Domesticating *Lebeckia ambigua:* solving the rhizobia issues.

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I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

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Abstract

Permanent dryland pastures are under-utilised in southern Australia (Angus and Peoples 2012), possibly due to the lack of well adapted perennial legume species that can fit into current farming systems. *Lebeckia ambigua* has been proposed as a candidate to fill this void with its adaptability to drought, acidic and infertile soils in low rainfall areas (Howieson et al. 2013). The research on *L. ambigua* has so far focussed on deep, sandy soils where cropping is problematic. Increasing the soil fertility in these previously low-profitable regions could provide mixed farming production with a comparative advantage over continuous cropping (Angus and Peoples 2012). However, the successful incorporation of *L. ambigua* into an agricultural system will require an understanding of its symbiont, *Burkholderia* species. Although *L. ambigua* and *Burkholderia* spp. have only recently been identified for domestication into agriculture (Howieson et al. 2013), researchers have had success with cultivating them throughout southern Western Australia (WA), except with inoculation.

There is a challenge to keep the inoculant *B.* spp. alive, in a peat carrier, when coated onto *L. ambigua* seed for sowing in a drying environment. Clay granules, as an alternative carrier, have previously been shown to be unable to carry high numbers of cells of *B.* spp. (Howieson et al. 2013). Field experiments with amended clay granules carrying *B.* spp. produced nodules on *L. ambigua*, albeit not in large number. Attempts at quantifying the numbers of cells in the amended granules, by resuscitating *B.* spp. from them using antibiotic media and plant infection techniques, were unsuccessful. However, antibiotic profiling of *B.* spp. strains identified chloramphenicol (20µg/ml) in YMA as an excellent media to suppress contaminants in the clay to facilitate enumeration.

Recently recovered strains of *B.* spp. were assessed alongside previous strains for tolerance to desiccation, which gave rise to a set of possible strains that could surpass the commonly used strain in this regard (WSM4204). Although clay granules were indicated to hold *B.* spp. cells sufficient for nodulation in the field, further studies must focus on the optimisation of a suitable inoculant technology for *L. ambigua*. The *B.* spp. and strain differences in tolerance to desiccation identified in this work may assist this target.

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Chapter 1: General review of literature

1.1. Introduction- advancements in rhizobial inoculation

1.1.1. The legume-rhizobia symbiosis

Legumes have been cultivated and consumed for centuries, and are well known for their ability to fix atmospheric nitrogen (N_2) in association with particular soil bacteria called rhizobia. This is a very specific relationship, with species of rhizobia closely matched with species of legumes. Rhizobia or root nodule bacteria are the terms given to bacteria that, after being established in legume roots or sometimes stems (e.g. *Frankia*), have the ability to fix N_2 (Sprent et al. 2017). Most rhizobia are not considered to be diazotrophic (the capacity to fix N_2 and grow without an external nitrogen source). However, some exceptions include strains of the genera *Burkholderia* and *Bradyrhizobium* (Sprent et al. 2017).

A legume is a plant from the Leguminosae family, and many (but not all) can form a relationship with rhizobia to take inert atmospheric nitrogen and transform it into amino acids and other N-containing metabolites, with the legume providing sugars (Giller et al. 2016). This association with rhizobia is referred to as a mutually symbiotic relationship because the legume receives ammonium (NH_4^+) reduced from N_2 by rhizobia in return for carbon translocated to the nodules, in which the rhizobia reside (Sprent 2009, Sprent et al. 2017). This process leaves a minimal carbon footprint, which makes it appealing to low input agricultural systems (Giller et al. 2016).

N_2 is almost inert due to the triple bond between the two nitrogen (N) atoms (Campbell et al. 2009) and before the development of fertiliser N in the 20th Century, legume-based N fixation was the biggest contributor to N inputs to the planet (Brockwell 2004). Such was the situation in Western Australia (WA) in the early development of agriculture. However, no agricultural legumes in WA are indigenous (Howieson et al. 2000b) and hence it was, and still is, essential to introduce their specific rhizobia at sowing. This application of symbiotic bacteria to a legume rhizosphere is termed inoculation, a process that if carried out successfully is advantageous to agricultural systems (Sprent et al. 2017).

1.1.2. Inoculant history

Inoculation is a relatively recent term, but farmers have actually been inoculating for centuries. When the soil was first tilled many thousands of years ago, legumes were used as a source of food and known as "soil improvers" (van Driel 1999). The Mesopotamians grew peas and beans in their early agricultural systems once they realised the benefits of higher yields and improved soil health (Drew et al. 2012). Theophrastus (372-287 B.C.) even wrote: "…the bean best reinvigorates the soil." (quoted from Fred et al. 1932). Diamond (1997) proposed a theory of how essential legume cultivation was to human development, as people switched from being nomads to farmers, without commenting on the key role of rhizobia. However, farmers of pre-history were inspired to spread or dust a field with the soil from the desired legume over a legume-free site, before sowing the specific legume (Date 1974, Drew et al. 2012).

The success of legumes in Australian ley-farming systems has relied on successfully introducing the specific rhizobia strains into Australian soils (Puckridge and French 1983, Bullard et al. 2005). As early as the late 19th Century, Guthrie (1896) noted that Australian

soils were depleted and lacked available N for adequate plant growth in an agricultural setting. His assessment may have been correct, as now three million tonnes of N is fixed per year by legumes to increase agricultural production (Drew et al. 2012), which equates to $3-4 billion in urea equivalents (Herridge 2008). Moreover, six million tonnes of N are needed annually in Australia for its agricultural output (Drew et al. 2012).

Marks (1905) built upon the knowledge of rhizobia that Guthrie published and with Guthrie's broth cultures set up the first field trials, where sand was added to the broth before being spread over the soil, prior to harrowing (Bullard et al. 2005). Non-private organisations were the main distributors of agar cultures and peat inoculants until 1965 when the expansion of the industry was inhibited by short shelf life of cultures and the fragile/bulky glass bottles that they were prepared in (Bullard et al. 2005). Private enterprises were encouraged to perform research and combat laboratory contamination and shelf-life issues. A key breakthrough was the recognition that cultures could be "carried" in finely ground peat. It was found that if the moisture content of the peats was kept below 35%, fungal contamination was reduced (Bullard et al. 2005), yet this allowed enough moisture for rhizobia to multiply to optimal numbers (10^9 CFU/g). This finding pressured the inoculant technology industry into research and development to focus on quality and sterility of peats (Roughley and Vincent 1967). Improvements focused on sterilisation by irradiating (gamma) the peat, and managing the salt content, to increase the quality of the product. Peat remained the preferred carrier for over half a century, but as cropping practices and sowing machinery evolved, new technologies such as freeze-dried cultures, liquids and granules appeared as replacements (Bullard et al. 2005).

1.1.3. The evolution of Australian farming

Famers globally have always strived for increased soil fertility and subsequently higher yields, which ultimately can be achieved by a legume-crop rotational system. This system was a successful and sustainable model between 1900 and the 1950's in Australia (Harrison 1936, Bath 1951), based mainly upon legume pastures. These pastures typically contained annual medics (*Medicago* spp.) and subterranean "sub" clover (*Trifolium subterraneum*) (Peoples et al. 1997). However, in the period from 1953 to the 2000's a swing in popularity occurred, with the demand for crop legumes (10% (1953) to 80% (2003)) overtaking the demand for pasture legumes (90% (1953) to 20% (2003)) (Bullard et al. 2005). In modern farms in WA, crop legumes have since been largely replaced by canola (*Brassica napus*) (Howieson 2015).

1.2. Domesticating non-native legumes for poor soils in Western Australia (WA)

1.2.1. New roles for pasture legumes- a changing environment

Ley-farming in southern WA is a low input agricultural system that relies on the dominance and productivity of pasture legumes to be successful (Nichols et al. 2007). This area of Australia is distinguished by having poor soils and low rainfall (Howieson et al. 2000b), which is reducing as portrayed in Figure 1.1. To remain successful in this region, farming practises must be able to adapt to changing environmental conditions. Changing conditions such as unreliable annual rainfall have caused a shift from traditional pasture legumes (e.g. sub clover) to new pasture legumes such as serradella (*Ornithopus* spp.) (Howieson and Loi 1994).

Self-regenerating pasture legumes (e.g. *Trifolium* spp.) with hard seeds that don't germinate every year have been important to Mediterranean-like environments for thousands of years (Howieson et al. 2000b). However, the hard seed impermeability that pasture legumes possess is yet to be fully understood. Hard-seed impermeability or hardseededness is an adaption to "false breaks" from unreliable autumn rainfall. This makes it a desirable trait when selecting new cultivars for dry-land agriculture (Chapman and Asseng 2001). Since ley farming systems rely on the regeneration of a pasture (often legume) after a cereal crop, the resilience of the legume to a false break is paramount (Chapman and Asseng 2001). However, adoption of new pasture legumes is often compromised by high seed costs and low germination from hard-seeds (Dear and Ewing 2008). New pasture legumes must overcome these constraints to be attractive to farming systems if they are to be successful.

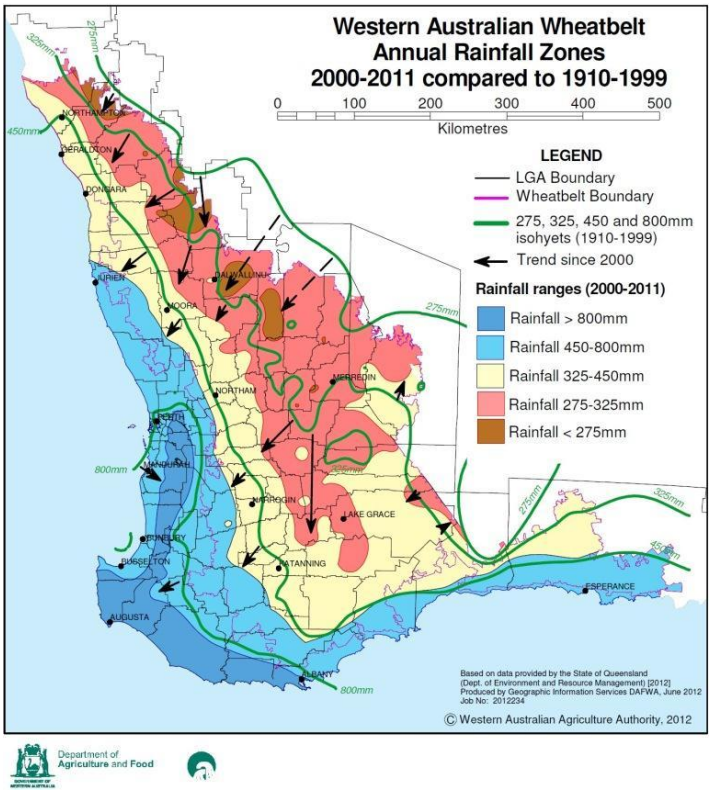

Figure 1.1: Annual rainfall for Western Australian Wheatbelt for years 1910 to 1999 compared with 2000 to 2011 (Department of Primary Industries and Regional Development 2012) .

1.2.2. Attributes for selection of improved pasture legumes

When managed appropriately, ley-farming systems can be superior to other farming systems such as continuous cropping, especially once improved pasture legumes have been incorporated. Benefits include increased soil fertility and weed control, and a decrease in disease (Howieson et al. 2000b, Loi et al. 2005). Attributes that are considered in the selection of improved pasture legumes are listed in Table 1.1, with some emphasis on seed characteristics. The most important advancement from the existing pasture legumes to the new is an increased understanding of the importance of hard seed breakdown, and selection of varieties with an increased level of hard seed (Nutt 2010). The hard seed property of a legume is advantageous because it can:

- allow the seed to pass through digestive tracts of ruminant animals
- allow seeds to be sown with a cereal crop (twin sowing) yet not germinate in that year or,
- allow seeds to be sown prior to the cropping program (summer sowing) when labour is less expensive (Loi and Nutt 2010).

Table 1.1: Desirable attributes in pasture legumes in the WA Wheatbelt. Adapted from Howieson et al. (2000b).

Desirable attribute	Old pasture legume (e.g. *Trifolium subterraneum* cv. Dalkeith)	Alternative pasture legume (e.g. *Biserrula pelecinus* cv. Casbah)
High seed yield	Y	Y
Hardseededness	N	Y
Seed survives ingestion	N	Y
Insect tolerance [a]	N	Y
Deep-roots	N	Y
Early flowering	Y	Y
Grazing tolerant	Y	Y
Robust rhizobia	Y	Y
Uses phosphate efficiently	N	Y
Tolerant to acid soils	N	Y
Easy to harvest	N	Y

Key: Y-does possess trait; N-does not possess trait

[a] *Red-legged earth mite, lucerne flea, blue green aphid, sitona weevil.*

1.2.3. The need for perennial legumes in WA agriculture

Perennial legumes live for two or more years. They can enhance ley-farming systems because they may close the "feed gap", which refers to the period of time (usually summer and autumn) where there is no green feed for animals (Moore et al. 2009). Perennial legumes with large extensive root systems can reach the water table and remain green

through summer (Dear et al. 2003). They may also increase water use efficiency in their vicinity and reduce the incidence of dryland salinity in low lying areas (Ridley and Pannell 2005). It is also thought that perennial plants are also more resilient to changing rainfall patterns (Howieson et al. 2013).

Native perennial legumes that exist in the south west of WA are not suitable for agriculture, due to their defence mechanisms to deter grazing, such as low-palatability, accumulation of toxins and pungent foliage (Cocks 2001). Therefore, researchers have examined other Mediterranean climates, such as South Africa, to find suitable plants (Howieson et al. 2013). The fynbos region of South Africa was identified as an area to research because of its low rainfall and deep acid sands that resemble closely the Kwongan heathlands of south west WA (Beard et al. 2000). In the past, research into potential perennial cultivars has focused on the legumes suited to wetter areas (Cocks 2001). However, developing perennial plants suited to dry areas with nutrient depleted sands is likely to become important for WA agriculture as cropping these regions becomes riskier.

1.2.4. *Lebeckia ambigua*- a suitable perennial plant for WA agriculture

The genus *Lebeckia* Thunb. dates back to Bentham (1844) and Harvey (1862) where 36 species of Papilionoid legumes were described from southern Africa (Boatwright et al. 2009). *L. ambigua* E.Mey. is a perennial suffrutescent legume from the western cape of South Africa (Figure 1.2). It is found in regions of low rainfall (<300mm) areas with poor soil fertility and a low pH (4.5-6.5) (Le Roux and Van Wyk 2007, Howieson et al. 2008). These challenging environments are consistent with the eastern Wheatbelt of WA (Hobbs et al. 1995). The rainfall isohyets in the south west of WA are transitioning in a south west direction (Figure 1.1) which supports the case for domestication of a drought tolerant

perennial legume (Li et al. 2008). Identification of *L. ambigua* surviving in the challenging edaphic conditions of the fynbos made it appealing to researchers on expeditions there between 2002-2007 (Howieson et al. 2008). It was identified as a potential agricultural plant during that period. However, any future success with this legume in WA is dependent on successfully pairing it with its symbiotic rhizobia.

