Novel *Burkholderia* bacteria isolated from *Lebeckia ambigua* - A perennial suffrutescent legume of the fynbos

John G. Howieson[a], Sofie E. De Meyer[a,] Anabel Vivas-Marfisi[a], Sunil Ratnayake[b], Julie K. Ardley[a], Ron J. Yates[a, c]

[a] Centre for Rhizobium Studies, Murdoch University, Murdoch WA 6150, Australia

[b] School of Botany, University of Melbourne, Victoria 3010, Australia

[c] Department of Agriculture Western Australia, Baron Hay Court, South Perth WA 6151, Australia

Abstract

We investigated symbiotic and physiological properties, and taxonomic position, of 23 bacterial strains isolated from *Lebeckia ambigua* root nodules collected from the South African fynbos region. The capacity for nodulation and nitrogen fixation on three provenances of *L. ambigua* was investigated for these strains together with several physiological characters, including growth rate on peat and in betonite clay, survival on polyethylene beads and pH tolerance. Additionally, the 16S rRNA gene phylogeny was determined. The root nodule bacteria isolated clustered in five different groups belonging to the genus *Burkholderia*, most closely related to *B. caledonica*, *B. graminis* and *B. tuberum*. Moreover there was a very strong influence of collection site on the taxonomy of

the *Burkholderia* strains. The physiological characterisation revealed two promising strains, WSM4174 and WSM4184, achieved rapid growth in normal media and reached high, stable numbers in sterile peat. However, there was a worrying susceptibility to desiccation amongst these*Burkholderia*. Additionally, evidence was found for isolation of non-symbiotic strains from the nodule material collected in South Africa.

Keywords: Betaproteobacteria; *Burkholderia*; *Lebeckia*; South Africa; Fynbos; Nodulation; Symbiosis; Nitrogen fixation

1. Introduction

Australian agriculture has relied upon N_2-fixation from rhizobia, as symbionts primarily of annual legumes, since European colonisation in the late 18thC. This N_2-fixation is currently estimated to be worth in excess of $2 billion per annum (Herridge et al., 2008). The south-west of Western Australia is a major agricultural region whose rainfall patterns appear to be changing, from a dry Mediterranean-type distribution to a generally reduced annual rainfall with a less predictable distribution (George et al., 2008). This change in climate mitigates against optimal growth from annual legumes because of a disruption to their pattern of germination and reproduction. Alternative perennial legume species are therefore being sought (Howieson et al., 2008). The target regions for our research include some 5 million ha in south-west WA receiving 250‑500 mm annual rainfall, of which a portion have already experienced a decline in precipitation of 10% between 1976 and 1999, followed by 15% in the subsequent seven years (George et al., 2008), with the driest winter for 100 years

recorded in 2010

(http://www.bom.gov.au/climate/current/season/wa/archive/201008.summary.shtml).

Lucerne (*Medicago sativa* L.) will not persist on many of our target soils, which are acid and infertile, and there is a paucity of well-adapted commercially available perennial, herbaceous forage legumes in regions which receive less than 500 mm annual rainfall (Howieson et al., 2008). As part of the search for legumes better adapted to these soils, we are attempting to domesticate species from southern Africa, including those from the acid, sandy soils of the fynbos biome in the Western Cape region of South Africa. We have collected seed and nodules of several genera of deep-rooted, suffrutescent, perennial legumes (e.g.*Rhynchosia*, *Lebeckia* and *Lessertia*), which although known to botanists (Boatwright et al., 2009; Van Wyk, 1991), are mostly new to agriculture. Our approach has focussed on assessing the nodule bacteria of these potential new agricultural legumes (Ardley et al., 2012; Garau et al., 2009; Gerding et al., 2012; Yates et al., 2007) because poorly adapted inoculants have historically restricted the success of new forage legumes introduced to WA soils (Parker, 1962). Several of the perennial legumes from southern Africa are nodulated by the relatively novel species of *Microvirga* (Ardley et al., 2012) or by Betaproteobacterial*Burkholderia* spp. (Garau et al., 2009; Phalane, 2008).

The Betaproteobacteria are a class of bacteria first reported to contain root nodule bacteria by Moulin et al. (2001). These are mainly from the genus *Burkholderia*, which are the dominant nodule occupants of species of the large sub-tropical genus *Mimosa* found in the Cerrado and the Caatinga (Bontemps et al., 2010; dos Reis et al., 2010).
Rhizobial *Burkholderia* have also been found in nodules of the papilionoid

legumes *Cyclopia* and the herbaceous, perennial legume *Rhynchosia ferulifolia*, both endemic to the western Cape of South Africa (Elliott et al., 2007a; Garau et al., 2009). In this manuscript we describe additional Betaproteobacteria nodule bacteria from the wild perennial fynbos legume *Lebeckia ambigua*, which we reveal are novel species of *Burkholderia*. Because rhizobial *Burkholderia* are increasingly being isolated from fynbos legumes, we raise the possibility of *Burkholderia* being nodule bacteria particularly well adapted to infertile sandy, acid soils.

2. Materials and methods

2.1. Acquisition of root-nodules, bacteria and host legumes

Four separate germplasm acquisition expeditions were made to the Western Cape of South Africa, between the latitudes of Cape Town and Springbok, from 2002 to 2007. Nodules and seed (where available) of herbaceous perennial legumes were collected and stored as previously described (Yates et al., 2004). Nodules from *L. ambigua* were collected from five sites (Table 1, Fig. S1) and returned to Australia for isolation. Bacteria were isolated from surface sterilised root nodules according to the procedure described by Yates et al. (2007). Pure cultures were deposited in the WSM Genebank (Centre for *Rhizobium* Studies, Murdoch University, Perth, Western Australia) after authentication. Seed of four representative provenances of *L. ambigua* were multiplied in the field plots at Murdoch University to provide seed for nodulation and effectiveness experiments described in this manuscript. Seed of the related legume *Lebeckia sepiaria* was provided by J.S. Boatwright, Kirstenbosch Botanical Gardens, Cape Town. The legumes used in the glasshouse experiments are listed in Table S1.

