than resident rhizobia. To achieve the best results in terms of sustainable survival, reproduction and nutrient cycling, matching more efficient nitrogen fixing, specific rhizobia to the host legume should be a priority.

As noted earlier, the mechanical process of topsoil transfer and direct respreading of topsoil has produced a flush of under- and middle storey plant growth, including legumes. This may be due to the physical scarification of the seeds contained in the seed bank, soil de-compaction and resulting improved root and water penetration and, or the non-regeneration of the canopy species (only two C. maculata trees in the first 38 ha of topsoil spreading) and reduction in competition for resources. The soil de-compaction will make soil sampling physically easier (as opposed to sampling in the Ravensworth State Forest itself) and may have resulted in a proliferation of rhizobia via the increase in legume germination and growth, or simply microbial mobility through such a medium. Thus sampling for rhizobia should start with the areas of re-spread topsoils. The Centre for Sustainable Ecosystem Restoration (CSER) has begun this
process using soil samples and trap plants (A. parvipinnula, A. saligna, M. atropurpureum and P. retusa) at the University of Newcastle plant growth facility.

Apart from the benefits to the revegetation program at Mount Owen that will accrue from these recommendations, building a collection of root-nodulating nitrogen fixing bacteria will be greatly beneficial for revegetation projects within the Hunter Valley. The knowledge gained from this collection of bacteria will increase understanding of microbial soil ecology in general through the discovery of several previously uncultured and undescribed strains of bacteria.

Further investigation of inoculum delivery and composition is also warranted. This study delivered inoculum by application of liquid culture to the base of the stem of seedlings. For a relatively small-scale field experiment this was feasible. Applying inoculum via a sticking agent to the seed itself has its limitations, especially with regards to applying sufficient numbers of rhizobia to small seeded species, for instance P. retusa. This issue could have particular impact upon multi-strain inocula, if these were to be used, where small seed size can impose physical limitations to numbers of rhizobia carried on the seed (Stephens and Rask, 2000). Powdered granular and liquid inoculation techniques are all used throughout the world, and all have limitations as to efficacy of delivery, longevity of rhizobia in inoculum, consistency of nodulation and cost of formulation (Stephens and Rask, 2000). These are technical issues that have no doubt been thoroughly investigated for agricultural applications, and can be overcome in conjunction with commercial contractors.

Inoculum composition, that is selection of rhizobia for use as an inoculum was one of the primary aims of this study. Four different approaches to inoculation were proposed in Chapter 3 (see Section 3.4.4), three of which follow a somewhat agricultural approach of attempting to produce the best outcome in terms of plant growth with the least number of strains of rhizobia.

1. A single broad-spectrum isolate used on all native legumes.
2. Selecting specific single strains of rhizobia for specific legumes.
3. Selecting a multi-strain inoculum, consisting of a two or three isolates.

These strategies will reduce costs associated with culturing of isolates and formulation of the inoculum. However, from a restoration viewpoint, only the second strategy has the potential to increase biodiversity greatly, if the decision is made to use different rhizobia for each species of native legume. The fourth strategy proposed a multi-strain inoculum whereby the composition of each inoculum (for each native legumes species
or for different areas of a revegetation project) would vary by one or two strains, introducing a mosaic pattern of rhizobial distribution, and as many different strains as possible. The question then becomes how to effectively deliver a diverse selection of rhizobia, or indeed if there is a cost-benefit limit to the number that should be re-introduced. Competition studies between rhizobial strains were not conducted as part of this study, but formulation of multi-strain inocula will require these to be performed to ensure that introduced rhizobia persist in numbers great enough to inoculate legume hosts.