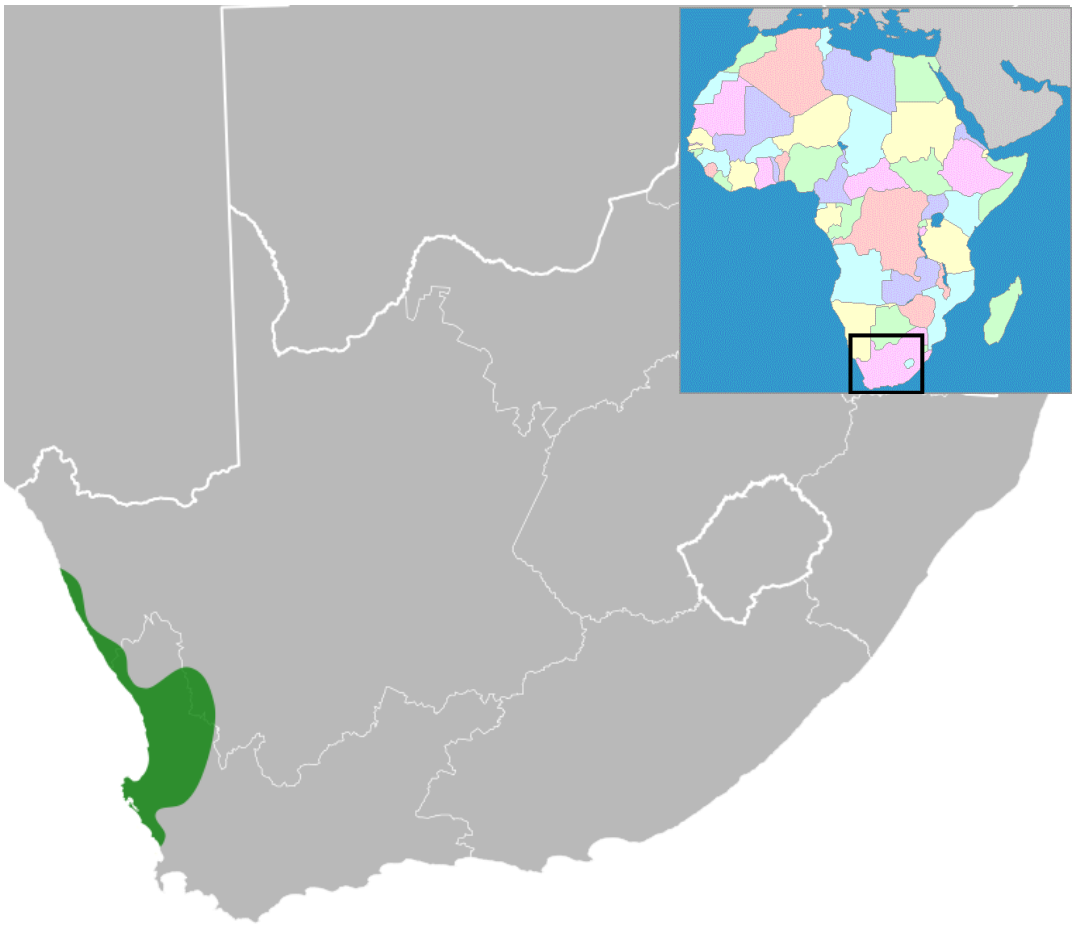

Figure 1.2: Main: Distribution of *L. ambigua* in the western cape of South Africa (https://en.wikipedia.org/wiki/Lebeckia_ambigua), insert: location of the main photo in Africa (http://www.yourchildlearns.com/africa_map.htm).

1.2.5. Attributes for successful adaptation of *L. ambigua* to WA

1.2.5.1. Tolerance of infertile soils

Poor soil fertility is a hindrance that plants must overcome to prosper in some areas of both the WA Wheatbelt and the western cape of South Africa. *L. ambigua* has adapted to low rainfall by producing a large tap root that can access the water table (where water is usually unused in WA), on these well drained, deep sands (Howieson et al. 2013). Other plant species such as Radiata pine (*Pinus radiata*) and Tagasaste (*Chamaecytisus proliferus*) can be sown on this land to access the unused water (Lefroy et al. 2001, Nichols et al. 2007). These plants may be long-term fixtures for 20 plus years, but are not easily grazed by sheep. However, *L. ambigua* may be grazed, and senesces after three to five years, which may also allow it to be included in a crop rotation system, especially once its high level of hardseededness (around 95%) and its breakdown is understood (Edwards 2015).

1.2.5.2. Tolerance to drought and infertility

Water conservation through reduced transpiration is an important plant adaption to hot dry summers (Ryel et al. 2004). This adaptation appears present in *L. ambigua* where a low surface area to volume ratio of its acicular leaves reduces transpiration (Le Roux and Van Wyk 2007).

To manage soil infertility, some plant species produce fine highly branched root hairs called cluster roots (Skene 1998). It has been speculated that *L. ambigua* possesses them (Edwards 2015) which may help this legume acquire scarce nutrients such as phosphorus (Skene 1998). Phosphorus is a limited resource both in the Fynbos and the eastern Wheatbelt of WA (Lamont 1982).

1.2.5.3 High levels of seed production

Perennial legumes are not known to produce much seed (Kowithayakorn and Hill 1982), however, *L. ambigua* has been reported to produce 25,000 seeds on some plants (Edwards 2015). This makes it a strong candidate for adaptation to the deep white sands of the eastern Wheatbelt of WA, because seed ecology will be a determinant of long term success. However, once we understand the complexity of its hardseededness and germinating conditions, the true potential of *L. ambigua* as a perennial legume in a crop rotational system will be examined.

1.3.5.4 Competent in symbiosis

*L. ambigua'*s symbiotic rhizobia must be inherently saprophytically competent (Chatel et al. 1973) to persist in the soil over time in the fynbos, to nodulate with new seedlings when seeds soften and germinate. This attribute will be essential in selecting appropriate strains for WA, and might also suit them to survival through a cropping phase in WA.

1.3. The rhizobial challenges of domestication of pasture legumes

As for the plant attributes, there is an important set of rhizobial attributes that are sought when domesticating new symbioses for Agriculture.

1.3.1. Attributes for rhizobia strain selection

Once a new legume has been selected to be a potential agricultural cultivar, the next step of the screening process is understanding its symbiotic rhizobia. Listed below are some characteristics of inoculant-quality rhizobia:

- an ability to fix high amounts of nitrogen with the intended host species,
- able to be adapt to the soil environment where the host thrives,

- genetic stability in the culture, storage and soil,
- easy to manufacture in terms of growth and survival and
- competitive with other soil rhizobia (Howieson et al. 2000a).

The success of a legume is dependent on its rhizobia possessing the characteristics above, otherwise the full benefits of the legume will never be fully exploited.

1.3.2. *Burkholderia*- the symbiont of *L. ambigua*

Burkholderia (beta-proteobacteria) was only recently shown to nodulate with legumes (Moulin et al. 2001), nine years after the description of the genus by Yabuuchi et al (1992) . Before this discovery it was considered that only rhizobia from the alpha-proteobacteria were able to form a symbiotic relationship with legumes (Parker et al. 2007). There is recent recognition of some *B.* spp. as the main nodulating partner with Brazilian *Mimosa* species, *L. ambigua* species, *Cyclopia* and *Rhynochosia ferulifolia,* which are endemic to the fynbos (Elliott et al. 2007, Garau et al. 2009, Bontemps et al. 2010, Howieson et al. 2013). Examination of the legumes of the fynbos region has revealed that a surprisingly large number of species nodulate with *B.* spp. (Garau et al. 2009).

Burkholderia has been identified as a genus well adapted to acid, infertile, coarse textured soils (Howieson et al. 2013), which is important when focusing on the selection of rhizobia for the soils of WA. The process of domesticating *L. ambigua* will rely heavily on development of successful inoculation technology for *B.* spp.

1.4. Inoculation

1.4.1. Culturing rhizobia for inoculation

Inoculation in an agricultural context refers to the administration of specific symbiotic rhizobia to the rhizosphere of the intended legume. The ancient method of inoculation (moving soil around) was practised until Hellriegel and Wilfarth (1888) discovered that bacteria created nitrogen-fixing nodules on plant roots. Beijerinck (1888) began isolating the bacteria from the nodules after this initial ground-breaking discovery. This finding encouraged a suitable media to be created for the cultivation of rhizobia before Nobbe and Hiltner (1896a, 1896b) commercialised agar inoculants and lodged patents in the USA and England in 1895 (Date 2001). Commercialised cultures soon evolved from agar-based, to incorporation into sterilised soil (Date 1974), then into peat (organic humus) (Fred et al. 1932).

Roughley and Vincent (1967) discovered the advantages of using a sterilised peat carrier, developed by flash-drying, autoclaving, gamma irradiation or chemical sterilisation of finely ground organic matter (Date 1974, Bullard et al. 2005).

As experience with inoculation increased, it was discovered that not all legumes responded to inoculation in all fields, and this began to confuse farmers and the industry. Allen and Allen (1961) developed a set of criteria for decision-making around introducing suitable rhizobia to the soil. The recommendations were to inoculate where:

- past paddock history indicated an absence of legumes,
- poor nodulation results were reported in previous years,
- when the legume followed a non-legume rotation, and

- when the land had been reclaimed after a clearing event

The approach to inoculation can be viewed as a safe investment in agricultural technology (Howieson et al. 2000b, Deaker et al. 2004, Herridge et al. 2008). This is backed by the argument that unnecessary inoculation is better than losing yield due to poor nodulation, and the added expense of buying fertiliser-N (Hynes et al. 1995, McKenzie et al. 2001).

1.4.2. The benefits of inoculation

In tackling the issue more deeply, Giller et al. (2016) proposed four scenarios to explain the need to inoculate (Figure 1.3). Case 1 shows that none of the treatments has an effect on legume productivity. This suggested there was another factor restricting plant growth, as yield had not been affected by inoculation or N fertiliser. This factor could be a deficiency in a nutrient such as phosphorus (P) or potassium (K). Case 2 displays a clearly N-limited soil but inoculation (+I, -N in Fig. 1) was not as successful as fertiliser application. This could be interpreted as an inoculation problem when sowing and administering rhizobia, a poor fixing rhizobial strain, competition from a resident rhizobial background or a combination of all. The resident background rhizobia might suggest a need for the development of competitive inoculant rhizobia. Case 3 illustrates a situation where a successful quality strain of rhizobia has been delivered and nodulated the target legume, to fix N_2 equivalent to the application of fertiliser N. Finally, Case 4 demonstrates successful nodulation; however, the control has also achieved 100% legume growth. This case suggests a background rhizobial population that was viable and abundant and effective at N fixation, which then produced an optimal symbiosis. Such a strain might be considered as a potential inoculant.

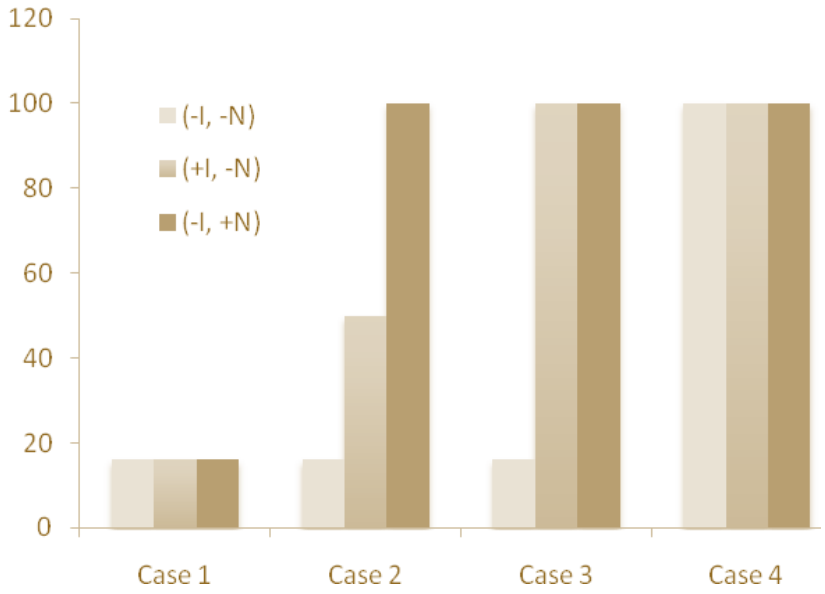

Figure 1.3: Showing the results from the 'need to inoculate' trial from Giller et al. (2016). The percentage of growth on the Y-axis and each case represented on the X-axis

The benefit of N fixation is dependent on the correct rhizobial species being administered to the specific crop or pasture legume (Giller and Cadisch 1995). If successful, the ability to use "free" N_2 is ecologically sustainable compared to the recent increased popularity in fertiliser N (Peoples et al. 1995, Herridge et al. 2008). Poor nodulation after inoculation is the reason why legume inoculant research units have been established around the developing world.

Crop and pasture legumes largely respond to inoculation once they have been introduced to new soils which lack the appropriate rhizobia (van Kessel and Hartley 2000). The ultimate benefit of inoculation as indicated in Figure 1.3 is increased growth without any artificial fertiliser N added.

Due to declining terms of trade in agriculture globally, there has been an increasing emphasis on inoculation and how to ensure it proceeds optimally. A good example is the Bill and Melinda Gates Foundation funded N2Africa program, which seeks to deliver the benefits of legume inoculation to sub-Saharan Africa (http://www.gatesfoundation.org/).

1.5. Techniques and inoculant carriers

1.5.1. Techniques of inoculation

Techniques of inoculation have substantially progressed since the rudimentary approach of transferring soil from fields containing well-nodulated legumes to recipient fields, albeit that some low socio-economic communities still use this technique. Brockwell (1977) summarised two main approaches to inoculation: seed inoculation and soil inoculation. Seed inoculation is where the rhizobia are adhered to the seed before planting by dusting, slurry, lime or phosphate pelleting, or vacuum impregnation. Whereas, soil inoculation refers to the addition of a carrier directly to the soil, with the carrier being either liquid or granular (Deaker et al. 2004).

Table 1.2 indicates the levels of cost, ease of application and effectiveness of various inoculation techniques, which is information that farmers may use to decide which is most applicable for their seeding operation. The inoculation success from the techniques in Table 1.2 is consistent with the Australian view of optimal application of rhizobia to be 1011 CFU (colony forming units) per hectare, with the minimum standard in peat to be ≥ 109 CFU (Bullard et al. 2005).

Table 1.2: Some techniques of inoculation with their cost and effectiveness.

Techniques	Application	Cost	Ease of application	Effectiveness
Peat slurry	Seed	Low	Easy	High
Soil dusting	Seed	Low	Easy	Low
Lime or phosphate pelleting	Seed/ soil	Low	Difficult	Low
Vacuum impregnation	Seed	High	Very difficult	High
Freeze dried powders	Seed	Medium	Moderately difficult	High
Pre-inoculated seed	Seed	High	Easy	Low*
Clay granules	Dry/wet soil	High	Medium	Medium
Peat granules	Wet soil	Very High	Easy	Medium
Liquid injection	Wet soil	Medium	Moderately difficult	High
Tea-bagging	Soil	Low	Easy	Low

*except for Lucerne (*Medicago sativa*) and other medics.

1.5.1.1. Dusting inoculation

Dusting inoculation describes where the peat inoculant is mixed with the seed, with no sticker or re-wetting involved (Deaker et al. 2004). However, this approach to inoculation can cause problems as bacterial desiccation occurs rapidly, and the seed delivery machinery collects most of the inoculant. Thus, its efficacy has been questioned. Although dusting is

not popular today, poorer countries such as Afghanistan still use this technique as farmers have not been exposed to modern inoculation technologies such as in Australia (Pain and Sutton 2007).

1.5.1.2. Slurry inoculation

The approach of mixing peat with a water solution and an adhesive (e.g. PVA) (Deaker et al. 2004), called peat slurry inoculation, allows for more rhizobia to attach to the seed coat. This application process is as easy and as cost effective as dusting, yet it results in better nodulation outcomes (Deaker et al. 2004). However, there can be problems with this inoculation technique. For example, when the slurry dries the inoculum can dislodge from the seed and become stranded in the seed hopper which can cause blockages (Gault 1987). Another example is that the inoculant slurry can become hard after drying which inhibits the ability of the seed to germinate, which then makes the inoculant less efficacious (Brockwell 1977).