2.2.Authentication of nodule isolates (Experiment 1)

Two studies of nodulation of *L. ambigua* were performed using the axenic sand-culture system described by Howieson et al. (1995). The experiments were conducted in pasteurised soil in sterilised polythene pots, covered with sterilised alkathene beads, held in a naturally lit phytotron maintained at 22 ° C during the day. This system of evaluation excludes air- and water-borne nodule bacteria from contaminating experiments.

In the first experiment, 23 strains of nodule bacteria listed in Table 1 were assessed for their capacity to nodulate three provenances of *L. ambigua* (CRSLAM-37, 39, 41, see Table S2). Three seedlings, representing each provenance, were placed in a 1 kg pot then inoculated with a single test strain as described by Yates et al. (2007). All treatments, plus uninoculated and nitrogen-supplied controls, were replicated four times in this split-plot design, and pots arranged in completely randomised blocks. The split-plot design allows the inclusion of more than one plant genotype in each pot which contains a single inoculant (Howieson et al., 1995; Yates et al., 2007). Sterile DI water was provided every second day and 20 ml of nutrient solution (Howieson et al., 1995) weekly through a sterilised watering tube inserted below the soil surface. After 56 days the plants were carefully removed, the roots washed free of soil and the presence of nodules recorded. Shoots were dried at 70 ° C then weighed to provide a preliminary indication of N_2-fixation.

2.3.DNA extraction and 16S rRNA gene sequence analysis

For 16S rRNA gene analysis, genomic DNA of all isolates was prepared using the GES method as described by Pitcher et al. (1989). Nearly full length amplicons for the 16S rRNA

gene were obtained for our strains using the primers and conditions described previously by Vancanneyt et al. (2004). All sequences from this study and the appropriate reference strains were aligned using the MEGA 5 software package (Tamura et al., 2011). A phylogenetic tree was constructed using the MEGA 5 software package with the Maximum likelihood method and the General time reversible model (Tamura et al., 2011). Bootstrap analysis with 1000 replicate data sets was performed to assess the support of the clusters.

Sequences of the 16S rRNA gene determined in this study have been deposited in the EMBL database (Accession numbers are shown in Table 1).

2.4.Nodulation and nitrogen fixation assay (Experiment 2)

In the second nodulation experiment, strains from experiment 1 that appeared to be effective for N_2-fixation were inoculated onto two seedlings of *L. ambigua* (CRSLAM-41, 50), and one seedling of *L. sepiaria*, again in a split-plot design in 1 kg pots of pasteurised soil. Water and nutrients were delivered as described in 2.2. After 56 days plants were cut at the hypocotyl, shoots removed, dried at 70 $^{\circ}$ C for 5 days and then weighed. The capacity for N_2-fixation (effectiveness) was determined by comparing yields of inoculated plants with +N controls (5 ml of 10 mM KNO_3 applied weekly). The strains were separated into three groups as described by Terpolilli et al. (2008); those considered effective (*E*) = >75% of +N control, partially effective (PE) = >20% but <75% of +N control, ineffective (*I*) = <20% of +N control or no nodulation (Nod-). General analyses of variance using a 5% least significant difference (LSD) were calculated on the data sets using GenStat 12® (Release 12.1, Lawes

Agricultural Trust, Rothamsted Experimental Station) and are shown in the figure legends where appropriate.

2.5. Physiological characterisation of the nodule bacteria from *L. ambigua*

The colonies of bacteria isolated from nodules of *L. ambigua* were gram stained and also visualised under a dissecting microscope. After authentication, growth and survival studies relevant to contemporary commercial applications were undertaken. The experiments included fermentation in small volumes, then growth in the common inoculant carriers - sterile peat and non-sterile bentonite clay, and survival when applied as inoculants to seed. The pH tolerance of selected isolates was also examined.

2.5.1. Growth rate in batch culture (Experiment 3)

Starter cultures of the effective strains WSM4174 and WSM4184 were serially diluted in saline by a factor of 10^{-6} then 20 μl of this dilution was used to inoculate 100 ml broth of ½ LA medium (Howieson, 1985) in 250 ml Erlenmeyer flasks (in duplicate). Flasks were placed on a shaker at 180 rpm and held at 28 $^{\circ}$ C. All flasks were shaken overnight and cell counts performed by sampling every 2 h for a period of 10 h the next day. Cultures were serially diluted then plated on ½ LA, incubated overnight at 28 $^{\circ}$ C, and colonies counted the next day.

2.5.2. Growth and survival in sterile peat and bentonite (Experiment 4)

Sterile peat (150 g, Becker-Underwood Australia) was inoculated with 70 ml of either WSM4174 or WSM4184 culture (grown in ½ LA broth) and incubated at 28 °C for 8 days. Cell counts were determined every one or two days for eight days by taking 1 g of peat and resuspending in 9 ml of saline (10^{-1} dilution). The samples were shaken (in a wrist shaker) for approximately 20 min, then serially diluted in saline and plated onto ½ LA plates for colony counts. Non-sterile bentonite clay was slowly dispersed into 250 ml of non-sterile water before adding inoculated peat carrying WSM4174 or WSM4184. The peat/bentonite mix was stirred for another 30 s then poured into trays which were placed in a 28 °C room and left to dry for one week. The dried mix was crushed to a particle size of 3 mm. Cell numbers were determined by suspending 1 g of crushed mix per strain in 9 ml of saline then shaken for 1 h (wrist shaker); serial dilutions of this suspension (dilution 10^{-1}) were then plated onto ½ LA with Chloramphenicol (40 μg/ml) and the fungicide Cyclohexamide (50 μg/ml) added to the media. All plates were incubated at 28 °C for 3-4 days.