5.6 Future Investigations Beyond Ravensworth State Forest Vegetation Complex (RSFVC)

With most native vegetation having been cleared from the central Hunter Valley, preservation and extension of native vegetation communities has been recognised as a priority (Peake, 2006). Given the intimate association between soil biota and vegetation that has become apparent in recent years (Reynolds et al., 2003; Wardle et al., 2004) preservation and reconstruction of a vertically structured ecosystem must take into account the soil microbial community as well as the vegetation. The Ravensworth State Forest is classified as Central Hunter Ironbark-Spotted Gum-Grey Box ecologically endangered ecosystem (Peake, 2006) and is distinguished from the Lower Hunter Ironbark-Spotted Gum-Grey Box ecologically endangered ecosystem primarily on the basis of understorey plants, including legumes. A comparison of the two ecosystems’ rhizobial community would greatly enhance knowledge of the differences (if any at this level) between the two systems. If it could be determined that differences were slight, or that as with the vegetation, there were many rhizobia (and other soil microbes) in common, it would greatly increase the area from which to source local strains.

These data could then be used as the reference for successful reconstruction of the soil when efforts are made to extend the forest/woodland habitat onto the soil replacement media that will need to be used at Mount Owen and indeed into other areas of the Hunter Valley. This could be included as one aspect of the completion criteria that the mine owners and operators should use at the termination of the mining lease. Composition of the rhizobial population could be used to indicate whether the restoration is on the right environmental trajectory, in terms of nitrogen acquisition and cycling by comparing forest soil to reconstructed soil.
Most studies of rhizobial distribution cited in this work examined large geographic areas in an attempt to ascertain broad distributions of native rhizobia. More intense studies on a smaller scale also have their place in filling in the gaps between these larger scale studies to determine if there are finer scale distributions of rhizobia and other soil microbes and whether there are any previously unknown microbes present. This study, small in geographic area, has found eight previously uncultured bacteria, one new strain of nodulating bacteria, and potentially more previously undescribed strains of nodulating bacteria (from the trapping program conducted on the Mount Owen rehabilitation area) that still require confirmation. To the best knowledge of this author, comprehensive investigations into the composition of Hunter Valley rhizobia have not been conducted before. Only limited numbers of native legumes have been used for revegetation projects and there is the likelihood of further discoveries being made.

Single strain specificity for legumes appears to be the exception rather than the rule in nature, with many if not most legumes able to nodulate with a number of strains. This strategy may be based on the limited mobility of rhizobia in the soil (Hamdi, 1971; Lowther and Patrick, 1993) resulting in heterogeneous distribution. Therefore, a question that could be addressed is: “Is there a correlation between the size of the root system, or the volume of soil that can be explored, and species promiscuity?” That is, do small legumes with small root systems have greater or lesser promiscuity than large shrubs or trees? And following from these questions, is there an upper limit to the number of rhizobia that can be successfully inoculated onto a host legume? Native legumes with a capacity for supporting multiple strains of rhizobia (that is more than normally used for successful multi-strain inocula) could be used as “spot” sources of rhizobia when introduced as seedlings, thus ensuring that biodiversity is introduced to reconstruction projects.

Microbial biodiversity has important implications for enhancing plant resource acquisition from the soil. Within such a heterogeneous environment, nutrients are sequestered in the soil in many different forms. Different bacteria and microbes producing different enzymes may assist in the mineralisation of different compounds making them available to plants (Reynolds et al., 2003). The focus of research in recent years has been moved beyond the study of single microbe-plant interactions to microbe-microbe-plant interactions and synergies and to community level interactions (Kumar-Saxena et al., 2006). While this may be seen as outside the direct scope of this study,
extending the concept of vertically structured ecosystems from vegetation into the soil, reconstruction on spoil or in any soil replacement media (or on a pasture system sterilized of native organisms by agriculture) would benefit not just from the introduction of nitrogen fixing organisms, but a raft of micro-organisms. Co-inoculation is not a new concept and examples of experimental manipulation of multiple symbionts in addition to rhizobia include mycorrhizal fungi (Rice et al., 1994; Lesueur et al., 2001; Weber et al., 2005) plant growth promoting rhizosphere bacteria (Bashan, 1998; Munro et al., 1999; Valverde et al., 2006) and protozoa (Clarholm, 1985; Bonkowski, 2004). The inclusion of other soil dwelling microbes such as saprophytic fungi that are instrumental to soil aggregation (C. Daynes, 2009, pers. comm.) and free-living nitrogen fixers is just beginning to be investigated by the CSER and will extend to include decomposer bacteria, soil dwelling and litter decomposing invertebrates. As suggested previously, the possibility of plant growth promoting root-nodule endophytes, further expands the possibilities for inocula formulation, with the aim of producing a comprehensive strategy of microbe inoculation for re-establishing soil function and through it self-sustaining ecosystems. This would be facilitated by the use of native legumes as “nurse” plants that when planted into a bare substrate such as spoil, or other replacement media, would provide carbon sources through root exudation, root turnover, root cell sloughing, and direct exchange, to support such a community of soil flora and fauna.