1.5.1.3. Lime pelleting

Fine limestone ($CaCO_3$) coated on the outside of the peat slurry to counteract the acid soil is called “lime-pelleting” (Deaker et al. 2004). Fertilisers such as superphosphate can cause the soil environment next to the seed to become acidic, which can decrease nodulation (Howieson and Ballard 2004). Therefore, delivering lime to the soil with the seed and rhizobia intact provides protection against these deleterious conditions (Deaker et al. 2004, Drew et al. 2012). Brockwell (1962) discovered that when the rhizobia and adhesive were combined and placed under the limestone coat on the seed, it provided a better chance of survival for rhizobia, especially if there was a delay in germination of the legume. Although, this may be true for sub clover, no other legumes were assessed in this study and the sub

clover inoculant has since been improved to an acid tolerant strain, WSM1325 (Yates 2009).However, this technique provided an ease of application in that it dried the peat slurry allowing for a separation of individual pellets for precision sowing. For the best results, sowing must occur immediately after lime-pelleting, but benefits do include increased calcium, which is proven to help nodulation (Vose and Gareth-Jones 1963, Howieson et al. 1993) and a localised increased soil pH (Roughley et al. 1966, Roughley 1970). This doesn't suit all legumes and rhizobia, especially species such as serradella and lupin, that require an acid environment (Tang and Robson 1993).

1.5.1.4. Freeze-dried powders

Freeze-dried powders can be applied to the soil utilising either water injection, or through slurry application to the seed, however placing rhizobia into dry soil should be avoided with this type of inoculation. Rhizobial cells desiccate rapidly in a dry environment and die (Deaker et al. 2004, Drew et al. 2012). A protectant is usually supplied with the powder to aid with re-absorption of water (Drew et al. 2012). Mcleod and Roughley (1961) reported that peat cultures perished more rapidly compared to freeze-dried cultures, especially at high temperatures (37°C). The process of freeze-drying is essentially very similar to the preservation measures of antibiotic-producing, food and other microbial cultures (McLeod and Roughley 1961). Many *Rhizobium* strains were proven by Appleman and Sears (1946) to be able to survive long periods of time after freeze drying. Following on from this research, Kosanke et al. (1992) reported that recovery of dried rhizobial populations was more successful if the rehydration process was slow. Rhizobial survival in a freeze-dried inoculant with the addition of either water, gum arabic or sugar solutions as adhesives has been shown to be low (Vincent 1965). Cells are harvested for freeze-dried inoculants by centrifugation.

McInnes and Date (2005) investigated the application of freeze-dried inoculants to seed of *Stylosanthes* and *Desmanthus* legumes in a hot environment, and reported that there was a significant loss in the viability for both inoculants from the first sampling time (directly after the freeze-drying process) and after 15 days. However, after sampling again 300+ days later, the death rate had decreased. This indicated that freeze-dried inoculants were surpassing the peat inoculants for the percentage of rhizobia surviving in the long term in these conditions. Replicated experiments (Kremer and Peterson 1983, McInnes and Date 2005) found that freeze-dried inoculants of rhizobia supported a higher count of cells than rhizobia stored in peat, at 60°C. Freeze-dried rhizobia products were more concentrated than peats, with 10^{10} CFU/g and 10^{9} CFU/g, respectively (McInnes and Date 2005). Moreover, these authors considered there was potential for seed pre-inoculated with freeze-dried cultures to provide survival of rhizobia in "farmer shed conditions" for up to five months (McInnes and Date 2005).

1.5.1.5. Pre-inoculated seed coating

Pre-inoculated seed describes a convenient off-farm inoculation procedure (Hartley et al. 2012). The desired seed is purchased with the rhizobia entrapped in a factory-applied seed-coating that may include insecticides, micronutrients and fungicides (Drew et al. 2012). Famers often dislike the extra step of on-farm seed inoculation (Williams 1994), therefore providing a seed coating off-farm can be attractive. It also allows for a range of plant growth and protection agents, polymers and dyes to be included in the formulation. Pre-inoculated seed has become a popular product for lucerne, and annual medics (Williams 1994, Drew et al. 2012) as their specific rhizobial genus, *Sinorhizobium*, survives well in this method of encapsulation. Polymers are integral to the encapsulation process and serve to protect the rhizobia during the reabsorption of water (Williams 1994, Hartley et al. 2012). However,

Vincent and Smith (1982) investigated many retail samples of pre-inoculated seed and concluded that they did not possess the desirable amount of rhizobial cells to be a viable inoculant. Additional testing in 1970-1974 (Brockwell and Whalley 1970, Brockwell et al. 1975) and 2001-2012 (Gemell 2002, Hartley et al. 2012) found poor survival of the rhizobia inoculant on many samples of clover, raising doubts about the efficacy of the pre-inoculating system for this legume. With the efficacy of pre-inoculated seed questioned (Hartley et al. 2012), seed coating techniques have progressed to forming pellets, where desired seeds are encapsulated in clay (Madsen et al. 2012), though there is no focus on rhizobial survival. Keeping rhizobia alive on seed is challenging and costly, hence research and development programs are needed to improve these products (Hartley et al. 2012).

1.5.1.6. Granular inoculants

Another simple way of introducing rhizobia to the soil is with granular inoculants. These have been found to be superior to liquid inoculants in some settings (Kutcher et al. 2002, Clayton et al. 2004). Granular inoculants can be manufactured by adding peat slurry to moist clay and mixing it together, then allowing the granules to dry. Alternatively, they can be made by suffusing liquid broth cultures onto ready-made granules which absorb the inoculant (Deaker et al. 2016). In very recent innovations, clay based granular inoculants have been successful when sown into dry soil, especially for twin or summer sowing of hard-seeded pasture legumes in the crop-pasture rotation (Loi and Nutt 2010). However, it is important to note soil temperature, moisture and time between inoculation and germination once the clay-based granules are in the ground, to gauge nodulation success. The bulk size of the granule causes handling disadvantages, including increased transport costs and the possibility to block up seeding equipment during application (Herridge 2008). Peat and bentonite or attapulgite (palygorskite) clay are the basic ingredients for making

granules for inoculation, and most manufacturers have a certain size limit to the granule to allow easy sowing. An advantage of dry granules is that they can be stored for up to six months without refrigeration (Drew et al. 2012). However, granular inoculants have fewer rhizobia per gram than peat inoculants (Drew et al. 2012), so higher application rates are necessary (e.g. 15kg/ha (Carr et al. 2006)). A further consideration is that granular inoculants can separate rhizobia from other chemicals applied to the seeds and seed exudates, which can be toxic, hence increasing survival of the rhizobia (Kutcher et al. 2002, Herridge 2008). There is also flexibility of the sowing time of clay granules in regards to twin or summer sowing (explained in section 1.2.2.). This alternate delivery of rhizobia can save costs when in the process of revitalising a pasture or introducing a new legume to the system, especially in a large scale sowing (Loi et al. 2008, Loi and Nutt 2010).

Clay granules are very well suited to the unpredictable conditions of southern Australia. However, a disadvantage with clay granules is that they cannot support rhizobial cell numbers at the same concentration per unit weight as peat (10^7 and 10^9 /g respectively) (Deaker et al. 2016). Yet, the flexibility that clay granules provide makes it a popular inoculation product for some farmers, especially when the desired legume is sown out of the reliable rainfall period of September-April.

Similar to clay granules, peat granules provide an easy flow method of inoculating legumes (Denton et al. 2017). They are essentially prilled, moist peat. These were not listed in Table 1.2 due to their similarities with the peat coating technique, with the only difference being that they reduce the risk of blockages in the seeder.

1.5.1.7. Liquid inoculation

Liquid injection refers to the peat culture being applied directly to the seedbed (Deaker et al. 2004). Seedbed inoculant injection places rhizobia in the soil at the time of sowing the seed. The peat or freeze-dried culture is mixed with water and polymers then sprayed into the row where the seed has been placed. This is a common technique for inoculating soybeans (Deaker et al. 2004). This direct inoculation works well in damp soils and avoids damaging the seed coats of some legumes. It also prevents the detrimental effects of both fungicides and pesticides on the rhizobia (Deaker et al. 2004, Drew et al. 2012). However, it is moderately expensive, cannot be undertaken in dry conditions and is a difficult process.

Establishing rhizobia in the soil before sowing of the legume is known as "alternative host delivery" (Thompson 1965, Diatloff 1969). Thompson (1965) in Australia administered rhizobia specific for clover into infertile, acidic soil by inoculating wheat and dead subterranean clover seed in the preceding season. This method of inoculation may enhance the survival of rhizobia because they are not in contact with any chemicals (insecticides, fungicides and herbicides) that are detrimental to their survival (Date 2001). This system has been improved by the invention of clay granules (Carr et al. 2006) which can be used in alternative host delivery or twin sowing (Loi et al. 2008). However the rhizobia must colonise the soil in the absence of their host, and not all rhizobial strains can achieve this (Chatel et al. 1973).

1.5.1.8. Tea bagging

Lastly, "tea bagging" is a process whereby a porous stocking containing inoculated peat is placed into a boom spray tank full of liquid and then left to allow the rhizobia to disperse

through the liquid before being sprayed onto the soil. However, this process doesn't always produce successful nodulation, for the reasons stated in Table 1.2.

A farmer's decision to use a particular method of inoculation depends on factors such as:

(i) legumes species,

(ii) rhizobia species,

(iii) their machinery,

(iv) the availability of inoculant technology and

(v) the environment into which they are sowing

For farmers to adapt to different and changing environments through the introduction of foreign legumes and rhizobia, new carriers and polymers must be manufactured to allow for this transition. A good example of the need for new technologies is for the inoculation of the new legume *L. ambigua*. The *B.* spp. associated with *L. ambigua* were shown to die very rapidly when coated onto seed with the normal technologies discussed above (Howieson et al. 2013).

1.5.2. Carriers and polymers

Certain criteria must be met for choosing a rhizobial carrier, such as the ability to retain a high amount of water, chemical and physical consistency, no toxicity to the rhizobia and environmental acceptability (Albareda et al. 2008).

There is a great deal of variation in how the different strains and species of rhizobia react to carriers. Peat is successful at maintaining high numbers for all rhizobia, especially the soybean symbiont *Bradyrhizobium japonicum* (Albareda et al. 2008). However, other rhizobia (such as *Burkholderia* spp.) do not survive well in substrates such as sepiolite,

attapulgite and amorphous silica compared to peat and cork substrate (Albareda et al. 2008, Deaker et al. 2016).

Polymers have been used in the inoculation process as an adhesion tool for peat based inoculants (Deaker et al. 2004, 2007). Freeze-dried and granular inoculants contain more stable efficacious rhizobia if they are encapsulated in polymers. However, a polymer must be water soluble, which is an important factor because it eventually has to release the rhizobia into the root zone of the legume seedling (Deaker et al. 2007). Table 1.3 describes characteristics of a range of water soluble polymers and whether they are advantageous as either an adhesive or a protectant against desiccation.

Polyethylene-glycol (PEG) and dextran have increased survival of rhizobia during the freeze-drying process due to their antifreeze qualities (Table 1.3) (Kim et al. 1988, Champagne et al. 1992). Other polymers such as gum arabic, PVP (polyvinyl-pyrrolidine) and cassava starch (tapioca flour) enhanced survival of bradyrhizobia compared to PEG and PVA (polyvinyl alcohol) 48 hours after inoculation (Tittabutr et al. 2007).Gelatine and xanthan gum offered protection during freeze-drying, and Xanthan gum was more able to aid survival than alginate for *Bradyrhizobium japonicum* (Jung et al. 1982). Trehalose, however, is the most successful polymer for increasing the survival of *B. japonicum* (Streeter 2003) even though *B. japonicum* is able to synthesise it (Cytryn et al. 2007).

PVP, PEG and gum arabic all have adhesive properties (Table 1.3) (Tittabutr et al. 2007)and have been reported to also protect rhizobial cells against toxic seed exudates (Vincent et al. 1962, Hale and Mathers 1977). Maltodextrins (hydrolysates of starch) bind water, which is important when creating a freeze-dried culture (Champagne et al. 1996). Initially, McInnes and Date (2005) designed experiments to include the osmoprotectant trehalose into a peat

Table 1.3: Useful characteristics of common polymers.

Polymers	***Useful characteristics***
Gum arabic	Complex carbohydrate adhesive derived from *Acacia*. However, it is expensive and not readily available
Methyl Cellulose	Highly hygroscopic adhesive powder used for pellet making and seed coating and benefits the survival of rhizobia
Polyacrylamide (PAM)	Binding resin used for the treatment of wastewater
Polyethylene glycol (PEG)	Hygroscopic white semi-solid adhesive polymer derived from antifreeze, which is successful for aiding the survival of rhizobia during freeze drying
Polyvinyl acetate (PVAc)	Synthetic adhesive resin, not as successful as PVA for aiding survival or rhizobia
Polyvinyl alcohol (PVA)	Highly soluble, more hydrophilic than PVAc. High hygroscopic PVAs are used in rhizobial entrapment
Polyvinyl pyrrolidone (PVP)	Highly hygroscopic polymer that can bind toxic seed exudates from legume seeds. A very successful seed coat adhesive and protectant
Trehalose	A natural disaccharide sugar that stabilises membrane structure in rhizobia cells
Xanthan gum	Is a stable polysaccharide secreted from certain bacteria that increases viscosity in liquids and aids as a protectant when freeze drying rhizobia

inoculant and by using a polyvinyl alcohol as an adhesive to increase the survival of rhizobia, which were both deemed unsuccessful. This study also showed that there was no statistical advantage of using peanut oil rather than methyl cellulose as an adhesive (McInnes and Date 2005).

Hydrolysis refers to the chemical breakdown due to the reaction with water. The level of chemical hydrolysis seems to be important when selecting a polymer to aid in rhizobial survival against desiccation. For example, PVA has a range of 86.5-89% hydrolysis, which protects desiccated cells more than polymers with lower (78.5-82%) or higher (98.5%) degrees of hydrolysis (Deaker et al. 2007). Poor survival was reported with rhizobia in PVP and methyl cellulose due to their highly hygroscopic nature (Deaker et al. 2007). Moisture sorption must be at optimum levels when designing a synthetic polymer as it can ensure the survival of rhizobia under desiccation pressure (Deaker et al. 2007). Dried *Rhizobium leguminosarum* bv. *trifolii* cells that were not accompanied by a polymer survived well if they remained in their dehydrated state, but these cells had diminished viability once relative humidity increased (Deaker et al. 2007). This suggests the reabsorption of water can be lethal unless rhizobia are entrapped in a polymer. A reduction in the differential between the internal and external cell water content is fundamental when increasing the survival of rhizobia (Bushby and Marshall 1977b, Deaker et al. 2007). Evidently, rhizobial survival cannot be attributed to a single property of a polymer (Deaker et al. 2007, Hartley et al. 2012). So, to achieve industry quality seed coating, advancements in polymeric substances are considered vital (Bullard et al. 2005).

1.5.3. Inoculation and cultivation of *L. ambigua*

There appears to be a vulnerability to desiccation for most *B.* spp. associated with *L. ambigua* (Howieson et al. 2013). Inoculant cells had complete mortality within one hour of seed inoculation, with a peat carrier. To avoid nodulation failure in experimental sowings, the inoculation and sowing of *L. ambigua* is a rapid process, whereby the seed is introduced into the wet soil within 30 minutes of inoculation via peat inoculant mixed with methocel. However, this time-frame isn't achievable for most farmers who usually choose to inoculate several hours or even days before seeding.