2.5.3. Survival of nodule bacteria in peat carrier when applied to the surface of polyethylene beads (Experiments 5 and 6)

Peat cultures of several effective strains selected from the nodulation and nitrogen fixation assays were prepared as described in 2.5.2 and inoculated onto the surface of 3 mm diameter polyethylene beads to determine how many cells could be applied to the surface of the beads, and for how long they survived. Approximately 0.1 g of peat was added to 1 ml of 2% methocel sticker in a sterile 10 ml McCartney bottle, thoroughly mixed, then coated onto 10 g of surface-sterilised beads. Coated beads were exposed to the air currents in a laminar flow

cabinet. Thirty minutes after application, and again after 5 h and 24 h, 1 g of coated beads were removed and serially diluted into sterile saline for plate counts as described in Section 2.5.1. Strains of nodule bacteria selected for comparison were RRI128 (*Sinorhizobium meliloti*), WSM471 (*Bradyrhizobium* sp.), STM815 (*Burkholderia phymatum*) and WSM3937 (*Burkholderia* sp.). In a subsequent experiment, the effect of cells being exposed to an air current (100 ml sterile container lid off) or protected (lid on) was examined.

2.5.4. pH tolerance (Experiment 7)

Three isolates from *Lebeckia* nodules (WSM4184, WSM4185 and WSM5005) and 10 other nodule bacteria representing 6 major genera (Table 2) were compared for their tolerance of acidity when grown on buffered solid ½ LA media adjusted to a range of five different pH values (4.5, 5.0, 5.5, 6, 7 and 8). Plates adjusted to pH 4.5 and 5.0 were buffered with 10 mM HOMOPIPES (Homopiperazine-N,N0-bis-2-ethane-sulfonic acid), at pH 5.5 and 6.0 with 10 mM MES (2-N-morpholinoi ethanesulphonic acid), and at pH 7 and 8 with 10 mM HEPES (4-(2-Hydroxyethyl)-1-piperazineethane sulfonic acid). Isolates were recovered from glycerol storage by culture onto ½ LA plates. A loop of culture was added to 1 ml of sterile water contained in a 1.5 ml microfuge vial and vortexed for 45 s. A sterile applicator stick was dipped into the vial and gently spotted onto the plate. This was repeated for three replicates at each pH. The colony diameter was measured 5 days after emergence of visible colonies.

2.6.Nodule morphology and sections

Light microscopy was used to examine the inner structure of *L. ambigua* nodules formed by WSM4174 by visualising sections embedded in Spurr's resin (Spurr, 1969). Nodules were fixed overnight at 4 ° C in 3% (v/v) gluteraldehyde in 25 mM phosphate buffer (pH 7.0), then procedures followed as described by Yates et al. (2007). The specimens were examined under an Olympus BX51 compound microscope and photographed with an Olympus DP70 digital camera.

3. Results

3.1.Authentication of nodule isolates (Experiment 1)

Twenty three isolates were obtained from *L. ambigua* root nodules collected from sites 5, 10, 11 and 14 (Table 1, Fig. S1). No nodules were collected at site 32. These 23 strains were assessed for nodulation and estimates of N_2-fixation on three provenances of *L. ambigua* (Table S2). Isolates WSM4178, 4182, 4289, 4290 and 4291 did not consistently form normal nodules, sometimes producing pseudo-nodules or white bumps, and have been recorded as nod-. Uninoculated and nitrogen fed plants also remained nodule free (Table S2). The weight and appearance of tops was used to estimate effectiveness for N_2-fixation in the nitrogen free sand-culture system. *B. phymatum* STM815^T and *Burkholderia* sp. WSM3937, were able to induce ineffective and partially effective nodules, respectively, on all provenances of *L. ambigua* (Table S2). The isolates from *L. ambigua* varied in their ability to fix nitrogen on the different provenances of this host, with only WSM5005 ranked as effective across all three provenances. Strain WSM4292 formed ineffective nodules on all three provenances, whilst WSM3560 was ineffective on host CRSLAM-37 (Table S2).

3.2.Identification of the isolates based on 16S rRNA gene sequences

Analysis of the 16S rRNA gene sequence of 17 strains isolated from *L. ambigua* placed them in the*Burkholderia* genus Figs. 1 and S2). Our strains formed five separate groups and shared sequence similarities between 99.9% and 94.9% with all recognised symbiotic *Burkholderia* species, between 96.5% and 93.8% with the other *Burkholderia* species and between 92.5% and 90.8% with *Cupriavidus* and*Ralstonia* species. The closest neighbour of group 1 was *Burkholderia caledonica* LMG 19076^{T} with 98.8% sequence similarity. Group 1 was separate from strains WSM3930 and WSM3937, which were previously isolated from *Rhynchosia* collected from site 2 in the Cape region (adjacent to site 5, see Fig. S1). Group 2 showed high sequence similarity (100%) to *Burkholderia graminis* LMG 18924^{T}, additional sequence analysis of the *recA* and *gyrB* housekeeping genes (Peter Vandamme Ghent University, personal communication) confirms that these strains are *B. graminis*. Group 3 showed high sequence similarity to*B. caledonica* LMG 19076^{T} (98.4%). The closest neighbour of group four and five was *Burkholderia tuberum* STM678^{T} with 98.1% and 99.9% sequence similarity, respectively.

Taxonomic groups were strongly aligned with site location. All group 1 isolates were collected from site 5, whilst all group 5 isolates were from site 14 (Table 1, Fig. 1). Group 4 isolates were primarily compiled from sites 10 and 11, which are separated by only 14 min of latitude (Table 1, Fig. S1). The only outlier in this group was WSM4205, collected from site 5 but clustering in group 4. Groups 2 and 3 comprise non-nodulating isolates from sites 11 and 14, respectively.

3.3.Nitrogen fixation (Experiment 2)

Six isolates from *L. ambigua* that nodulated and appeared effective on at least one provenance in experiment 1, plus control strains of *Burkholderia*, were assessed for nodulation and nitrogen fixation on two *L. ambigua* genotypes and compared for their reaction on the related legume *L. sepiaria* (Fig. 2). All isolates were able to nodulate *L. sepiaria*, with WSM3618, WSM4177 and the *R. ferulifolia* isolate WSM3937 fixing nitrogen effectively (Fig. 2). However, none of the strains could be considered effective across both provenances of *L. ambigua* and *L. sepiaria*. Strains WSM4174 and WSM4184 appeared effective on *L. ambigua* whereas WSM5005 appeared ineffective upon CRSLAM-50, despite being effective upon three provenances CRSLAM-37, CRSLAM-39 and CRSLAM-41 in experiment 1. The interaction between host and strain for top dry weight was significant ($P < 0.05$).