This study investigated one aspect of nutrient deficiency found in spoil and other non-soil media at open cut coal mines, but it is equally relevant to any restoration effort in any degraded soil, such as damaged pasture lands. With open cut mining expected to continue to expand within the Hunter Valley, and given that 54% of continental Australia has been used for grazing at some stage since European settlement (Bell, 2001), restoration to native vegetation will be aided by, and in some cases crucially so, by improved understanding of native legume-rhizobia symbioses and the role of other soil biota in the function of soil and in how we regain and reconstruct soil ecology.
References


### Appendices

#### Appendix 1. Flora List of two Hunter Ecosystems sampled for potential native rhizobia.

(From Department of Environment and Climate Change website http://threatenedspecies.environment.nsw.gov.au/tsprofile/index.aspx)

1) Central Hunter Ironbark-Spotted Gum-Grey Box Forest

<table>
<thead>
<tr>
<th>Flora</th>
<th>Flora</th>
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<tr>
<td><em>Acacia falcata</em></td>
<td><em>Acacia parvipinnula</em></td>
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<tr>
<td><em>Allocasuarina luehmanii</em></td>
<td><em>Brachyscome multifida</em></td>
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<tr>
<td><em>Breynia oblongifolia</em></td>
<td><em>Brunoniella australis</em></td>
</tr>
<tr>
<td><em>Bursaria spinosa subsp. spinosa</em></td>
<td><em>Calotis cuneifolia</em></td>
</tr>
<tr>
<td><em>Cheilanthes sieberi subsp. sieberi</em></td>
<td><em>Chrysocephalum apiculatum</em></td>
</tr>
<tr>
<td><em>Corymbia maculata</em></td>
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<tr>
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<td><em>Dichondra repens</em></td>
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<tr>
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<td><em>Hypericum gramineum</em></td>
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<td><em>Microlaena stipoides var. stipoides</em></td>
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<td><em>Vemonia cinerea var. cinerea</em></td>
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<tr>
<td></td>
<td><em>Wahlenbergia gracilis</em></td>
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#### Additional Flora

- *Glycine clandestina*
- *Glycine tabacina*
- *Hakea sericea*
- *Indigofera australis*
- *Laxmannia gracilis*
- *Lomandra multiflora subsp. multiflora*
- *Melichrus urceolatus*
- *Paspalidium distans*
- *Pratia purpurascens*
- *Solanum prinophyllum*
- *Themeda australis*
- *Wahlenbergia communis*
2) Lower Hunter Spotted Gum-Ironbark Forest

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<tr>
<th>Species</th>
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<td><em>Bursaria spinosa subsp. spinosa</em></td>
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<td><em>Davesia leptophylla</em></td>
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<td><em>Eucalyptus punctata</em></td>
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<td><em>Eucalyptus sparsifolia</em></td>
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<td><em>Goodenia hederacea subsp. hederacea</em></td>
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<td><em>Lissanthe strigosa</em></td>
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<td><em>Vernonia cinerea</em></td>
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Appendix 2. Growth Solutions

Yeast Mannitol Agar (YMA) and Yeast Mannitol Broth (YMB) Solution used to culture bacterial isolate strains.

K$_2$HPO$_4$  0.5g  
MgSO$_4$.7H$_2$O  0.2g  
NaCl  0.1g  
Mannitol  10g  
Yeast Extract  0.4g  
Agar  15g  

The above chemicals are added to 1L of Milli-Q water, the pH adjusted to 7 using 1M HCl, and autoclaved at 121°C. YMB is the same formulation with the agar omitted.

Modified Hoagland’s Nutrient Solution (-nitrogen) used to grow Macroptilium atropurpureum seedlings.