L. ambigua is sown in early spring (a date that depends on the region of the state) because soil temperatures of above 20°C seem to be necessary for its germination and early vigour, and allows for maximum weed control. Hence there is some variability in optimal seeding times around WA: the northern Wheatbelt temperatures increase after winter before those in the south. Across all regions, however, this is an awkward time for sowing legumes, as the following rainfall diminishes in reliability. *L. ambigua* also has a small seed (Le Roux and Van Wyk 2007, Edwards 2015) so it has to be sown in the top 10mm of the surface for successful emergence, which exposes them to drying conditions (Howieson et al. 1995, Loi et al. 2005). Combining the required seeding depth with the optimal time of year means that there is not abundant moisture for sowing *L. ambigua* seeds into, and for keeping peat slurry inoculants alive. Clay granules may therefore be the best formulation of inoculant carrier for this symbiosis, and, given the problems already identified with this form of carrier (Howieson et al. 2013), the subject of the experimental chapters of this thesis.

1.6. Aims of thesis

The aims of this thesis include:

- Formulating an inoculant delivery system suitable for the challenging moisture conditions faced by *L. ambigua* at sowing,
- Developing methods for enumerating *B.* spp. in non-sterile clay granules using an antibiotic profile and plant infection techniques, and
- Seeking evidence of variation in tolerance to desiccation in the *Burkholderia* genus.

Chapter 2: Modifying clay granules to enhance the survival of *Burkholderia* spp.

2.1. Introduction:

2.1.1. Inoculation and sowing of *L. ambigua*

Soil temperatures above 20°C seem to be necessary for the germination and early vigour of *L. ambigua* (Edwards 2015). Hence, *L. ambigua* is sown between August and October in early Spring in WA, depending on the region of the state. This is an awkward time for sowing legumes as the reliable rainfall is hard to predict. Further, *L. ambigua* has a small seed (less than 1 mg) (Le Roux and Van Wyk 2007, Edwards 2015) so it has to be sown in the top 10mm of the soil surface for successful emergence, like other small seeded new pasture species. Combining the seeding depth with the optimal time of year for germination means that there is a high risk of moisture stress soon after seeding. When these factors are further combined with the target non-wetting sands, it is clear there is a small window of moisture availability for sowing *L. ambigua* seeds into. Given these factors, it is likely the inoculant will be exposed to dry soils shortly after seeding, so it essential that it is protected from desiccation.

Peat inoculants lose moisture immediately when they are applied to seed (Deaker et al. 2016) and thus cannot be sown into dry soils. Clay granules, which have lower moisture loss, appear as the best option for carrying *B.* spp. for successful inoculation into a drying environment. However, there is a worrying vulnerability to desiccation for most *B.* spp. that nodulate *L. ambigua* which have been so far investigated (Howieson et al. 2013). Thus, the

aim of this research is to amend clay granules, through different additives, peat levels and drying temperatures to aid in the survival of inoculant strains of *B.* spp.

2.1.2. Aims

The aims of this chapter include:

- The formulation of clay granules with amendments that create an environment that increases the survival of *B.* spp. strains over that described by Howieson et al. (2013).
- The determination of antibiotic resistance patterns in *B.* spp. to assist in enumeration under non-sterile conditions
- The enumeration of strain survival dynamics in the clay granules using antibiotic media to create a successful counting method for *B.* spp. embedded in clay granules.

2.2. Methods:

2.2.1. Antibiotic resistance patterns in *B.* spp.

B. spp. and control strains of alpha-rhizobia were exposed to different antibiotics to identify any natural resistance that could be used to help enumerate strains in experiments.

Four *B. dilworthii* strains, eight *B. graminis* strains, two *B. sprentiae* strains and three control rhizobia (*Ensifer medicae, Rhizobium leguminosarum* bv. *trifolii* and *Rhizobium sullae*) were resuscitated from glycerol stocks held at -180°C (collection located at the Centre for *Rhizobium* Studies) and streaked on yeast mannitol agar (YMA) (Appendix A (Hungria et al. 2016)). The strains are listed in Table 2.1.

Table 2.1: Species and strain identification

Species	Strain
Burkholderia dilworthii	308*
B. dilworthii	WSM3556
B. dilworthii	WSM4204
B. dilworthii	WSM4206
B. graminis	303*
B. graminis	WSM4181
B. graminis	WSM4182
B. graminis	WSM4629
B. graminis	WSM4627
B. graminis	WSM4628
B. graminis	WSM4633
B. graminis	WSM4634
B. sprentiae	WSM4184
B. sprentiae	WSM5005
Sinorhizobium medicae	WSM419
Rhizobium leguminosarum	WSM1325
R. sullae	WSM1592

*Strain 308 and 303 have not been assigned WSM (Western Soil Microbiology) numbers yet.

Antibiotic susceptibility tests were performed on YMA medium amended with the antibiotic Sensi-disc dispenser system (Oxoid), with bio-discs (Oxoid) containing ampicillin (10, 25

µg/ml), chloramphenicol (30, 50 µg/ml), erythromycin (30 µg/ml), gentamicin (10, 30 µg/ml), kanamycin (30 µg/ml), neomycin (10 µg/ml), penicillin U (10 µg/ml), streptomycin (10, 25 µg/ml) and tetracycline (30 µg/ml), modified from De Meyer et al., (2013, 2014). The modifications included using YMA instead of ½LA (Hungria et al. 2016). Petri dishes that contained YMA media were seeded with each specific rhizobia, with two replications. Four different antibiotic discs were placed on each replicate with the sensoid disc dispenser and then pushed gently onto the plate with sterile forceps. There were 136 plates (17 strains x 13 antibiotic treatments x 2 replications) with 3 plates containing 4 antibiotics and 1 plate containing 1 antibiotic for each strain of rhizobia. The plates were then inverted and incubated at 28°C. Plates were evaluated between two and five days later, with the resistance "halo" measured in millimetres.

Each rhizobial strain was assessed for susceptibility to each antibiotic by measuring the resistance zone around the sensoid disc. The results were then evaluated by using an Oxoid Ltd. Antimicrobial Susceptibility Test Chart, which gives the level of either: susceptible, intermediate or resistant.

2.2.2. Confirmation of antibiotic resistance in *B.* spp. strains (WSM) 4184, 4204 and 5005.

The preliminary antibiotic experiment (2.2.1) indicated there was a similarity of reaction between species. Thus, further antibiotic resistance experiments were performed with strains (WSM) 4184, 5005 (*B. sprentiae),* 4204 (*B. dilworthii*) and WSM1325 (*Rhizobium leguminosarum* bv. *trifolii*) to attempt to more clearly define differences.

Chloramphenicol (CAM) (30mg/ml) and neomycin (Nm) (10mg/ml) were combined in molten YMA with a sterile pipette before pouring into petri plates, with two replications for

each treatment. Further combined treatments at lower concentrations were produced by lowering the antibiotic levels by 25% each time (i.e. 100%, 75%, 50%, 25% and 0%) to produce a growth profile in the strains as the concentration decreased. Controls were provided for the individual antibiotics separately at three concentrations: CAM (at 10, 20 and 30mg/ml) and Nm (at 10, 20 and 30mg/ml). The test strains were streaked onto YMA media, grown for three days then re-streaked onto antibiotic plates with the varying antibiotic concentrations and combinations.

Strains were assessed by comparing the antibiotic treatment plates to the control plates (no-antibiotics in the media as shown in Figure 2.2).

2.2.3. Peat formulation

To create clay granules, *B.* spp. must be first stabilised in peat.

Finely ground pre-sterilised (gamma-irradiated) peat sealed in plastic pouches is the most common carrier for rhizobia (Deaker et al. 2016). Sterile peats (100g) were obtained from the manufacturer for incubation of *B.* spp. strains WSM5005, WSM4184 and WSM4204.

The strains were firstly prepared as an agar culture (YMA), with a loop-full of the pure culture from refrigerated glycerol streaked onto solid media. After 48 hrs of growth and having establishing purity by visualisation under a dissecting microscope, a loop-full of cells was transferred to YMA broth. The broths (50mL) in flasks (250mL) were placed on the orbital shaker for 2-3 days. Within the laminar flow cabinet, the necks of the flasks were flamed, and then 50ml of broth was removed by drawing into a sterile syringe through a needle. An entry point for injection into the peat was selected and sterilised by swabbing with ethanol (70%). The needle was then inserted through the plastic and into the peat with

25ml injected into one direction and then turned 90 degrees with the remaining 25ml injected in the other direction to get an even distribution. Ethanol (70%) was then swabbed over the injection site and allowed to evaporate before placing a sticky label and tape over the site to seal the hole. Once sealed the peat packet was massaged, so the liquid reached all four corners of the packet to ensure it was completely blended. The peat was then incubated at 28°C for ten days to mature.

After the period of maturation, the peat was removed, counted by the "Miles and Misra drop plate method" (O'Hara et al. 2016) and checked for contaminants. Plates containing YMA growth medium were divided into six by marking sections on the petri dish. The point of entry of the packet of peat was again sterilised using an ethanol (70%) swab. It was then cut with a sterile scalpel blade to make an incision big enough to insert a sterilised measuring spoon. One gram of peat was taken from the packet and placed into sterile saline solution (0.89% in sterile DI water) and mixed on a vortex shaker for 10 seconds. One ml of the first dilution was transferred into the second dilution, and this was repeated eight times to give a dilution series (with each dilution shaken). Once the dilution series was completed, two drops of 25µL from the highest dilution were placed onto one section of two petri dishes to give two reps per treatment to count. This was repeated for the lower dilutions. The pipette was calibrated on a sensitive scale to give an accurate reading of the droplet size. After absorption of the liquid drops, the plates were inverted and sealed then placed in a 28°C oven for 24 hrs. The emerging colonies were then counted with a marker pen over two consecutive days under a dissecting microscope. This method was repeated for all *B.* spp. strains.

The numbers of CFU (colony forming units) in the peats were calculated by the following equation from O'Hara et al. (2016):

Average number of CFU per drop x the area of the section which gave the reading (e.g. 10^7) x the corrected volume of the drop (e.g. 40, for the drop of 25µl) = the amount of CFU/ml.

2.2.4. Clay granule formulation

Clay granules were formed with the help of Chris Poole of ALOSCA Pty. Ltd. under a confidentiality agreement pertaining to the methods. Because clays differ slightly in their composition, variants were sourced from different pit areas at the Watheroo deposit of bentonite (30.324765 S., 115.982186 E.). The moisture content of the clay samples was adjusted with DI (deionised) water (Table 2.2), then a polymer (PVA-poly vinyl acetate) added, and finally a range of levels of peat (in suspension) carrying the *B.* spp. were mixed into the clays. PVA was chosen due to its reported enhancement of survival relative to other polymers (Deaker et al. 2007). The peat suspensions were made by first diluting the peat (15g) into sterile DI water (Table 2.2) before adding it to the clay. This mixture was thoroughly stirred until the peat was dispersed evenly through the clay slurry. After mixing was complete, treatments including a polymeric additive, citric acid, increased peat, or a combination of all treatments was added to the clay slurry to begin investigations of rhizobial survival (Table 2.2). The clay slurry was then placed into shallow plastic 500 mL containers for air-drying in a room at a constant temperature. Two temperatures of drying were examined; 25°C or 28°C. The clays remained for three weeks at these temperatures (drying step 1). The containers were then placed in a naturally lit phytotron glasshouse for another five weeks for final drying. After the slurries had hardened, they were placed between two heavy-duty sheets of plastic and milled with a mallet until the shards or

granules were able to be sieved to a size between 0.5mm and 2.5mm (the size of the commercial granule) (Figure 2.1).

Table 2.2: Granule treatments and additives (a- dried at 28°C, b- dried at 25°C). Clays A and E supplied from ALOSCA™.

Treatment	Clay	Peat	DI water	Additives/methods
1a,b	A	WSM4184	750ml	Control from Howieson et al. 2013
2a,b	A	WSM4184	750ml	PVA
3a,b	A	WSM4184	750ml	Citric acid
4a,b	A	WSM4184	750ml	PVA and citric acid
5a,b	E	WSM4184	1000ml	
6a,b	A+E	WSM4184	750ml	
7a,b	A	WSM4184	750ml	Double amount of peat
8a,b	A	WSM4204	750ml	
9a,b	A	WSM5005	750ml	

Figure 2.1: Clay granules made with peat containing *B.* spp. and crushed to the market size of between 0.5-2.5mm.

2.2.5. Enumeration of strains embedded in clay granules using antibiotics to suppress contaminants

YMA in petri dish plates amended with CAM (20mg/ml) were prepared based on results from sections 2.2.3 and 2.2.4. An ALOSCA™ technologies procedure to disburse the clay granules was acquired (Howieson et al. 2013). This procedure was altered by diluting 1g of crushed clay granules three times (i.e. 10^{-1}-10^{-3}) instead of seven times, shaking on a wrist shaker for 50 minutes instead of 30 minutes, and by using YMA media instead of ½LA media. 25µl of the milky clay liquid was placed into the middle of the plate then spread in a circular motion with a glass stick. The plates were checked meticulously between one and four days

after streaking and growth was recorded as percentage growth compared to the control (e.g. Figure 2.2).

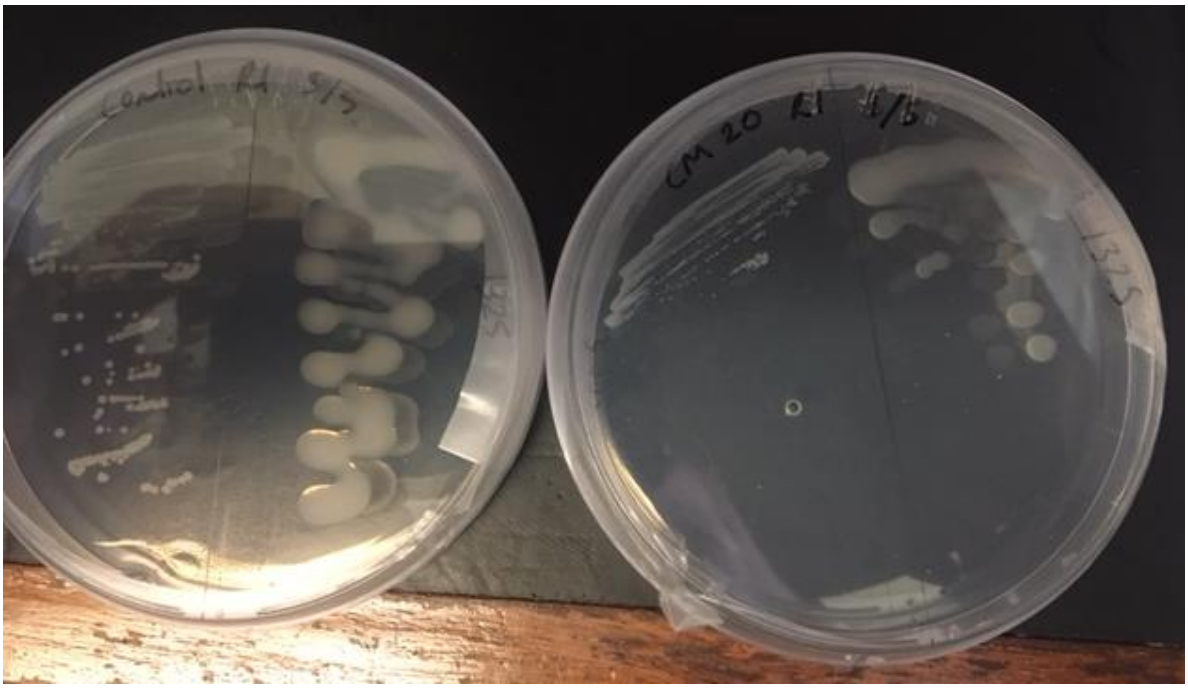

Figure 2.2: WSM1325 (*R. leguminarosarum bv. trifolii*) and WSM5005 (*B. sprentiae*) grown on YMA (left) and YMA+ CAM (20µg/ml) (right).