3.4.Physiological characterisation of the nodule bacteria from *L. ambigua*

Burkholderia strains from *Lebeckia* nodules were consistently very fast growing on ½ LA medium, with isolated colonies appearing overnight. These colonies were approximately circular with a slightly rough and watery edge, glistening but only slightly elevated, and with a yellow/brown central tinge when viewed through a dissecting microscope with light from below (. S3). In appearance and growth rate they resembled the *Burkholderia* isolates from *Rhynchosia* described by Garau et al. (2009), but were very different from the more widely described nodule bacteria (Brenner et al., 2005). Strains WSM3556, WSM4204 and WSM4206 (taxonomic group 1) slightly differed from the other isolates. They were smaller (0.2‑1.5 mm in diameter) and more white than yellow.

3.4.1. Growth rate in broth (Experiment 3) and in sterile peat (Experiment 4)

Strains WSM4174 and WSM4184 produced a mean generation time of 1.5 h and 1.6 h respectively when grown on ½ LA at 28 °C in 100 ml shaking batch culture (Fig. S4), but grew poorly on nutrient agar at 37 °C (data not shown). Cultures also grew at 2% NaCl and displayed resistance to Chloramphenicol (40 μg/ml) and Ampicillin (50 μg/ml, data not shown). Both strains achieved >10 fold increase in cell numbers within 48 h after introduction as broth cultures into sterile peat (Fig. S5). Maximum numbers of $>2 \times 10^9$ were reached, and these stabilised after five days.

3.4.2. Growth and survival in dry bentonite clay (Experiment 5)

Neither strain WSM4147 nor WSM4184 were recovered from bentonite clay (data not shown). This experiment was repeated several times.

3.4.3. Survival of nodule bacteria in peat cultures inoculated onto the surface of sterile polyethylene beads (Experiment 6)

For all inoculants except WSM4205, approximately 10^7 cells were present per 1 g of beads when counted 30 min after the inoculation procedure. Within 5 h, all the nodule bacteria isolated originally from*L. ambigua* had reduced in number to less than 10^6 per g of beads, with only the *Sinorhizobium* and*Bradyrhizobium* control treatments above this value (Fig. 3). After a further 24 h of drying, the decline in numbers of the *Burkholderia* from *Rhynchosia* and both the *Sinorhizobium* and *Bradyrhizobium* had ceased, whilst all *Burkholderia* from *Lebeckia* were below 10^5 cells per g beads. At this time,

WSM4184 and WSM3618 from *Lebeckia*, and *B. phymatum* STM815^T had maintained a cell number of approximately 10^4 cells per g of beads, whilst WSM4174 and WSM4205 were unrecoverable (Fig. 3).

When the experiment was repeated, initial numbers of rhizobia coated to the beads were again above 10^7per g of beads after 30 min, and these numbers remained stable if the lid remained in place (Fig. 4). However, where the lids were removed and the inoculants exposed to an air flow, cell numbers of the*Burkholderia* strains from *L. ambigua* were below 10^4 per g of beads after 5 h (Fig. 4). As in the previous experiment, cell numbers of WSM4174 continued to decline and were unrecoverable after 24 h, whereas WSM4184 numbers stabilised at just below 10^4 cells per g of beads. *Burkholderia* sp. WSM3937 from*Rhynchosia* maintained a higher cell number than the *Lebeckia Burkholderia*, again reflecting the results of the previous experiment.

3.4.4. Response of *Lebeckia Burkholderia* and major nodule bacteria genera to pH (Experiment 7)

Burkholderia sp. WSM5005, WSM4185 and WSM4184 from *Lebeckia*, along with *Burkholderia* sp. WSM3937 from *Rhynchosia* and *Rhizobium tropici* strain CIAT899 were the only cultures of 13 strains (representing the major genera of nodule bacteria) able to grow across the full pH range (pH 4.5‑pH 8.0). Within 5 days, colonies from these cultures had grown to be visible, and had expanded to be >3 mm in diameter. The colonies from *Burkholderia* strains isolated from *Lebeckia* expanded to be >6 mm diameter at all pH values (Table 2).

3.5.Nodule morphology and sections

Both *L. ambigua* and *L. sepiaria* developed indeterminate nodules on the main and lateral roots of inoculated glasshouse grown plants (Fig. 5c). There were 2‑10 nodules per plant, and some nodules were bifurcated on 8 week old plants. Sections of effective nodules harvested from 56 day old plants showed a mass of uniformly infected central tissue with no uninfected interstitial cells present. Ineffective nodules were small and white

4. Discussion

The *Lebeckia* isolates grew overnight, and in appearance were distinctively different from the commonly described nodule bacteria. Rather than white or opaque and semi-domed, entire colonies, most of these isolates were yellow‑brown, with a rough margin, and were flat. This description is consistent with that of Garrity et al. (2005) for the genus *Burkholderia*. The 16S rRNA gene phylogeny clearly shows that the strains do belong to the *Burkholderia* genus. This is, therefore, the third record of *Burkholderia* species associated with perennial legumes from the Western Cape of South Africa, after isolates have been reported nodulating *Cyclopia* spp. and *R. ferulifolia* (Elliott et al., 2007a; Garau et al., 2009).

The *Lebeckia* isolates were placed in 5 distinct groups in the *Burkholderia* genus (Figs. 1 and S2). The strains from *Rhynchosia* (WSM3930 and WSM3937), which were also isolated in our laboratories, form a separate, although closely related cluster, to taxonomic group 1 of the *Lebeckia Burkholderia*. This group arose from *Lebeckia* nodules collected from site 5, which is adjacent to the site of collection of the*Rhynchosia* nodules (Site 2, see Fig. S1). Strains belonging to group 1 appear to have *B. caledonica* LMG 19076^{T} as their

closest neighbour (Fig. 1). *B. caledonica* LMG 19076^T was isolated from rhizosphere soil from Edinburgh, UK (Coenye et al., 2001) and no nodulation data exist in the literature for this strain.