KCl  10mM  
CaCl$_2$  3mM  
KH$_2$PO$_4$  2mM  
MgSO$_4$.7H$_2$O  2mM  
Na$_2$FeEDTA  0.2mM  
Micro Nutrients  0.2mM  
Agar  15g/L
Appendix 3.

Sequence data for the 16S rDNA used for the identification of the bacterial isolates presented in Chapter 2 and the type strains used to anchor each cluster of the dendrogram in Fig 2.5.

>MOKr
AGACGCTGGCGGCGGTGCTGCTACTAAATACATGCAAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTACCTGCTACTTCTGGGCTGAGTGGCGAACGGGTGAGTAATACATCGGAACATGGCTCTGTAGTGGGGGATAGCCCGGCGAAAGCCGGATTAATACCGCATACGATCTACGGATGAAGCGGGGGACCTTCGGGCCTCGCGCTATAGGGTTGGCCGCGTGATTAGCTACCTTGGTGGGTAAAGGCCTACCAAGGGCAACGATGCTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACAACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATTTCGGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGGAAGGCCTTAGGGTTGTAAAGCTCTTTCACCGGAAGAAGATAATGACGGTATCCGGAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAAATAC

>BAst1
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>MOIa1
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>MOIa3
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>RSFAp1
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>B cenocepacia
AU1054
AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCATGCCTTACACATGCAAGTCGAACGGCAGCACGGGTGCTTGCACCTGGTGGCGAGTGGCGAACGGGTGAGTAATACATCGGAACATGTCCTGTAGTGGGGGATAGCCCGGCGAAAGCCGGATTAATACCGCATACGATCTACGGATGAAAGCGGGGGACCTTCGGGCCTCGCGCTATAGGGTTGGCCGATGGCTGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCAGTAGCTGGTCTGAGAGGACGACCAGCACACTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGA

> Cenocepacia ATCC25465
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> C. septicum
ATCC25465
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> C. violaceum
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> Sinorhizobium meliloti
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> C. septicum
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> C. violaceum
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>Bacillus cereus ATCC14579

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E. coli ATCC 8739

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>P aeruginosa LESB58

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>paracoccus

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### Appendix 4. Seed Sources Used in Specificity Trial

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<td>Aust Seed Co</td>
<td>Scarification &amp; HWST</td>
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<tr>
<td></td>
<td>100</td>
<td>Harvest Seeds</td>
<td>Scarification &amp; HWST</td>
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<tr>
<td></td>
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</tr>
<tr>
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<td>200</td>
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<td></td>
<td>100</td>
<td>Aust Seed Co</td>
<td>Scarification &amp; HWST</td>
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<td><em>I. australis</em></td>
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<td>Oven 80°C x 3 mins &amp; HWST</td>
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Appendix 5. Primary Data From Specificity Trial.

*A. parvipinnula*

Relative efficiencies of the nodulating isolates for *A. parvipinnula*. The average shoot:nodule growth rate ratio was 7.48, correlation $r = 0.80$. The effective isolates are numbered 1 – 12 as follows: 1 - MOAsal1, 2 - BAp2, 3 – MOIa3, 4 – RSFAp2, 5 – Bas, 6 – MOKr, 7 – BAp1, 8 – MOHv, 9 – RSFAp1, 10 – MOAsal2, 11 – MOIa3, 12 – MOAdecor, 13-MOIa2.

Relative efficiencies of the effective isolates and the worst performed isolate (bold) for *A. parvipinnula* listed in descending of shoot growth rate. Numbers in far left column correspond to position of isolates in Fig. 3.10.