2.3. Results

2.3.1. Antibiotic resistance patterns of *B.* spp. strains

The strains were differentiated primarily by their reaction to ampicillin (AMP) at 25µl/ml delivered by sensoid discs (Table 2.3). Whereas *B. sprentiae* gave an intermediate reaction to AMP at 10µl/ml, the *B. graminis* and *B. dilworthii* strains varied. All strains were resistant to CAM up to 50µl/ml, penicillin (PEN) 10µl/ml, and susceptible to streptomycin (STR) as low as 10µl/ml and tetracycline (TCN) at 30µl/ml.

On the basis of these results we chose four strains with which to continue the antibiotic study: *B.* spp. strains (WSM5005, 4204 and 4184) and a control *R. leguminosarum* bv. *trifolii* (WSM1325).

When antibiotics were injected into molten agar, resistance patterns varied and these are shown in Table 2.4. The *B. sprentiae* strains behaved similarly and could be differentiated from *B. dilworthii* on the basis of their reaction to CAM 15µl/ml, Nm 5µl/ml. WSM 1325 was tolerant of most of the combinations.

CAM at 20µg/ml gave the best result for the enumeration of *B.* spp. cells (Table 2.4) and hence was chosen for future experiments. *B.* spp. strains tested appeared to be susceptible to low levels of Nm which resulted with the antibiotic resenting an insurmountable stress on cells.

Table 2.3: Antibiotic resistance patterns of rhizobial species grown on YMA plates. R= resistant, I= intermediate and S= susceptible.

Species	Strain	Ampicillin 10	Ampicillin 25	Chloramphenicol 30	Chloramphenicol 50	Gentamycin 10	Gentamycin 30	Erythromycin 30	Kanamycin 30	Neomycin 10	Penicillin U10	Streptomycin 10	Streptomycin 25	Tetracycline 30
Burkholderia dilworthii	308	I	S	R	R	S	S	S	S	I	R	S	S	S
Burkholderia dilworthii	WSM3556	R	R	R	R	S	S	S	S	R	R	S	S	S
Burkholderia dilworthii	WSM4204	R	R	R	R	S	S	S	S	R	R	S	S	S
Burkholderia dilworthii	WSM4206	I	S	R	R	S	S	S	S	R	R	S	S	S
Burkholderia graminis	303	R	R	R	R	S	S	S	S	R	R	S	S	S
Burkholderia graminis	WSM4181	R	R	R	R	S	S	S	S	R	R	S	S	S
Burkholderia graminis	WSM4182	R	R	R	R	S	S	S	S	R	R	S	S	S
Burkholderia graminis	WSM4629	R	R	R	R	S	S	S	S	R	R	S	S	S
Burkholderia graminis	WSM4627	R	R	R	R	S	S	S	S	I	R	S	S	S
Burkholderia graminis	WSM4628	R	I	R	R	S	S	S	S	R	R	S	S	S
Burkholderia graminis	WSM4633	R	S	R	R	S	S	S	S	R	R	S	S	S
Burkholderia graminis	WSM4634	R	I	R	R	S	S	S	S	R	R	S	S	S
Burkholderia sprentiae	WSM4184	R	I	R	R	S	S	S	S	R	R	S	S	S
Burkholderia sprentiae	WSM5005	R	I	R	R	S	S	S	S	R	R	S	S	S
Sinorhizobium medicae	WSM419	R	I	R	R	R	S	R	S	R	R	I	S	S
Rhizobium leguminosarum	WSM1325	R	I	R	R	S	S	S	S	I	R	S	S	S
Rhizobium sullae	WSM1592	R	I	R	R	R	S	S	R	R	R	S	S	S

Table 2.4: Antibiotic resistance of *B.* spp. strains (WSM 5005, 4204 and 4184) (green) and control *R. leguminosarum bv. trifolii* (WSM 1325) (black). * indicates growth compared to the control *****. The red font indicates the chosen antibiotic and level. CAM- Chloramphenicol, Nm- Neomycin.

Antibiotic (µg/ml)	5005	4204	4184	1325
CAM30, N10				*
CAM22.5, N7.5				*
CAM15, N5		**		***
CAM7.5, N2.5	*		*	*****
CAM30				
CAM20	*****	****	***	***
CAM10	*****	*****	****	***
Nm30				
Nm20				*
Nm10	*		**	**
Control	*****	*****	*****	*****

2.3.2. Peat numbers

ALIRU quality standards for peat inoculants recommend a minimum 1 x 10^9 cells/g (Bullard et al. 2005). (WSM) 5005 and 4184 reached this count, however WSM4204 was marginally under (Table 2.5).

Table 2.5: *B*. spp. cells in peat: the number of cells per 1g of peat for each strain of *B*. spp.

Strain in peat	CFUs per gram of peat
WSM4184	$1.25x10^9$
WSM4204	$7.5x10^8$
WSM5005	$1x10^9$

2.3.3. Enumeration of strains embedded in clay granules using antibiotics to suppress contaminants

No *B.* spp. strains were recovered from the clay granules after streaking them onto CAM (20µg/ml) following the ALOSCA™ reabsorption method (Howieson et al. 2013). Clay granules streaked onto YMA media with no antibiotics present proved difficult to enumerate as a number of different bacteria and fungi appeared on the plate, obscuring the growth of rhizobia. Figure 2.3 presents the challenges of identification of different rhizobia after treatment 7b was streaked onto YMA media and grown for two days.

Figure 2.3: Treatment 7b streaked onto media lacking antibiotics as a control comparison.

2.4. Discussion

B. spp. were differentiated by the antibiotic resistance patterns in relation to AMP tolerance. All *B.* spp. and strains were tolerant of CAM at 20µg/ml, which is consistent with the literature (De Meyer et al. 2013, Howieson et al. 2013, De Meyer et al. 2014). *B.* spp. strains (WSM) 4184, 4204 and 5005 grew well in peat, but when they were introduced to clay, no *B.* spp. were recovered. These results are consistent with Howieson et al. (2013), so unfortunately, none of the amendments to the clay or drying process seemed to provide an increase in survival.

This may be explained in a number of ways. Firstly, it has been historically difficult to de-sorb rhizobia cells from dried inoculants (Kosanke et al. 1992, Howieson et al. 2013) and despite following a recommended protocol, it is possible we were unable to do so. However, many rhizobia are able to enter a state of anhydrobiosis (Kosanke et al. 1992), and it is possible that *B.* spp. may also (Anandham et al. 2007). Although little is known about the recently discovered *B.* spp. genus (Yabuuchi et al. 1992) it may be the cells become anhydrobiotic and are not easily recovered from this state.

A further reason why *B.* spp. were not recovered from the clay may be due to sampling error. The small amount of clay granules chosen from the initial amount may not have been representative of cells in the clay *per se*, especially if spatial distribution was not even. It is also possible that the stress of rehydration from clay, combined with growing on CAM at 20µg/ml, was too great for the cells to survive. However, at the moment there is only weak evidence (if any) to suggest that *B.* spp. survive in clay.

Laboratory and glasshouse conditions differ to field conditions, so to fully examine the survival of *B.* spp. in clay, it may be necessary to place the granules into the field and assess

whether they can produce nodulation of *L. ambigua*. This would allow a longer time for the clay particles to break down under soil conditions, and may enable surviving *B.* spp. to enter and colonise the soil.

Chapter 3: Efficacy of clay granules as inoculants for *L. ambigua* – field studies.

Dr Sofie De Meyer contributed to 10% of this chapter (sections 3.2.5, 3.2.6, 3.3.2.1 and 3.3.3).

3.1. Introduction

In the fynbos region of South Africa, where *L. ambigua* and its micro-symbiont (*Burkholderia* spp.) naturally occur, the environment is prone to bush fires, soils are seasonally dry, the summers hot, and yet both organisms persist in the deep sands (Howieson et al. 2013). All of these factors should regularly expose *B.* spp. cells to desiccation, which they clearly tolerate at some level. However, desiccation of standard (Howieson et al. 2013) and altered ALOSCA clay granules (Ch. 2) appears to kill these bacteria.

Attempts to resuscitate *B.* spp. from the clay granules and enumerate them in the presence of antibiotics were unsuccessful. Antibiotics were necessary to suppress contaminants overgrowing *B.* spp. because the clay granules were not sterile. These conditions did not provide a successful method for counting the *B.* spp., which may have been an artefact of laboratory procedures; hence we decided to evaluate them for efficacy *in situ*. As part of this, we examined an old trial site for persistence of the *B.* spp. to provide evidence of saprophytic competence of the strains. The rationale for this was that if we could prove the *B.* spp. had survived in the field, but could not resuscitate them from our clay granules *via* a nodulation bio-assay, then we would have evidence that it was not the field conditions *per se* that precluded survival.

3.1.1. Aims

The aims of this chapter were:

- To assess if *B.* spp. strains survived in modified clay granules, by placing them into soil in the glasshouse in a "nodulation bio-assay",
- To assess if *B.* spp. strains survived in modified clay granules, by placing them directly into a field environment, as an alternative nodulation bio-assay
- To determine if any *L. ambigua* symbionts exist naturally at this site (which would confound results) and,
- To determine if *L. ambigua* symbionts had persisted over three years in a similar soil at a different field site, as a guide to their saprophytic abilities.

3.2. Methods

3.2.1. Background information for the Corrigin and Brookton sites

Corrigin, Western Australia (32.465508 S, 117.676509 E) has an annual rainfall of 371mm and Brookton 445mm (Australian Government- Bureau of Meteorology). The soil types of both sites are known as "Christmas tree sands" and have a pH between 5.4 and 6.0 (Table 3.1). Both are the prospective sands for cultivation of *L. ambigua* (Howieson et al. 2013). The Corrigin site was chosen because of the nearby location of an existing *L. ambigua* experimental site, good July/August rainfall and excellent weed control. The soil at the particular site was white non-wetting sand transitioning to yellow sand at around 15cm.

Table 3.1: Soil parameters from two different depths at Corrigin and Brookton *.

	pH ($CaCl_2$)	Colwell P (mg/Kg)	Colwell K (mg/Kg)	Sulphur (mg/Kg)	Organic Carbon (%)	Conductivity dS/m	Exc. Aluminium Meq/100g
Corrigin 0-5cm	6.0	15	32	8.4	0.5	0.044	0.051
Corrigin 5-20cm	5.4	10	20	1.3	0.18	0.015	0.180
Brookton 0-5cm	6.1	25	28	8.0	0.6	0.052	0.033
Brookton 5-20cm	5.7	12	22	3.1	0.1	0.001	0.220

* *analysed by CSBP (Bibra Lake Laboratory)*

3.2.2. Soil trapping from Corrigin to assess presence of background *L. ambigua*-nodulating *B.* spp. (Experiment 1)

The soil from Corrigin with no known history of *L. ambigua* cultivation was collected from 0-5 cm by placing a pogo sampler sterilised by 70% ethanol into the ground at ten random positions along a 100m transect. Soil was removed from the pogo and placed into a sterile bag before snap frozen on dry ice and placed in an esky. Soils at the above sites collected at 5-15cm were taken with a coring pole and then repeating the process above to preserve possible rhizobia at this depth in the soil.

3.2.2.1. Layering method

Field soils were examined for possible symbionts using the layering *in situ* trapping method (Howieson et al. 2016). The layering method was amended as follows: Sterile sand medium (Yates et al. 2007) (50% yellow sand, 50% coarse river sand) was added to the pot (3.5L) at 60% of the pot volume and then the soil from Corrigin filled the pot to 80% (i.e. 20% extra). The potentially rhizobia rich material was then covered with a shallow layer of sterile soil and then moistened with sterile DI water. Seeds of *L. ambigua* were germinated as described (3.2.3.) and sown into the top sterile layer so the germinated radicle would grow through the trapped soil to allow nodulation. Finally, the seeds were covered with alkathene beads and a watering tube was inserted to aid in the sterile administration of water and nutrients, as in Yates et al. 2016a. After eight weeks, the plants were removed and examined for nodules and top dry weight (TDW).

3.2.3. *L. ambigua* seed preparation, sterilisation and germination

L. ambigua seed was scarified with an abrasion mechanism equipped with a diamond grinding stone (courtesy of Dr Neil Ballard- Global Pasture Consultants) to break the hard seed coat to allow maximum germination (Edwards 2015). *L. ambigua* seed was selected for uniformity (0.7-1mm long and yellow in colour) then surface-sterilised by immersion in ethanol (70%, 1min), transferred to hypochlorite (3%, 3 mins) and finally rinsed six times in sterile DI water before being placed on water agar and inverted (Howieson et al. 1995, Yates et al. 2016b). Plates were then left in aluminium foil (complete darkness) and at room temperature for three days.

3.2.4. Inoculation of *L. ambigua in situ* with modified clay granules containing *B.* spp. (Experiment 2)

3.2.4.1. Preparation for the Corrigin site

Due to the low productivity of the soil, tagasaste had previously been sown, yet deemed unsuccessful and removed. Prior to sowing *L. ambigua*, wheat (*Triticum sp*. cv. Mace) was grown as a cover crop and background subterranean clover (*Trifolium subterranean* cv. Geraldton, Dalkeith) had returned. The site was prepared by spraying a knock-down herbicide (Paraquat™ and Diquat™) at 1.6L/ha on the cover crop with an application of Talstar™ (Bifenthrin) at 50ml/ha to control insects. Pests such as rabbits and kangaroos were controlled by the farmer.

3.2.4.2. Field experiment

On September 13th 2016, 96 plots of *L. ambigua* (4 replications of 24 treatments in randomised blocks) were established at the Corrigin field site (Figure 3.1) using the block design described in Yates et al. (2016b) with a 2m buffer between blocks. Wooden pegs were placed 0.5 m spacing to represent the start of the line for each plot. Using a sterile rod, 5cm deep furrows were made 0.5 m long from each peg.

In the laboratory, sterile seeds of *L. ambigua* were placed together with 0.5g of a clay granule treatment in an envelope. The seeds and granules were then placed into the furrows by tapping the envelope along the furrow then covering with surrounding soil; care was taken between each treatment by washing hands in 70% ethanol.

Figure 3.1: Corrigin randomised block field experiment with surrounding wheat crop.

3.2.4.3. Data collection

Data was obtained on four separate occasions, with the first recording the number of plants emerged, nodules formed and top dry weights (TDW) of individual plants, the second inspecting number of plants, TDW and height, the third focused on counting the number of mature plants and measuring their height and finally the last measurement was estimating colour. Transitioning data collection from destructive sampling to observational methodology was necessary because of the inaccessibility of the nodules and difficulty of plant excavation as the soils dried. The first observation was six weeks after sowing (Figure 3.1), the second nine weeks after sowing, the third was 14 weeks and the final observation was 38 weeks post sowing.

For the first assessment twelve individual plants were removed from each plot using a small spade to excavate the entire root system. Roots were scored for any nodules (after washing the dirt off the roots) and then recorded as the number of plants with at least one nodule.

The nine-week data collection followed the method above, however only four plants were taken from each plot. If there were less than four plants, half of the plants from the plot were taken to allow for the next sample/observation (at 14 weeks). Finally, a measure of greenness was taken after 38 weeks. The greenness indication method was undertaken by focusing on the level of greenness in a plant, with brown ranked as a "1", and "5" being a healthy green (Figure 3.2). The reduction in the number of plants available for excavation was due to rabbit activity and the death of plants in some treatments. General analysis of variance using a 5% least significant difference (LSD) was calculated on the data sets using GenStat® 18 VSN (Release 18, Part 1 VSN International 2015, Hemel Hempstead, UK).