Strains WSM4181 and WSM4182 (which could not be authenticated on *L. ambigua*) form group two, and show very high similarity (100%) in 16S rRNA, *recA* and *gyrB* to *B. graminis* LMG 18924^T (Peter Vandamme Ghent University, personal communication). *B. graminis* strains were isolated from the rhizospheres of wheat, corn and pasture grasses from South Australia and Côte Saint André in France (Viallard et al., 1998). Although *B. graminis* strains are not able to nodulate legumes, they are known for their nematode reduction capacity in sugarcane (Omarjee et al., 2008). Strain WSM4178 did not nodulate its original host, and seems to constitute a separate group in our 16S rRNA gene phylogenetic tree, close to *B. caledonica* LMG 19076^T (Group 3, see Figs. 1 and S2). da Silva et al. (2012) report several *Burkholderia* strains from acid Amazon soils, isolated using legume trap plants, but which were mostly unable to achieve nodulation in subsequent tests. They suggest that these strains are most likely to be rhizospheric bacteria able to colonise host plants. The fact that five of our strains (WSM4178, 4182, 4289, 4290 and 4291) ostensibly isolated from nodules, could not induce nodules on their original host, raises several possibilities. Firstly, it is possible that they were isolated from the rhizosphere of *L. ambigua* through incomplete surface sterilisation of the nodules. Secondly, they may have resided intercellularly in the nodule tissue and were protected from the sterilant (Barreto et al., 2012; Dudeja et al., 2012). Thirdly, they may have entered the nodule as co-inoculants with other nodulating strains (Bai et al., 2003; Denton et al., 2003; Dudeja et al., 2012; Egamberdieva et al., 2010; Sturz et al., 1997). Finally they may be rhizobial strains which have lost the capacity to nodulate upon sub-culture (Howieson et al.,

2000b; Sachs et al., 2010). Two non-nodulating strains (WSM4181 and WSM4182) arose from the same nodule as the effective strain WSM4186 (data not shown) which suggests that co-occupation could be a possibility. Further studies are needed to clarify this.

Burkholderia strains in groups four and five showed a close relationship to *B. tuberum* strains STM678^{T}and DUS833 (Figs. 1 and S2), previously isolated from *Aspalathus carnosus* (Moulin et al., 2001;Vandamme et al., 2002) and shown to be effective on several *Cyclopia* species (Elliott et al., 2007a). All strains in group four, except WSM4205, originate from sites 10 and 11. All strains in group five originated from nodules collected at site 14 and appear to form a third distinct species of nodulating *Burkholderia*isolated from *L. ambigua*.

Currently, six *Burkholderia* species, including *B. mimosarum* (Chen et al., 2006), *B. nodosa* (Chen et al., 2007), *B. phymatum* (Vandamme et al., 2002), *B. sabiae* (Chen et al., 2008), *B. symbiotica* (Sheu et al., 2012) and *B. tuberum* (Vandamme et al., 2002) have been confirmed to nodulate and fix nitrogen in symbiosis with legumes. Although sharing the ability to nodulate they are dispersed throughout the 16S rRNA gene phylogenetic tree (Figs. 1 and S2) and show no clear, close relationship. This indicates that the 16S rRNA gene phylogeny does not reflect nodulation capacity and interaction with the host plant, as previously indicated by several studies (Dresler-Nurmi et al., 2007; Haukka et al., 1998; Steenkamp et al., 2008). Experiments on host range and the presence of nodulation and nitrogen fixation genes of the new isolates from *Lebeckia* reported here are currently under way and will provide more clarity on this matter.

There was a very strong influence of collection site on the taxonomy of the *Burkholderia* we recovered from*L. ambigua*. Although site 14 (taxonomic group 5) is geographically close to sites 10 and 11 (taxonomic group 4), it is a different biome, being some 400 m higher in elevation and with greater rainfall (Fig. S1). Site 5 is geographically separated from both. Brazilian rhizobial *Burkholderia* species have also been found to have distinct geographical distributions (Bontemps et al., 2010). The species distribution of the*Lebeckia* isolates may thus reflect physical differences in the environment. On the other hand, the provenances of *L. ambigua* from all these sites differ substantially in pod characteristics, seed shape, and growth habit (J. G. Howieson, Murdoch University, 2012), although they are currently considered the same species (B. E. van Wyk, Johannesburg, pers. commu., 2011). It may be that the variable provenances of*L. ambigua* at these sites explain the presence of the different species of bacteria recovered from their nodules. In support of this, there did seem to be an effect of site (and thus *Burkholderia* species) on capacity for nitrogen fixation; many isolates from site 5 were poorly effective on provenances from sites 11 and 32 (Table S1 and S2). It is clear that a broader assessment of strain versus host compatibility is required before strains can be selected to support further agronomic research with *L. ambigua*.

There appears to be some capacity for the fynbos legumes to share their symbionts, based on the partial effectiveness of the *R. ferulifolia* strain WSM3937 on three provenances of *L. ambigua* (Table S2). Taxonomically diverse Brazilian *Mimosa* spp. growing in the same geographic location are also reported to nodulate with the same set of *Burkholderia* rhizobia (Bontemps et al., 2010). Rhizobial *Burkholderia*therefore seem to be associated more with a physical niche, in this case acid, sandy, infertile soils, than with a particular legume phylogeny. In the case of the fynbos legumes, this is even more striking, as *R. ferulifolia* belongs to the tribe Phaseoleae, whereas *Lebeckia* is in the tribe Crotalarieae.

The *Lebeckia* nodules are typically crotalarioid, being indeterminate, with a central mass of uniformly infected tissue and no uninfected interstitial cells (Fig. 5). This is consistent with the morphology and structure which are found in nodules of other crotalarioid legumes (Renier et al., 2011; Sprent, 2009) and have been proposed to have evolutionary significance (Sprent, 2007).