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<th>Isolate</th>
<th>Shoot DW/wk (g)</th>
<th>% Δ from Isolate 1</th>
<th>Nodule DW/wk (g)</th>
<th>% Δ from Isolate 1</th>
<th>Shoot:Nodule Ratio</th>
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<td>1 MOAsal1</td>
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<td>0</td>
<td>0.0063</td>
<td>0</td>
<td>10.71</td>
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<tr>
<td>2 BAp2</td>
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<td>0.0088</td>
<td>+39.68</td>
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<td>3 RSFAp2</td>
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<td>-26.69</td>
<td>0.0051</td>
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<td>4 MOIa3</td>
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<td>5 Bas</td>
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<tr>
<td>8 MOHv</td>
<td>0.0354</td>
<td>-47.81</td>
<td>0.0049</td>
<td>-22.60</td>
<td>7.22</td>
</tr>
<tr>
<td>9 RSFAp1</td>
<td>0.0353</td>
<td>-41.89</td>
<td>0.0093</td>
<td>+46.79</td>
<td>3.80</td>
</tr>
<tr>
<td>10 MOAsal2</td>
<td>0.0351</td>
<td>-48.13</td>
<td>0.0024</td>
<td>-61.70</td>
<td>14.50</td>
</tr>
<tr>
<td>11 MOIa3</td>
<td>0.0338</td>
<td>-50.17</td>
<td>0.0052</td>
<td>-17.65</td>
<td>5.93</td>
</tr>
<tr>
<td>12 MOAdecor</td>
<td>0.0333</td>
<td>-50.87</td>
<td>0.0050</td>
<td>-21.54</td>
<td>6.70</td>
</tr>
<tr>
<td>13 MOIa2</td>
<td>0.0309</td>
<td>-54.41</td>
<td>0.0040</td>
<td>-36.78</td>
<td>7.77</td>
</tr>
<tr>
<td>MOGclan3</td>
<td>0.0040</td>
<td>-94.16</td>
<td>0.0006</td>
<td>-90.96</td>
<td>6.72</td>
</tr>
</tbody>
</table>
The relative efficiencies of the nodulating isolates for *H. violacea*. The average shoot:nodule growth rate ratio was 9.17, correlation $r = 0.85$. Effective isolates are numbered 1 to 12 as follows; 1 – MOX, 2 – MOAsal2, 3 – MOKr, 4 – BAp2, 5 – BAs, 6 – MOAsal1, 7 – RSFAp2, 8 – BDu1, 9 – MOHv, 10-MOia3, 11- MOia2, 12 – RSFAamb2.

Relative efficiencies of the effective isolates and the worst performed isolate (bold) for *H. violacea* listed in descending order of shoot growth rate. The numbers in the far left column correspond to the isolates numbered in Fig 3.15.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Shoot DW/wk (g)</th>
<th>% Δ from Isolate 1</th>
<th>Nodule DW/wk (g)</th>
<th>% Δ from Isolate 1</th>
<th>Shoot:Nodule Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MOX</td>
<td>0.0655</td>
<td>0</td>
<td>0.0052</td>
<td>12.53</td>
</tr>
<tr>
<td>2</td>
<td>MOAsal2</td>
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<td>-9.77</td>
<td>0.0035</td>
<td>-32.69</td>
</tr>
<tr>
<td>3</td>
<td>MOKr</td>
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<td>-11.76</td>
<td>0.0077</td>
<td>+48.08</td>
</tr>
<tr>
<td>4</td>
<td>BAp2</td>
<td>0.0477</td>
<td>-27.18</td>
<td>0.0050</td>
<td>-3.85</td>
</tr>
<tr>
<td>5</td>
<td>BAs</td>
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<td>-34.35</td>
<td>0.0056</td>
<td>+7.69</td>
</tr>
<tr>
<td>6</td>
<td>MOAsal1</td>
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<td>0.0032</td>
<td>-38.21</td>
</tr>
<tr>
<td>7</td>
<td>RSFAp2</td>
<td>0.0367</td>
<td>-43.91</td>
<td>0.0067</td>
<td>+27.78</td>
</tr>
<tr>
<td>8</td>
<td>BDu1</td>
<td>0.0367</td>
<td>-43.99</td>
<td>0.0039</td>
<td>-25.96</td>
</tr>
<tr>
<td>9</td>
<td>MOHv</td>
<td>0.0342</td>
<td>-47.71</td>
<td>0.0039</td>
<td>-26.06</td>
</tr>
<tr>
<td>10</td>
<td>MOia3</td>
<td>0.0325</td>
<td>-50.36</td>
<td>0.0043</td>
<td>-18.39</td>
</tr>
<tr>
<td>11</td>
<td>MOia2</td>
<td>0.0311</td>
<td>-52.45</td>
<td>0.0045</td>
<td>-14.60</td>
</tr>
<tr>
<td>12</td>
<td>RSFAamb2</td>
<td>0.0264</td>
<td>-59.61</td>
<td>0.0024</td>
<td>-54.86</td>
</tr>
<tr>
<td>MOGclan3</td>
<td>0.0029</td>
<td>-95.53</td>
<td>0.00004</td>
<td>-99.23</td>
<td>73.13</td>
</tr>
</tbody>
</table>
Relative efficiencies of the effective isolates and the worst performed isolate (bold) for *I. australis* listed in descending order of shoot growth rate. Numbers in far left column correspond to numbered isolates in Fig 3.12.