Table 3.2 shows the controls added to the randomised block design with the different granule treatments described in chapter 2. The sealed and unsealed envelopes were added to identify if *L. ambigua* still formed nodules after 18hrs of desiccation and if the sealing added to the survivability of rhizobia.

Figure 3.2: *L. ambigua* plant difference in colour. The plant on the left was rated 1. Plant on the top right was rated 4.5 and the bottom right plant was rated 3.5.

Table 3.2: Granule treatments and additives from Chapter 2 selected for Experiment 3.2 (a- dried at 28°C, b- dried at 25°C). Clay sourced by ALOSCA™ (A and E). Treatments 19-24 are the control treatments sown with peat instead of granules.

Treatment	Clay	Strain	DI water	Additives/methods
1a,b	A	WSM4184	750ml	Control used in Howieson et al. 2013
2a,b	A	WSM4184	750ml	PVA
3a,b	A	WSM4184	750ml	Citric acid
4a,b	A	WSM4184	750ml	PVA and citric acid
5a,b	E	WSM4184	1000ml	
6a,b	A+E	WSM4184	750ml	
7a,b	A	WSM4184	750ml	Double amount of peat
8a,b	A	WSM4204	750ml	
9a,b	A	WSM5005	750ml	
19	-	WSM4184	-	Inoculated 18h prior and sealed
20	-	WSM4184	-	Inoculated 18h prior and unsealed
21	-	WSM4204	-	Inoculated 18h prior and sealed
22	-	WSM4204	-	Inoculated 18h prior and unsealed
23	-	WSM4204	-	Fresh inoculated in field (common practice)
24	-	-	-	Uninoculated

3.2.5. Identifying nodules using MALDI ID

Nodules were collected at the first 2 sampling dates and their occupants cultured for analysis of bacterial species. Isolation of bacteria from the nodules followed the procedure outlined in Hungria et al (2016). Two nodules from each of the five treatments (four replicates) were sterilised by immersion in ethanol (70%) for one minute then sodium hypochlorite (4%) for three minutes then washed through six changes of sterile water. Following on from the last rinse, the nodule was crushed with sterile forceps and the milky liquid was dropped onto a plate YMA growth agar. Using a glass rod, the drop was aseptically streaked across the growth medium. The plate was inverted once liquid had settled and placed in a growth chamber (28°C). MALDI ID was provided the plates after two days and ribosomal protein markers acted as fingerprints for identifying species and strains of rhizobia (Ziegler et al. 2015).

3.2.6. Assessment of saprophytic competence of *L. ambigua*-nodulating *B.* spp. (Brookton) (Experiment 3)

Soils from Brookton (32.278452 S, 116.838136 E) were collected as described in 3.2.1. This experiment was established by CRS staff three years prior (2014) to sampling, as a "cross row" experiment to evaluate strains for persistence. *L. ambigua* plants were well established at the site, however some older plants had senesced. The sampling zone was located 5cm away from the base of the senesced plants and care was taken to avoid contamination between each strain by washing equipment in 70% ethanol between samples. Four replicates were taken from each cross-row plot and placed into a sterile zip lock bag. The bags were placed in a container with ice and transferred back to the glasshouse in the procedure outlined in 3.2.1. There were three sowings in Brookton, each

one-year apart, and three replicates were sampled for soil with three different sampling points around each plant/plot. *L. ambigua* seeds were planted in pots and followed the soil trapping experiment described in section 3.2.2. After the isolation procedure, *L. ambigua* plants that produced nodules were given to Dr Sofie De Meyer for identification by a 16S rDNA sequence.

3.3. Results

3.3.1. Soil trapping (Experiment 1)

None of the plants nodulated in the soil collected from the Corrigin site and assayed in the glasshouse. All plants were pale green and grew to the same height as the uninoculated plants. Small white nodules or bumps were found on the roots of two *L. ambigua* plants, but they were deemed ineffective pseudo-nodules.

3.3.2. Nodulation of *L. ambigua in-situ* after application of granular inoculants. (Experiment 2)

Granules sown alongside *L. ambigua* seed at the Corrigin field site did cause nodules (Table 3.3). The percentage of plants nodulated at the first sampling time indicated WSM4184 inoculated 18 hours before sowing and sealed from the air (treatment 19) had the most plants that nodulated. The uninoculated treatment (24) had no plants that produced nodules. The second sampling time was measured by cuts for TDW and not nodule measurements due to the drying soil and the possibility of nodules located deeper on the *L. ambigua* root system and any excavation could destroy the rest of the experiment.

Rabbit and kangaroos were observed on the area which could have changed the results from each sample. However, majority of the treatments after 38 weeks revealed a significant difference in colour over the uninoculated treatment.

Table 3.3: Data from each sampling time (weeks) of *L. ambigua* inoculated with different formulations of clay granules. Red and shaded indicates a significant difference from the uninoculated treatment (24).

	Sampling time			
Treatment	6 weeks % of plants nodulated	9 weeks Mean Biomass (g)	14 weeks Mean Height (mm)	38 weeks Mean greenness indicator
1a	0%	0.102	72.0	5.80
1b	8%	0.093	54.6	4.63
2a	0%	0.093	62.1	7.50
2b	25%	0.117	84.0	4.63
3a	8%	0.061	75.9	6.25
3b	0%	0.101	69.7	5.63
4a	0%	0.030	52.1	4.25
4b	50%	0.111	65.7	6.38
5a	25%	0.046	87.5	6.13
5b	0%	0.104	69	7.00
6a	0%	0.122	60.8	7.17
6b	0%	0.095	58.8	7.00
7a	42%	0.175	65.2	6.63
7b	17%	0.144	58.6	7.75
8a	17%	0.087	75.7	3.75
8b	0%	0.142	54.3	3.38
9a	25%	0.052	5.6	5.88
9b	33%	0.094	65.3	0.75
19	100%	0.103	63.5	7.50
20	0%	0.080	63.2	6.50
21	17%	0.052	28.3	7.13
22	25%	0.121	63.4	4.67
23	8%	0.120	59.8	5.00
24	0%	0.038	19.9	0.00
L.S.D. (5%)	**26%**	**0.078**	**38.58**	**3.318**

Treatment 7 consistently produced an increase in nodulation and growth parameters relative to the control, with significant increases at the 4 different sampling times. Treatment 5a produced the tallest plants after 14 weeks of growth, when most of the treatments were significantly different from the uninoculated plots (24). Treatment 8b was the only formulation that did not have a greenness indicator value significantly different to the uninoculated plots. Treatments 4b, 9b, 7a and 19 had a significant weight difference (data not shown) as well as percentage of plants nodulated compared to the uninoculated plot after six weeks.

3.3.2.1. *B.* spp. species strains identified from Corrigin field nodules

B. sprentiae occupied nodules that were harvested from the treatment plots that were administered with clay granules containing either WSM 4184 or 5005 (both *B. sprentiae*) based upon the MALDI ID assessment (Table 3.4). This provided strong evidence that there was no contamination between the plots. The rest of the nodules that were collected contained WSM 4204, which is consistent with the initial inoculant that was administered to that plot.

Table 3.4: MALDI-ID analysis of *L. ambigua* nodules after been sown with specific clay granules containing different *B*. spp.

Treatment	Strain/ species in clay granule	Species located by MALDI-ID
4b	*B. sprentiae* (WSM4184)	*B. sprentiae*
7a	*B. sprentiae* (WSM4184)	*B. sprentiae*
8b	*B. dilworthii* (WSM4204)	*B. dilworthii*
9b	*B. sprentiae* (WSM5005)	*B. sprentiae*
19	*B. sprentiae* (WSM4184)	*B. sprentiae*

3.3.3. Saprophytic competence (Brookton) (Experiment 3)

Of the 40 soil samples taken and used to trap inoculants, five yielded nodules from the 1st and 2nd sowings, and ten from the 3rd sowing. Colonies that appeared and were used for identification were white, smooth, round with entire margins, as described in the literature (De Meyer et al. 2013, De Meyer et al. 2014).

Table 3.5: Nodule occupants trapped from Brookton soil in the glasshouse (Experiment 2) and identified by 16S rDNA (Dr Sofie De Meyer).

Location and original strain	Identified nodule occupant
Brookton 1st sowing WSM4184 (0-5cm)	*Burkholderia dilworthii* WSM3556 (99.93%)
Brookton 1st sowing WSM4184 (5-10cm)	*Rhizobium anhuiense* CCBAU 23252 (99.79%)
Brookton 1st sowing WSM4204 (0-5cm)	*B. dilworthii* WSM3556 (100%)
Brookton 1st sowing WSM4204 (5-10cm)	*B. dilworthii* WSM3556 (100%)
Brookton 2nd sowing WSM4184 (0-5cm)	*B. graminis* C4D1M (99.72%)
Brookton 2nd sowing WSM4184 (0-5cm)	*B. sprentiae* WSM5005 (100%)
Brookton 2nd sowing WSM4204 (0-5cm)	*B. dilworthii* WSM3556 (100%)
Brookton 2nd sowing WSM4204 (0-5cm)	*R. anhuiense* CCBAU 23252 (99.79%)
Brookton 3rd sowing WSM4184 (0-5cm)	*B. sprentiae* WSM5005 (100%)
Brookton 3rd sowing WSM4184 (5-10cm)	*B. sprentiae* WSM5005 (100%)
Brookton 3rd sowing WSM4204 (0-5cm)	*B. dilworthii* WSM3556 (100%)
Brookton 3rd sowing WSM4204 (5-10cm)	*B. dilworthii* WSM3556 (100%)
Brookton 3rd sowing WSM4204 (5-10cm)	*B. graminis* C4D1M (99.72%)

B. spp. identified from the soils from Brookton is shown in Table 3.5. *B. dilworthii* and *B. sprentiae* were both inoculated in the initial experiment. However, *Rhizobium anhuiense* and *B. graminis* were not. The 16S rDNA sequencing indicated they were present in the nodules of *L. ambigua* grown in the glasshouse from soil sampled at the site. These are not known as *L. ambigua* nodulating species of bacteria.

3.4. Discussion

None of the granular treatments formulated as inoculant for *L. ambigua* and applied in the soil at Corrigin produced nodules on field grown *L. ambigua* for all of the plants sampled (i.e. non-yielded 100% nodulation). The best treatment was 7a, which produced 42% of plants nodulated. In contrast, the optimal peat inoculant (WSM4184) treatment (19) produced nodules on 100% of plants sampled. This provides strong evidence that although the clay granules as developed in this experiment produced some nodulated plants, they are not a suitable carrier of *B.* spp. as inoculants for *L. ambigua*. Treatment 7a (WSM4184, Clay A, double peat added) presented as the most successful clay granule modification across all sampling times, and this can possibly be attributed to double the amount of peat added during the clay granule formulation.

Under normal circumstances, inoculants carriers are formulated to produce nodulation on 100% of plants, and this is the standard sought by regulatory authorities (Deaker et al. 2016). Notwithstanding this, 10 of the 18 granular treatments produced nodules on at least some of the *L. ambigua* plants sampled, whilst the uninoculated controls did not, which strongly suggests that bentonite clay does carry some live *B.* spp.

Inoculants have previously been found to deliver inconsistent results (Denton et al. 2009) and this usually interpreted as other factors being present to impact nodulation such as salinity (Singleton and Bohlool 1984, Rao et al. 2002, Manchanda and Garg 2008), low pH, aluminium toxicity and other associated edaphic stresses (Hungria and Vargas 2000, Howieson and Ballard 2004) such as waterlogging, excessive heat, drought, and soil antibiosis (Diatloff 1970, Hungria and Franco 1993, Bordeleau and Prévost 1994). However, in this case, the peat controls delivered 100% of plants nodulated under the same edaphic circumstances as the granule treatments, so it is highly likely that extraneous factors did not contribute to the variable nodulation. Nevertheless, all but one treatment (8b) of clay granule formulation developed a significant difference in colour and height relative to the uninoculated plots after 38 weeks, primarily as all the plants in the uninoculated and 8b plots had deceased.

The data, overall, indicated a strong possibility that the formulations of clay granules were successful in carrying live *B.* spp. into the rhizosphere of *L. ambigua*, and it may be possible that nodules were dislodged from the root systems during the excavation procedure. Further, *L. ambigua* has a long tap root (Le Roux and Van Wyk 2007, Edwards 2015) and it is possible many of the nodules weren't formed at the surface, especially because the soil was completely dry. In this context, it has also been recorded that granular inoculants are more likely to produce lateral root nodulation in legumes rather than crown nodulation (Kyei-Boahen et al. 2002, Valverde and Wall 2002, Remmler et al. 2014). So, as the soil became dry (leading into summer) the *B.* spp. cells could have followed the declining soil moisture to depth, to prevent desiccation, and nodulated along the lateral or tap roots and been difficult to recover.

Results from Brookton indicated that *B.* spp. persisted well in the soil over three years. This was achieved in some plots without live *L. ambigua* present. This information allows us to confidently pursue the development of new systems to manage *L. ambigua,* such as a self-regenerating perennial ley-system, once the hard seed impermeabilty and germination dynamics are better understood.

The field nodulation data has presented a conundrum. Neither the trapping experiments, nor the counting methodologies, have been able to provide any evidence that the granules hold live *B.* spp. Yet, plants nodulated in the field and the MALDI-ID seems to have confirmed that the nodule occupants from these plants were highly correlated with the inoculant strains. Further methods of quantification of *B.* spp*.* are explored in the next chapter.

Chapter 4: Establishing the viable cell number of *Burkholderia* spp. in clay by plant infection

4.1. Introduction:

Clay granules made from bentonite have emerged as a useful carrier material for inoculant rhizobia, particularly in the genera *Sinorhizobium* and *Rhizobium* (Hackney 2017). Clay granules with varying formulations were prepared as a carrier for *Burkholderia* spp. to inoculate *L. ambigua* in the field, producing inconsistent results which were somewhat in conflict with (Howieson et al. 2013). Whilst this was a positive outcome, the level of colony forming units (CFUs) held in the granules is still unknown.

There are few ways of counting rhizobia in non-sterile media, either in laboratory or under glasshouse conditions. Cells in sterile carriers can be diluted in the laboratory and plated on growth media in the traditional dilution counting methods described by O'Hara et al (2016). For non-sterile conditions the Most Probable Number (MPN) method offers an indirect means of counting based upon plant-infection (Brockwell 1963, Woomer et al. 1988). This plant infection technique is traditionally undertaken by serially diluting soil containing an unknown quantity of rhizobia (Brockwell 1963, Weaver and Frederick 1972) rather than clay granules.

MPNs are based on the probability of the presence or absence of the rhizobia in the medium to be diluted (Woomer et al. 1988). It relies on two assumptions: organisms are distributed randomly in the medium and one or more rhizobia are capable of causing

nodules (Woomer et al. 1988). However, as new carriers and new genera of rhizobia are becoming available, techniques must be constantly revised.

4.1.1. Aims

The aims of this chapter were to:

- Quantify the numbers of *B.* spp. in the clay granules using a plant infection technique, and
- Establish if any *B.* spp. were alive in the clay granules but didn't satisfy the assumptions of the MPN plant infection technique.

4.2. Methods

4.2.1. Enumeration of rhizobial cells in bentonite by the MPN method.

Bentonite clay carrying two species of *Burkholderia*- (WSM) 4184, 5005 (*B. sprentiae*) and WSM4204 (*B. dilworthii*) were serially diluted then applied to *L. ambigua* seedlings growing in pasteurised soil in sterilised polythene pots.