There was promise displayed by the *Burkholderia* species represented by taxonomic group 4 in some key attributes essential for manufacturing nodule bacteria as legume inoculants. In addition to a high level of effectiveness for N_2-fixation, strain WSM4184 (group 4) achieved rapid growth in normal media and reached high, stable numbers in sterile peat (Figs. S4 and S5). Strains WSM4184 and WSM3618 from group 4 were more tolerant of desiccation than WSM4174 (group 5, see Fig. 3). Strain WSM4205 (group 4) was also intolerant of desiccation, but appears to be an outlier in this group based upon its site of collection, as already noted. However, overall there appears to be a susceptibility to desiccation in the *Burkholderia*strains isolated from *L. ambigua*, as evidenced by death in dry bentonite, and during drying as peat-borne inocula on the surface of beads. The fynbos vegetation occurs in soils that are seasonally dry, fire prone and of low clay content, all of which we would expect to frequently expose these bacteria to desiccation. This, again, must be addressed in support of the agronomic evaluation of *Lebeckia*. It could be speculated that the perennial rhizospheres of these legumes provide some protection to nodule bacteria from drying.

A further valuable trait in commercial inocula is acid tolerance (O'Hara et al., 2002). As previously noted, there are very few perennial, agricultural legume symbioses adapted for dry and acid soils in Mediterranean climates. If the ability to grow on acid agar reflects acid soil

tolerance, then these new species of *Burkholderia* are amongst the most acid tolerant nodule bacteria so far described (Table 2). Nodulating *Burkholderia* strains have previously been noted as micro-organisms adapted to acid and infertile soils (Garau et al., 2009; Gyaneshwar et al., 2011). However, growth on acidified media is not always indicative of success in acid soil, and edaphic evaluation must be conducted *in situ* (Howieson et al., 1988).

Our exploration of the legume flora of South Africa, in search of species with domestication attributes (Howieson et al., 2000a, 2008), and adapted to the variable climate of Mediterranean Australia, has revealed a suite of unusual nodule bacteria. The novel species of *Microvirga* and *Methylobacterium*associated with *Listia* spp., together with the several new species of *Burkholderia* identified in this work highlight the microbiological challenges facing legume domestication.

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Table 1. Origin of root-nodule bacteria used in this study.

Origin of root-nodule bacteria used in this study.						
Strain	**Collection site**	**Latitude and longitude**	**Legume host**	**Exp.**	**Accession numberb**	**Reference**
Burkholderia sp.						
WSM3556	5	33° 29′21″; 18° 19′36″	L. ambigua	1	HQ698908	This work
WSM3558	5	33° 29′21″; 18° 19′36″	L. ambigua	1	N.A.	This work
WSM3560	5	33° 29′21″; 18° 19′36″	L. ambigua	1	N.A.	This work
WSM4204	5	33° 29′21″; 18° 19′36″	L. ambigua	1	HQ698906	This work
WSM4205	5	33° 29′21″; 18° 19′36″	L. ambigua	1,2	HE862280	This work
WSM4206	5	33° 29′21″; 18° 19′36″	L. ambigua	1	HQ698907	This work
WSM4289	5	33° 29′21″; 18° 19′36″	L. ambigua	1	N.A.	This work
WSM4290	5	33° 29′21″; 18° 19′36″	L. ambigua	1	N.A.	This work
WSM4291	5	33° 29′21″; 18° 19′36″	L. ambigua	1	N.A.	This work
WSM4292	5	33° 29′21″; 18° 19′36″	L. ambigua	1	N.A.	This work
WSM4293	5	33° 29′21″; 18° 19′36″	L. ambigua	1	N.A.	This work
WSM3617	10	32° 01′56″; 18° 47′38″	L. ambigua	1	HQ698903	This work
WSM3618	10	32° 01′56″; 18° 47′38″	L. ambigua	1,2	HE862276	This work
WSM4182	11	31° 47′59″; 18° 37′16″	L. ambigua	1	HQ698910	This work
WSM4184	11	31° 47′59″; 18° 37′16″	L. ambigua	1,2,4	HE965764	This work
WSM4185	11	31° 47′59″; 18° 37′16″	L. ambigua	1,4	HE965765	This work
WSM5005	11	31° 47′59″; 18° 37′16″	L. ambigua	1,2,4	HF549035	This work
WSM4174	14	31° 26′47″; 19° 8′41″	L. ambigua	1,2	HQ698904	This work

WSM4175	14	31° 26′47″; 19° 8′41″	L. ambigua	1	HE962574	This work
WSM4176	14	31° 26′47″; 19° 8′41″	L. ambigua	1	HQ698909	This work
WSM4177	14	31° 26′47″; 19° 8′41″	L. ambigua	1, 2	HE862275	This work
WSM4178	14	31° 26′47″; 19° 8′41″	L. ambigua	1	HE862279	This work
WSM4180	14	31° 26′47″; 19° 8′41″	L. ambigua	1	HE862274	This work
WSM3937	2	33° 17′33″; 18° 26′26″	Rhynchosia ferulifolia	2,4	EU219865	Garau et al., 2009
B. phymatum STM815	French Guiana	Mimosa sp. a	2	AJ302312	Moulin et al., 2001	
Brad. sp. WSM471	Albany W. Australia	Ornithopus pinnatus	4	Gi06491	Howieson et al., 1994	
M. loti WSM1293	Greece		Lotus orn.	4	Gi08882	Howieson et al., 2011
M. sp. WSM1497	Greece		Bis. pelecinus	4	AF178964	Nandasena et al., 2001
Methylobacterium sp. WSM2598	South Africa	Listia bainesii	4	DQ838527	Yates et al., 2007	
R. leg. bv. trifolii WSM1325	Serifos<comma> Greece	Trifolium sp.	4	Gc01039	Yates et al., 2005	
R. leg. bv. viciae WSM1455	Mykonos<comma > Greece	Pisum sativum	4	Gi06482	Howieson et al., 2000a	
R. tropici CIAT899	Colombia		Ph. vulgaris	4	Gi05744	Graham et al., 1994

[a] Originally reported as isolated from *Machaerium lunatum* but not authenticated on this host, effectively nodulates *Mimosa* (Elliott et al., 2007b; Mishra et al., 2012).

[b] Either 16S rRNA gene accession number or Gold Card number, N.A. = Not Available, Exp. = Experiment, *L* = *Lebeckia*, *Bis.* = *Bisurella*, *Ph.* = *Phaseolus*,*B.* = *Burkholderia*, *Brad.* = *Br adyrhizobium*, *M.* = *Mesorhizobium*, *R.* = *Rhizobium*, *leg.* = *leguminosarum*, *orn.* = *ornithopod ioides*.