The relative efficiencies of the nodulating isolates for *I. australis*. The average shoot:nodule growth rate ratio was 11.34, correlation $r = 0.69$. Effective isolates are numbered 1 to 8 as follows; 1 – MOAsal1, 2 – MOIa3, 3 – MOKr, 4 – MOAsal2, 5 – BAs, 6 – MOIa2, 7 – BAp2, 8 – RSFAp1

Relative efficiencies of the effective isolates and the worst performed isolate (bold) for *I. australis* listed in descending order of shoot growth rate. Numbers in far left column correspond to numbered isolates in Fig 3.12.
The relative efficiencies of the nodulating isolates for *A. amblygona*. The average shoot:nodule growth rate ratio was 20.06, correlation \( r = 0.94 \). The effective isolates have been labelled.

Relative efficiencies of the effective isolates and the two worst performed isolates (bold) for *A. amblygona* listed in descending order of shoot growth rate.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Shoot DW/wk (g)</th>
<th>% Δ from Isolate 1</th>
<th>Nodule DW/wk (g)</th>
<th>% Δ from Isolate 1</th>
<th>Shoot:Nodule Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOKr</td>
<td>0.1045</td>
<td>0</td>
<td>0.0060</td>
<td>0</td>
<td>17.47</td>
</tr>
<tr>
<td>RSFAp1</td>
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<td>0.0029</td>
<td>-51.67</td>
<td>31.75</td>
</tr>
<tr>
<td>MOAimp2</td>
<td>0.0896</td>
<td>-14.26</td>
<td>0.0032</td>
<td>-46.67</td>
<td>27.99</td>
</tr>
<tr>
<td>MOAsal1</td>
<td>0.0766</td>
<td>-26.70</td>
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<td>16.32</td>
</tr>
<tr>
<td>RSFAp2</td>
<td>0.0638</td>
<td>-38.95</td>
<td>0.0029</td>
<td>-51.67</td>
<td>22.20</td>
</tr>
<tr>
<td>MOTs1</td>
<td><strong>0.0018</strong></td>
<td><strong>-98.29</strong></td>
<td><strong>0.000005</strong></td>
<td><strong>-99.92</strong></td>
<td><strong>393.00</strong></td>
</tr>
<tr>
<td>MODu1</td>
<td><strong>0.0013</strong></td>
<td><strong>-98.77</strong></td>
<td><strong>0.000003</strong></td>
<td><strong>-99.95</strong></td>
<td><strong>437.00</strong></td>
</tr>
</tbody>
</table>
The relative efficiencies of the nodulating isolates for *D. ulicifolia*. The average shoot:nodule ratio was 11.75, correlation r = 0.71. The effective isolates have been labelled.

The relative efficiencies of the effective isolates and the worst performing isolate (bold) for *D. ulicifolia* listed in descending shoot growth rate.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Shoot DW/wk (g)</th>
<th>% Δ from Isolate 1</th>
<th>Nodule DW/wk (g)</th>
<th>% Δ from Isolate 1</th>
<th>Shoot:Nodule Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOKr</td>
<td>0.0953</td>
<td>0</td>
<td>0.0078</td>
<td>0</td>
<td>12.22</td>
</tr>
<tr>
<td>MOAimp2</td>
<td>0.0666</td>
<td>-30.12</td>
<td>0.0019</td>
<td>-75.64</td>
<td>35.05</td>
</tr>
<tr>
<td>RSFAp2</td>
<td>0.0661</td>
<td>-30.64</td>
<td>0.0043</td>
<td>-44.87</td>
<td>15.37</td>
</tr>
<tr>
<td>MOAsal2</td>
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</tr>
<tr>
<td>MOAdcor</td>
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<td>0.0027</td>
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<td>15.56</td>
</tr>
<tr>
<td>MOGclan3</td>
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</table>
The relative efficiencies of the nodulating isolates for *P. retusa*. The average shoot:nodule ratio was 7.67, correlation $r = 0.99$. The effective isolates have been labelled.