4.2.1.1 Serial dilution of bentonite

One gram of bentonite clay was randomly selected from the bulk product used in previous experiments (Chapters 2 and 3) and diluted in 10 ml of sterile DI water. This was then shaken for 30 minutes on a wrist action shaker set at medium speed (five shakes per second). Using a disposable tip with its end removed (to ensure it was not blocked by clay particles), 1 ml was removed from the 1st dilution and placed into 9 ml of DI water. This was vortex-mixed for 30 seconds before the dilution step was repeated. The original bentonite sample was diluted in this manner five times, and then repeated for the next strain.

L. ambigua seed was selected for uniformity, sterilised and germinated as previously described (section 3.2.3). Four seeds per pot were planted before *L. ambigua* seedlings were inoculated with the dispersed clay liquid from the dilution series described above. The soil was then covered with sterilised polyalkathene beads. Perspex shields were placed over the pots to provide further protection from contamination. These "MPN pots" were placed in a naturally lit phytotron glasshouse for five weeks with the temperature maintained at 22°C during the day. This experimental system prevents air and water borne root nodule bacteria entering the experiment (Howieson et al. 2013, O'Hara et al. 2016). Blocks of pots were arranged in a completely randomised design after seedling emergence, and moistened with sterile DI water every second day, with 5ml of nutrient solution added once per week (Howieson et al. 1995). Liquids were transferred into the pot via a sterilised watering tube positioned under the soil. After five weeks the plants in each treatment and every dilution were inspected for the presence or absence of nodules. The number of positive dilutions was entered into the MPN tables provided by O'Hara et al (2016) and Woomer (1994) and the number of cells in the clay was then calculated.

4.2.2. Detecting *B.* spp. embedded in clay by a modified trapping method

A modified method of detecting the presence of *B.* spp. in clay granules was developed to research the possibility that live cells were embedded in the clay, but could not be removed from the clay in a viable state during the MPN dilutions. This was a modification of the layering technique described in Chapter 3. Pots were filled to 80% capacity with a pasteurised sand mixture (steamed 50% yellow sand and 50% coarse river sand) and then placed in the glasshouse. A flush of boiling sterilised DI water was then poured through all

the pots. Once pots had cooled, the clay granules from treatments 1, 4, 7, 8 (Table 3.1) were added to the surface of the pots.

The granules were then incorporated into the top 5cm of the pot by using a sterile fork to scratch the granules into the sterilised sand and mix thoroughly. Care was taken to sterilise the fork with 70% ethanol in between each pot to stop the chance of contamination. This incorporation method was adopted so the clay didn't act as a collar that could have affected the germinating seed when moistened. Fresh, sterile sand mix was then layered over the granules. An inoculated control treatment (WSM4204 as a broth culture) and an uninoculated control (sterile DI water) were included in the design.

Granule treatments 4, 7 and 8 were chosen as they were thought to have the highest probability of causing nodules based on results from Chapter 3. Treatment 1 was also included because it is the exact formulation used by Howieson et al. (2013). All treatments chosen were those dried at 25°C (b). There were two different amounts of clay granules incorporated into each pot; 0.1g and 1g, to increase the sample size of the clay granules. *L. ambigua* seed was surface sterilised, germinated and sown following the same procedure as section 4, above. Four seeds were planted per pot and sterile polyalkathene beads were placed on top to prevent contamination.

Nine weeks after sowing the *L. ambigua,* the plants were harvested (Figure 4.1). Pots were carefully shaken out to catch the full root system of the *L. ambigua* plant and to avoid losing any nodules. Nodules were counted and the top of the plant cut at the transition zone from green to white to separate the roots from the tops to assess dry weight.

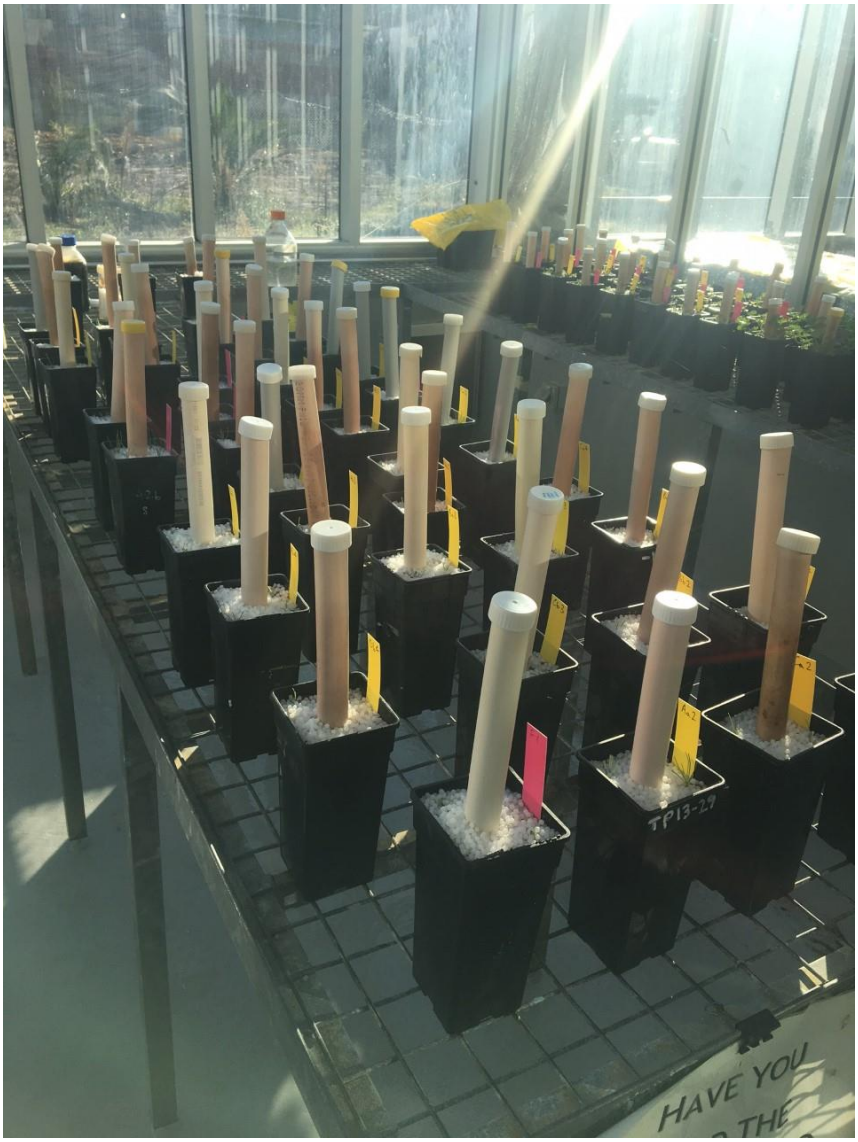

Figure 4.1: Modified clay granule enumeration experiment as arranged in the glasshouse (4.2.2.).

4.3. Results

4.3.1. Enumeration of *B.* spp. cells in bentonite by the MPN method.

L. ambigua did not form any nodules with the clay granule dilutions that were administered following the MPN dilution methodology. However, varying levels of greenness of *L. ambigua* were observed in all the treatments, similar to the plants in Figure 4.2.

Figure 4.2: MPN of *L. ambigua* inoculated with five dilutions of clay granule treatment 1a. Dilutions increased from left to right.

4.3.2. Detecting *B.* spp. embedded in clay by a modified trapping method

L. ambigua seedlings sown into the granules mixed through the top few cm of sand did not form any nodules with any of the treatments (data not shown). When the roots were washed carefully from the pots there were no clay granules recovered, which indicated the clay had been broken down and disseminated through the soil. The plants that were recovered from this experiment were all pale and yellow, except the N+ control, compared to the healthy green plants described in 4.3.1.

Figure 4.3*: L. ambigua* plants from the modified clay granule experiment (section 4.2.2.). No nodulation was observed in treatment F (left) compared to the inoculated control (E) on the right. Pink tags are 12.5cm long.

The TDW of plants in the granular treatments is shown in Figure 4.4 relative to the peat inoculant control WSM 4184 (treatment F, red bar) and the uninoculated (negative) control. The negative control didn't nodulate. Granular treatments did not produce a greater TDW than the uninoculated control. There was no difference between the two quantities of clay granules (0.1g and 1g): neither produced nodulation.

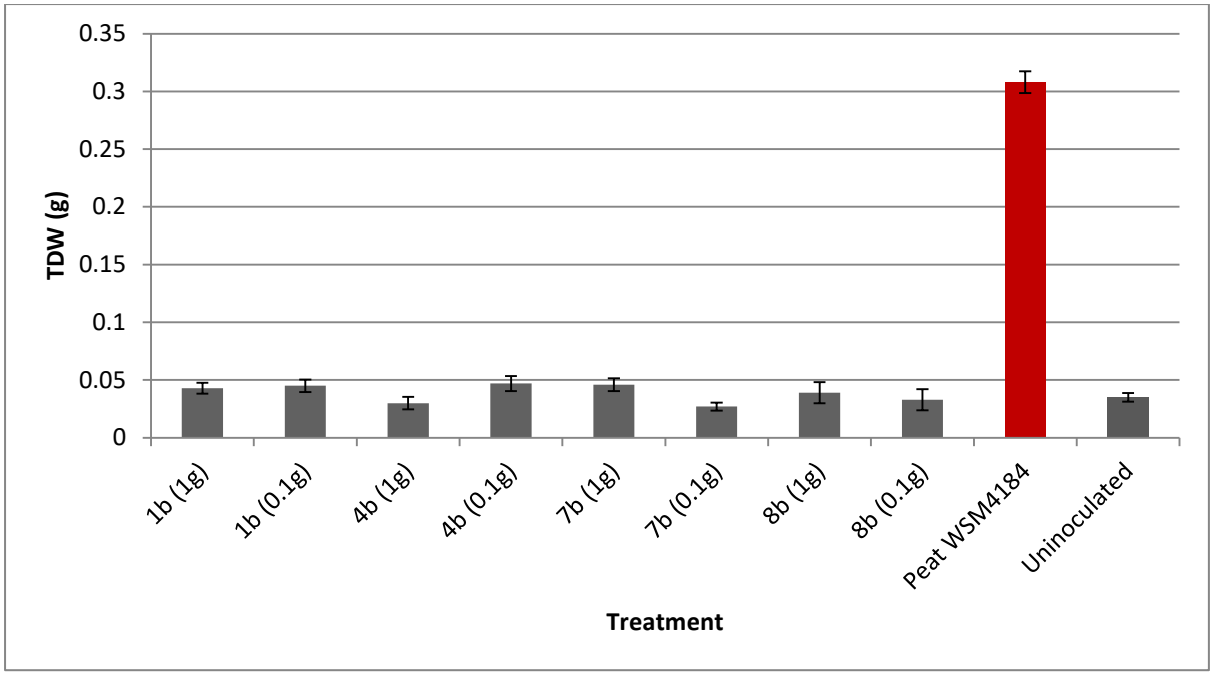

Figure 4.4: Top dry weights (TDW) of *L. ambigua* from the modified clay granule experiment (4.2.2.). Error bars indicate the standard error of the means. Peat WSM4184 inoculated control (red bar) was the only treatment with nodules.

4.4. Discussion

B. spp. capable of forming nodules on *L. ambigua* were undetected in either the MPN or the modified trapping experiment. MPN experiments sometimes produce inconsistent data if the assumptions aren't met (Woomer et al. 1988). The assumption that all organisms are randomly distributed (and equally dilutable) may not be met where *B.* spp. are housed in, and extracted from, a clay matrix because the cells may be tightly bound to clay particles (Bakken and Lindahl 1995). It is also possible the clay matrix could have coated the cells and prevented them from being dissolved randomly through the dilution series (Bushby and Marshall 1977a, Salema et al. 1982, Deaker et al. 2007). A different explanation for the lack of nodulation could be that there was significant osmotic shock during the rehydration of rhizobial cells from the (dry) clay granules into the diluent.

This chapter introduced a new technique for inducing plant infection with *B.* spp. held in clay granules. It was expected that "passive" movement from the granules to the soil would allow *B.* spp. to enter the rhizosphere of *L. ambigua* to form nodules, thereby replicating the field environment. However, this process failed to release live and infecting *B.* spp. from the clay as judged by complete lack of nodulation.

Clay granules are recommended to be sown at 10-15kg per hectare (Carr et al. 2006). The clay granules that were applied to *L. ambigua* in the modified glasshouse clay granule trapping experiment (section 4.2.2.) were sown at an effective rate of 100kg/ha for 0.1g and 1000kg/ha for 1g of clay granules, based on the surface area of the pots. This is well in excess of recommended rate. The clay granules used in this experiment were five months old, so it is possible *B.* spp. in the clay may have already perished, although the shelf life of commercial formulations is six months (Carr et al. 2006).

Given that *L. ambigua* did not produce any nodules after applying the MPN dilution method, the varying level of greenness seen could be accredited to some nutrition from the diluted clay granules that were administered to the small (4.5 cm^2) pot (used in the MPN experiment), compared to the large (8cm^2) pot (used in the modified trapping experiment). However, the varying level of greenness should have decreased as the dilution increased, which it did not. This suggests that there may have been a toxicity in the clay granules that was diluted out, causing the greenness to increase with dilution. This toxicity could have been aluminium tri-oxide (Al_2O_3), (Ritchie 1989) which is part of the clay granules (2-5%). Al_2O_3 is known to be detrimental to plant growth, and can inhibit phosphate uptake, cause calcium deficiency as well as reduced root elongation (Munns 1965). Plants may be affected at only 20µM Al in a soil solution (Carr et al. 1991) which is present at up to 5% (50,000

ppm) in the bentonite. Against this, the clay granules administered in this experiment were alkaline (7-8.5), which would not normally allow Al_2O_3 to become soluble. However it is possible that Al_2O_3 became soluble after it was placed into the acid soil medium (Carr et al. 1991) as used in the experiments. So, it is possible to hypothesise that the initial dilutions placed on the seed of *L. ambigua* contained a high amount of Al_2O_3 a fraction of which solubilised when in the acid environment, but which was diluted through the MPN method.

A further possible reason why *L. ambigua* did not cause nodules in the MPN experiment could have been the timing of harvest. Plants were harvested when peat inoculated seedlings (sown as sacrificial plants for sampling) had nodulated. Nodules do appear earlier from peat slurry inoculation than in-furrow clay granule inoculation (Yates 2017). More research needs to focus on the period of time for *L. ambigua*, and other legumes, to nodulate under glasshouse conditions when inoculated with dry granules.

In summary, *B.* spp. did survive in clay granules as evidenced by nodulation at Corrigin; however, they did not produce nodules subsequent to the Corrigin field work. Granular inoculants manufactured by ALOSCA™ report the shelf life to be six months (Carr et al. 2006) and since these experiments were undertaken five months after granules being manufactured, it is possible that all the *B.* spp. cells housed in the granules had either deceased or were not liberated sufficiently to cause nodulation in the dilution systems employed.

Chapter 5: Evaluation of *Burkholderia* spp. strains for tolerance to desiccation *in-vivo*

5.1. Introduction

Clay granules appear to carry viable *Burkholderia* spp. cells, however their efficacy and enumeration has been problematic. Because this may have been related to intolerance to desiccation, a wider group of strains, including several recovered from some of the original inoculation sites around WA, were examined.

5.1.1. Aims

The aim of this chapter was to seek evidence of variation in desiccation tolerance in *L. ambigua* nodulating strains of *B.* spp.

5.2. Methods

Strains assessed and their origins are shown in Table 5.1. The reisolates from WA soils were recovered through trapping from soil during 2015/16 (De Meyer 2016).

Table 5.1: *B.* spp., their origin and sowing year introduced into WA soils.