Table 2. Droplet size (mean diameter mm, including EPS when present) of root-nodule bacteria strains 5 d after growth was first detected, when grown on buffered ½ LA agar plates adjusted to pH levels ranging from 8 to 4.5. NG (no growth); · >3 mm colony diameter; • ≥ 3 mm and <6 mm colony diameter; ● >6 mm colony diameter.

Strain	Genus	pH 4.5	pH 5	pH 5.5	pH 6	pH 7	pH 8
WSM419	Ensifer	NG	NG	·	●	●	●
WSM1455	Rhizobium	NG	NG	•	●	●	●
WSM1325	Rhizobium	NG	·	•	●	●	●
CIAT899	Rhizobium	•	•	●	●	●	●
WSM1592	Rhizobium	NG	NG	•	•	●	●
WSM1293	Mesorhizobium	NG	NG	·	●	•	•
WSM1497	Mesorhizobium	NG	•	•	•	•	●
WSM471	Bradyrhizobium	NG	NG	•	·	•	•
WSM2598	Methylobacterium	NG	NG	•	•	•	•
WSM3937	Burkholderia	●	●	●	•	●	●
WSM4184	Burkholderia	●	●	●	●	●	●
WSM4185	Burkholderia	●	●	●	●	●	●
WSM5005	Burkholderia	●	●	●	●	●	●

Fig. 1. Maximum likelihood tree based on the 16S rRNA gene sequences of the strains isolated in this study and several type strains in the genus *Burkholderia*. Strain number, sequence accession or Gold Card numbers and collection site are listed. Percentage bootstrap values based on 1000 replicates are given at the nodes. The sequence of several *Ralstonia* and *Cupriavidus* strains were included as outgroup. Type strains are indicated with a superscript T and the strains isolated in this study are marked in bold. Strains marked with star are able to nodulate and fix nitrogen is association with legumes. Bar, 1% substitution per nucleotide position.

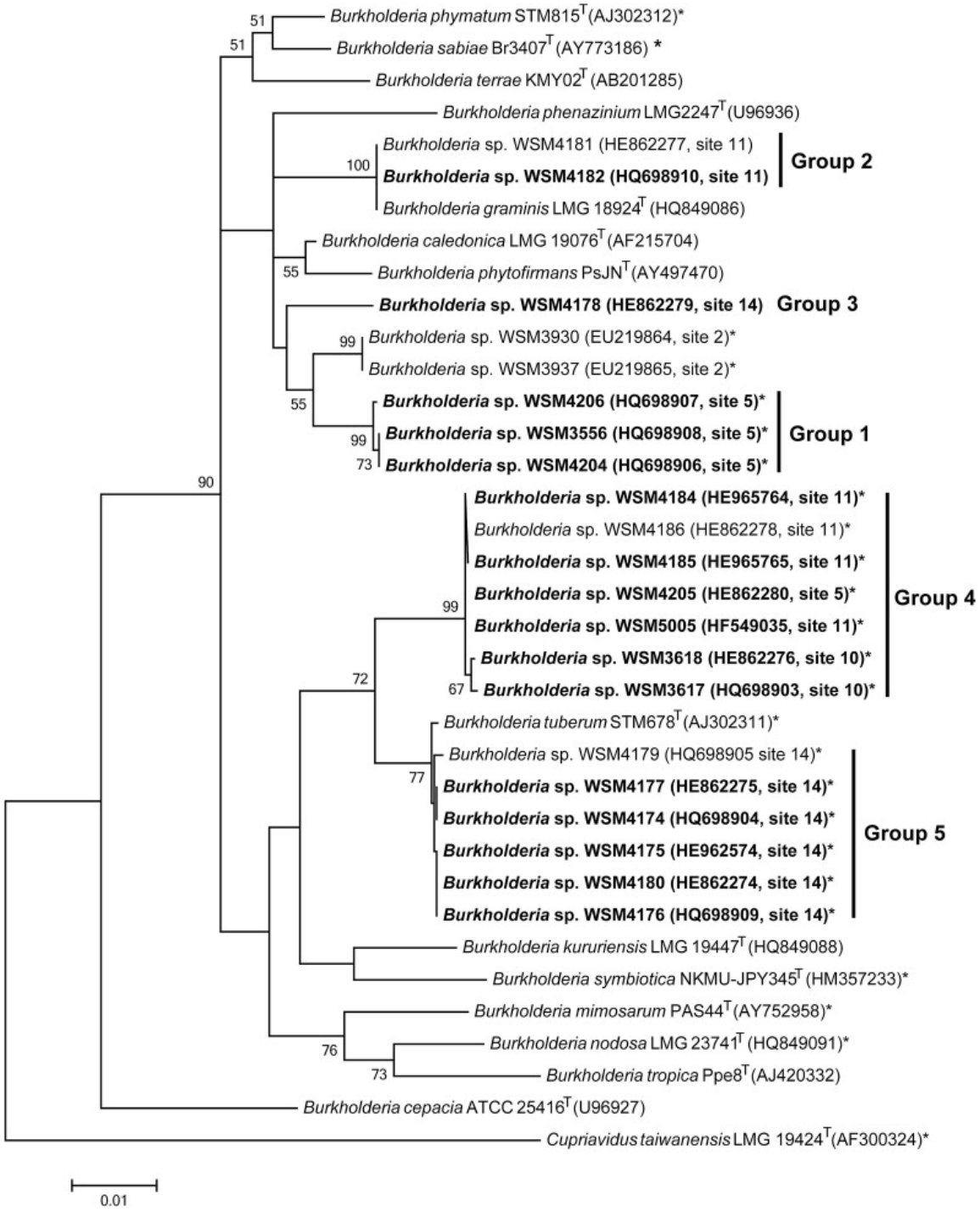

Fig. 2. Total shoot dry weight (g plant^{-1}) produced by *Lebeckia ambigua* provenances CRSLAM-41 and CRSLAM-50, and *L. sepiaria*(KBLS1) when inoculated separately with eight strains of root-nodule bacteria (Table 1). N- uninoculated nitrogen-free control; N+, nitrogen-fed control; (●), no nodulation; (○) partially effective nodulation; (□) ineffective nodulation. Absence of symbols indicates effective nodulation. LSD ($p < 0.05 = 0.0314$).