The relative efficiencies of the effective isolates and the worst performing isolate (bold) for *P. retusa* listed in descending shoot growth rate.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Shoot DW/wk (g)</th>
<th>% Δ from Isolate 1</th>
<th>Nodule DW/wk (g)</th>
<th>% Δ from Isolate 1</th>
<th>Shoot:Nodule Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOAdcor</td>
<td>0.00632</td>
<td>0</td>
<td>0.00092</td>
<td>0</td>
<td>6.86</td>
</tr>
<tr>
<td>MOAimp2</td>
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<td>0.00078</td>
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<td>7.06</td>
</tr>
<tr>
<td>BDu2</td>
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<td>7.11</td>
</tr>
<tr>
<td>RSFAp2</td>
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<td>0.00051</td>
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</tr>
<tr>
<td>MOAm1</td>
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<td>0.00035</td>
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<td>6.60</td>
</tr>
<tr>
<td>RSFAamb2</td>
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<td>0.00030</td>
<td>-67.14</td>
<td>7.41</td>
</tr>
<tr>
<td>RSFAp1</td>
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<tr>
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<tr>
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<tr>
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<td>7.32</td>
</tr>
<tr>
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<tr>
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<tr>
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</tr>
<tr>
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<tr>
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</tr>
<tr>
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<td>-91.85</td>
<td>12.67</td>
</tr>
<tr>
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<td>-85.16</td>
<td>0.00003</td>
<td>-97.28</td>
<td>37.50</td>
</tr>
<tr>
<td>MOKr</td>
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<td>-87.44</td>
<td>0.00007</td>
<td>-92.75</td>
<td>11.90</td>
</tr>
<tr>
<td>MOIa2</td>
<td>0.00074</td>
<td>-88.28</td>
<td>0.00008</td>
<td>-91.30</td>
<td>9.25</td>
</tr>
</tbody>
</table>
The relative effectiveness of the nodulating isolates as measured by average shoot dry weight/week for the host species *A. parvipinnula*. Isolates are divided into levels of effectiveness by the vertical bars. Columns with the same letter are not significantly different at the 5% level. The bars at top right indicate least significant differences at the levels shown.

The relative effectiveness of the nodulating isolates as measured by average shoot dry weight/week for the host species *H. violacea*. Isolates are divided into levels of effectiveness by the vertical bars. Columns with the same letter are not significantly different at the 5% level. The bars at top right indicate least significant differences at the levels shown.
The relative effectiveness of the nodulating isolates as measured by the shoot dry weight/week for *I. australis*. Isolates are divided into levels of effectiveness by the vertical bars. Columns with the same letters are not significantly different at the 5% level. Bars at the top right indicate LSD’s.

The relative effectiveness of the nodulating isolates as measured by the shoot dry weight/week for *A. amblygona*. Isolates are divided into levels of effectiveness by the vertical bars. Columns have not had letters added as there is no significant different between the isolates at the 5% level. Bar at the top right indicates LSD at the 5% level.
The relative effectiveness of the nodulating isolates as measured by the shoot dry weight/week for *D. ulicifolia*. Isolates are divided into levels of effectiveness by the vertical bars. Columns with the same letters are not significantly different at the 5% level. Bars at the top right indicate least significant differences at the levels shown.

The relative effectiveness of the nodulating isolates as measured by the shoot dry weight/week for *P. retusa*. Isolates are divided into levels of effectiveness by the vertical bars. Columns have not had letters added as there was no significant different between the isolates at the 5% level. The bar at the top right indicates LSD at the 5% level.