Strain ID	Species	Origin	Sown in WA
309	*Burkholderia dilworthii*	Brookton, WA	2014
346	*B. sprentiae*	Gillingarra, WA	2010
WSM4184	*B. sprentiae*	Klawer, RSA	-
WSM4204	*B. dilworthii*	Modder, RSA	-
WSM4629	*B. graminis*	Tincurrin, WA	2014
WSM4630	*B. sprentiae*	Murdoch University, WA	2004
WSM5005	*B. sprentiae*	Klawer, RSA	-

Key: WA= Western Australia, RSA= South Africa

5.2.1. Adherence of strains to polyethylene beads

B. spp. strains were prepared in peats as previously described (Ch. 2 section 2.2.3) and adhered to the surface of polyethylene beads (3mm diameter) in a method modified from Howieson et al. (2013). Sterile screw-topped polycarbonate vials (SPV) (50ml) were prepared containing 10g of autoclaved polyethylene beads. 0.1g of peat was weighed on scales within the sterile laminar flow cabinet and placed into the SPV with 1ml of SeedStikA adherent (ALOSCA™). The lid was placed on the SPV and then shaken until there was an even coating of peat on the beads. The lid was then removed so the sterile air from the laminar flow could enter the jar (Figure 5.1).

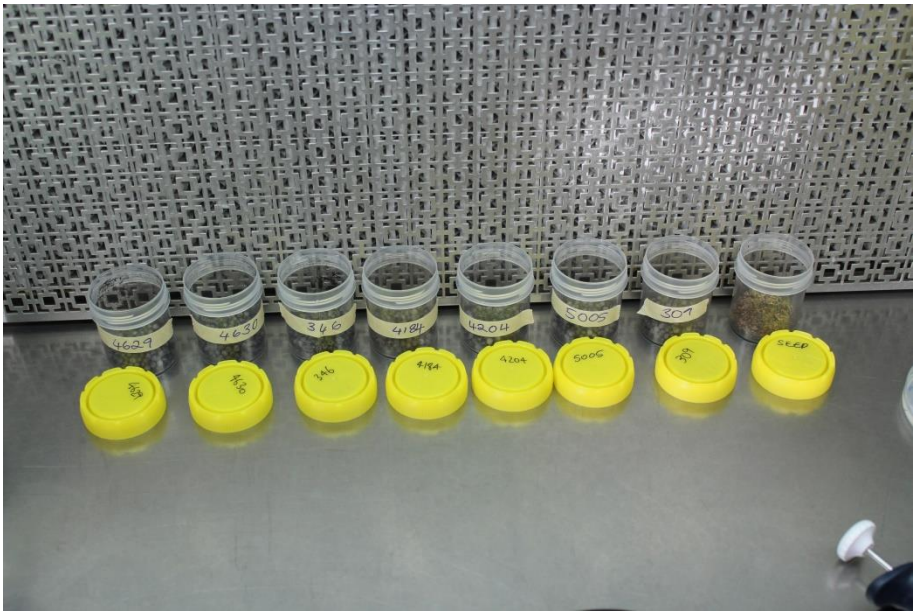

Figure 5.1: Peat containing *B*. spp. strains inoculated onto sterile polyethylene beads with SeedStikA™ (commercial polymeric sticker) in a SPV and left to dry under sterile air in a laminar flow.

After 30 mins, 1g of peat coated beads was removed from each SPV and placed into 9ml of saline solution (0.89%) in a second sterile SPV, then placed onto the wrist shaker for 10 mins. Ten µl of the suspended liquid was then taken from the jar and serially diluted seven times in an epindorph tube (containing 100µl of saline solution) with each tube shaken on a vortex mixer between each dilution to keep the cells in suspention. Two plates of YMA (Appendix A) were prepared for each strain with lines drawn on the bottom to indicate sections (dilution 1-7). Two drops (20µl) were placed into each dilution section with a sterile pipette. All plates were then wrapped in parafilm once the solutions had been absorbed into the agar, inverted and placed in the incubator for two days.This process was repeated after 300 mins (5 hrs), and then again after 1440 mins (24 hrs).

General analysis of variance using a 5% least significant difference (LSD) was calculated on the data sets using GenStat® 18 VSN (Release 18, Part 1 VSN International 2015, Hemel Hempstead, UK).

5.3. Results

B. spp. strains differed in the rate at which they died after adherence to the beads (Figure 5.2). *B. sprentiae* strain 309 survived poorly compared to other strains of *B.* spp. with a rapid and continuous drop off in cell number from the first count, ending with 0.0004% of cells alive after 1440 mins. However, strains 4629 (*B. graminis*) and 346 (*B. sprentiae*) died more slowly than the other strains, with only approximately 40% loss of viable cells after 300 mins, and approximately 0.5% and 0.24% respectively still alive after 1440 mins. After 1440mins there were 625,000 and 300,000 cells alive respectively, compared with only a few cells of 309 recovered on the beads.

As a group, the *B. sprentiae* strains followed a similar exponential decay curve, except for strain 346, which had a slower death rate, very similar to *B. graminis*. As a group the *B. dilworthii* species exhibited contrasting behaviour - the death rate of WSM 4204 appeared to slow between 30mins and 300mins, whereas 309 exhibited a steady decay.

The performance of all *B.* spp. strains differed markedly to the controls from Howieson et al. (2013) (RRI128, WSM471), which still had 1.3% and 12.5% of cells alive after 1440mins (Figure 5.2). The best *B.* spp., strain WSM 4629, had 0.05% of cells still alive after this elapsed time.

There was no significant difference ($p>0.05$) between *B.* spp. strains in the initial counts on the peats (data not presented), whereas differences between strains at subsequent sampling times were significant ($P<0.001$).

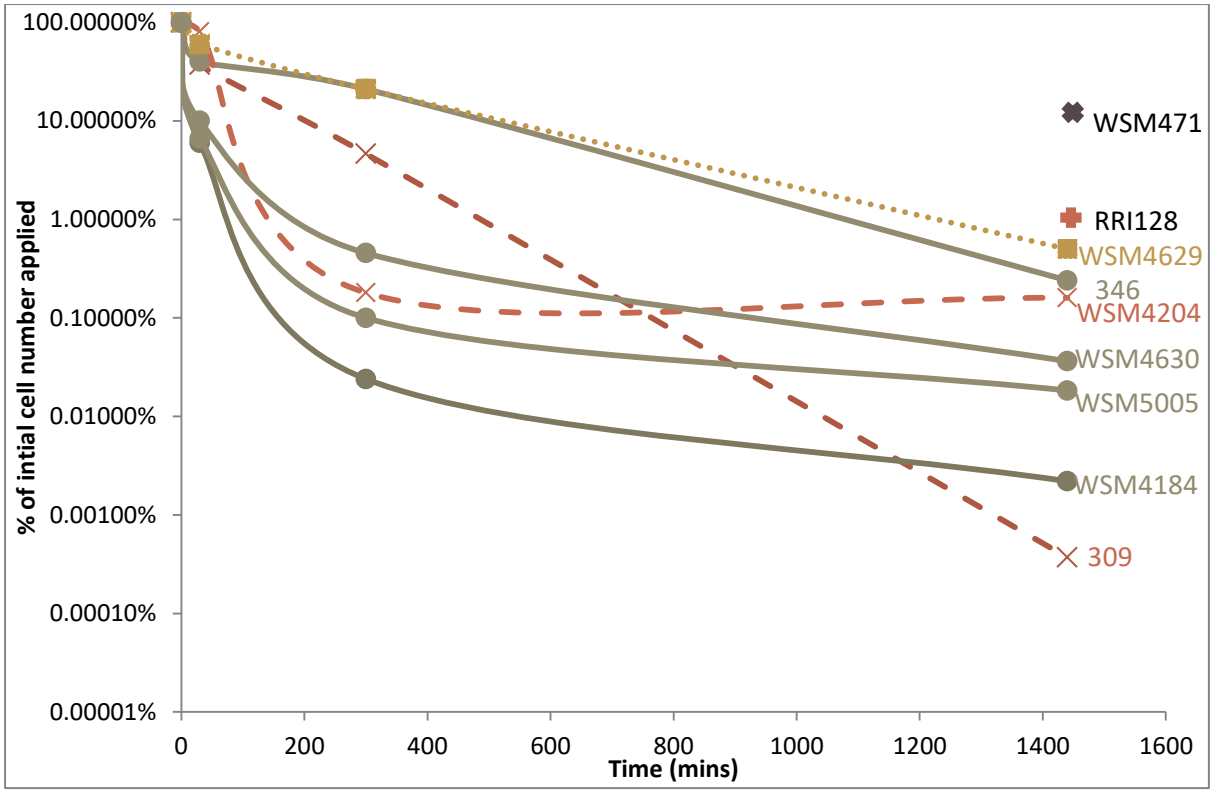

Figure 5.2: Death of *B.* spp. strain cells over time represented as a proportion of the initial peat numbers applied to beads. The grey lines represent *B. sprentiae*, the yellow dotted line represents *B. graminis* and the red dashed lines represent *B. dilworthii*. The x and + signs represent controls (WSM471 and RRI128) from Howieson et al. (2013) after 1440mins.

5.4. Discussion

There are several attributes considered to be essential when selecting an inoculant strain. These include manufacturability, saprophytic competence and high levels of nitrogen fixation (Roughley 1970, Halliday 1984, Deaker et al. 2016). Very few authors have reported on the characteristic of strain survival in a peat carrier after removal from storage. The data presented here and previously (Howieson et al. 2013) strongly indicate that when dealing with *B.* spp. this characteristic must be added to the essential selection criteria. This is because, farmers will generally inoculate *L. ambigua* in a window of 5- 24 hrs before

seeding, by which time with current technologies most strains will have less than 1% of cells alive (which will likely cause nodulation failure).

It has previously been highlighted that the *B*. spp. are rhizobia that die more rapidly when compared to the current alpha rhizobia inoculants. For example, the current inoculants for serradella, WSM471 (*Bradyrhizobium* sp.) and lucerne RRI128 (*Sinorhizobium meliloti*) had above 1% cell survival after 24 hrs (Howieson et al. 2013). However, this current work has demonstrated that there may be important differences between *B.* spp in this characteristic. The seven strains investigated in respect to tolerance to desiccation, measured in terms of cell death over time, varied significantly. WSM4629 and 346 proved to tolerate desiccation stress better than the other *Burkholderia* spp. strains examined in this chapter; in particular, WSM4629 exhibited a significant difference to other strains in the desiccation experiment across every sample time.

Unfortunately, WSM4629 has been identified as *B. graminis*, and is incapable of fixing nitrogen optimally with *L. ambigua* (Appendix B and Garau et al. (2009). Furthermore, the best surviving *B*. spp. in the work of Howieson et al. (2013) were WSM3937 which nodulates *Rhynchosia ferulifolia*, and STM815 which nodulates *Mimosa* spp. Yet, none of these strains effectively fix N after nodulating *L. ambigua*, so it may be that the *B.* spp. isolated from this legume are inherently intolerant of desiccation when in a peat carrier.

This experiment has identified *B.* spp. strains that survive better than WSM4184, which was used in field experiments between 2012 and 2015. In this experiment, WSM4184 was one of the least resilient *B.* spp. strains in contrast to Howieson et al. (2013) where it was one of the best. However, WSM4184 tolerated desiccation better in Howieson et al. (2013) than in this experiment (5h=0.13%, 24h=0.016% surviving cells in Howieson et al. (2013) compared

with 5h=0.024%, 24h=0.002%). Slight differences in protocol may explain this variation, however, it needs further examination, perhaps best undertaken in a comprehensive screening of all available high N fixing strains.

The considerable variation between the seven *B.* spp. strains investigated in the present work indicates a strong potential for identifying an elite strain which has the ability to tolerate desiccation. This should coincide with research on saprophytic competence and maintaining genetic stability.

General discussion

L. ambigua, a suffrutescent legume, and its rhizobia *B.* spp*.,* have been selected from the fynbos region of South Africa, an environment similar to the sands of the Wheatbelt of WA (Howieson et al. 2013). It is likely that this combination of legume and inoculant can improve the profitability of farming the acid, infertile sands common to south west WA.

Due to its germination and growth patterns, *L. ambigua* is best sown in spring, when the ground is drying. This drying is deleterious for rhizobial survival as a peat-based inoculant, so clay granules (which keep bacteria alive in dry environments) were investigated as an option for developing an inoculation carrier. *B.* spp*.* were previously shown to survive poorly in non-sterile clay granules (Howieson et al. 2013), however in this study the granule formulation was amended in an attempt to increase the survival of *B.* spp. An antibiotic profile was established to overcome the contamination encountered when counting *B.* spp*.* in a non-sterile environment. Chloramphenicol at 20µg/ml was the most appropriate antibiotic examined and was used to reduce contamination after the clay granules were diluted and streaked onto bacterial growth media. However, no *B.* spp*.* were recovered from the clay granules using this approach.

Manipulation of the components of the clay granules, such as the addition of extra peat and protective polymers during the manufacturing phase, produced nodulation in field sown *L. ambigua*, although the results were variable. On average, less than 50% of plants were nodulated, a level too low to be considered successful (Deaker et al. 2016). This was in contrast to peat inoculants producing 100% of plants nodulated. In trying to elucidate the cause of this variability attempts were made to enumerate the number of live cells in the clay granules. Standard enumeration techniques such as MPN (Woomer 1994) were

followed, however they failed to provide quantification of live cells, or even evidence of any live cells in five month-aged inoculants.

Closer inspection of the content of the clay granules indicated a high amount of bound Al_2O_3 (2-5%) which can be extremely toxic to plants (Carr et al. 1991) and more specifically legumes (Munns 1965). Around 20µM of Al_2O_3 can inhibit root growth and cause deficiencies of phosphorus and calcium (Munns 1965, Carr et al. 1991). Root trimming and elongation can also be a negative side effect of Al_2O_3 (Munns 1965) which could explain the reason why *L. ambigua* failed to nodulate and grow in the glasshouse conditions.

A broader range of *B.* spp. strains from South Africa and WA field sites were then examined in respect to tolerance of desiccation stress. Large variability, in terms of cell death, between strains of *B.* spp. was identified, which indicates more strains should be collected and examined before formulating an inoculant. However, comparing the survival of these *B.* spp. strains relative to the control rhizobia reported in Howieson et al (2013), highlighted *B.* spp. as universally intolerant of desiccation. This indicates the ongoing challenges of working with *B.* spp. especially in an agricultural inoculation context.

This intolerance to desiccation in *L. ambigua* nodulating *B.* spp. is incongruous when considering that the *B.* spp. strains in this study were collected from soils that are seasonally dry, prone to fire and high in sand content. These edaphic conditions would suggest that the bacteria under study are regularly exposed to desiccation in their natural environment (Vanderlinde et al. 2010, Howieson et al. 2013). This highlights the unforeseen challenges of rhizobial domestication.

It appears that the strains which nodulate *L. ambigua* effectively are poorer than other *B.* spp. in tolerance to desiccation. To consolidate this evidence, further research should hence focus on a wider collection of new *B.* spp. strains from *L. ambigua* along with studies of saprophytic competence, nitrogen fixation and genetic stability. Once these factors have been addressed the limitations of inoculating *L. ambigua* should be overcome. Incorporating *L. ambigua* into a ley-farming system will be successful only if the inoculant bacteria is able to be delivered alive and in high numbers.

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Appendix

A

Yeast mannitol agar

Component	Quantity/L
K_2HPO_4	0.5g
$MgSO_4.7H_2O$	0.2g
NaCl	0.1g
Mannitol	5g
Yeast extract	0.4g
DI water to complete 1L	
Agar	12-15g

Autoclaved at 121°C for 15mins

B

Effectiveness of *Burkholderia* spp. on *L. ambigua* host, error bars represent standard error of the means and controls are represented as uninoculated and added N. Different species are presented in different colours.