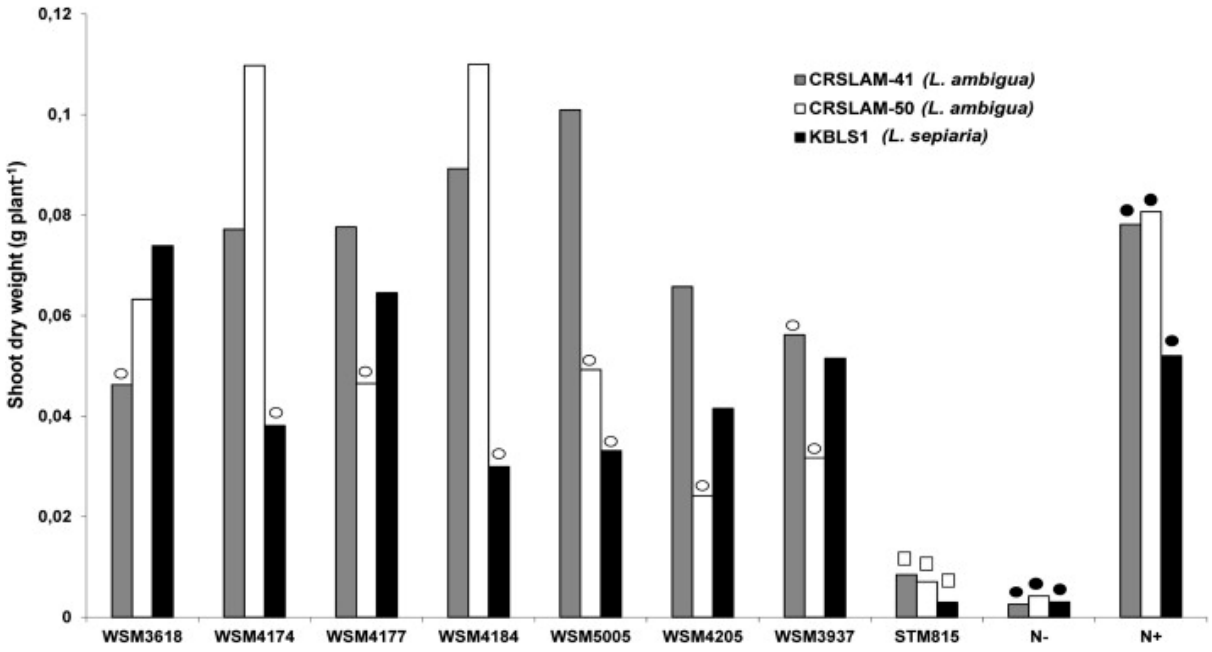

Fig. 3. The survival of *Burkholderia* nodule bacteria from *Lebeckia* and *Rhynchosia* when inoculated as peat cultures onto the surface of polyethylene beads. *Sinorhizobium* strain RRI128, *Bradyrhizobium* strain WSM471 and *Burkholderia* strain STM815 are included as comparators.

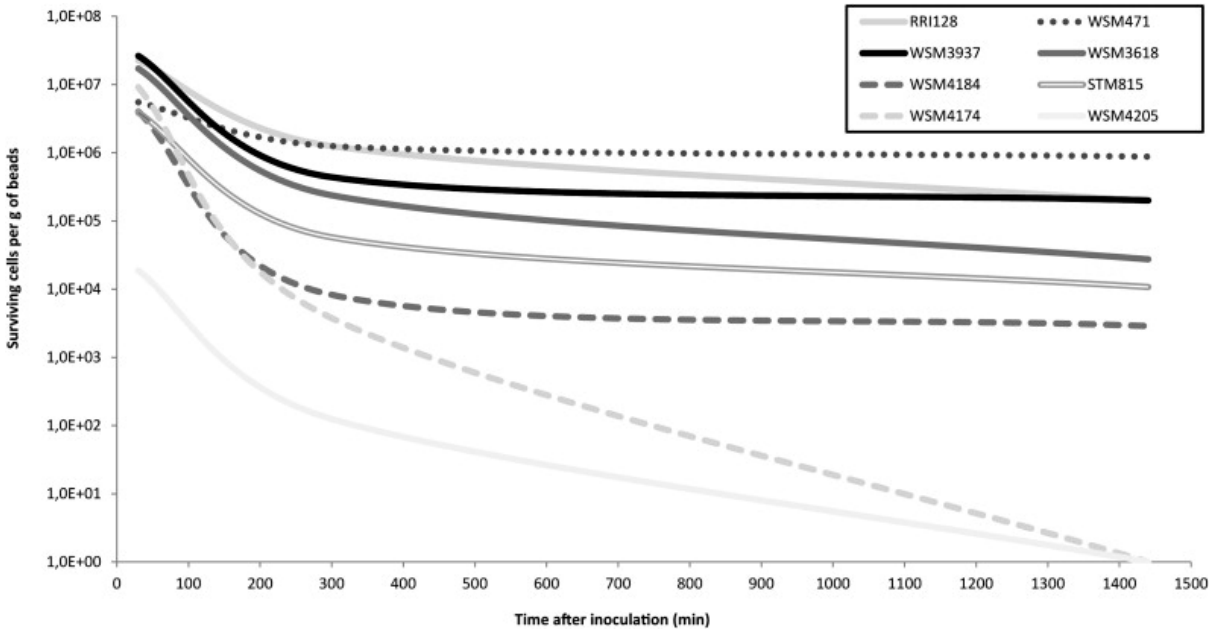

Fig. 4. The survival of *Burkholderia* root nodule bacteria
from *Lebeckia* and *Rhynchosia* when inoculated as peat cultures onto the surface of polyethylene beads. Beads were stored in petri plates in a laminar flow cabinet with (not exposed), or without lids on (exposed).

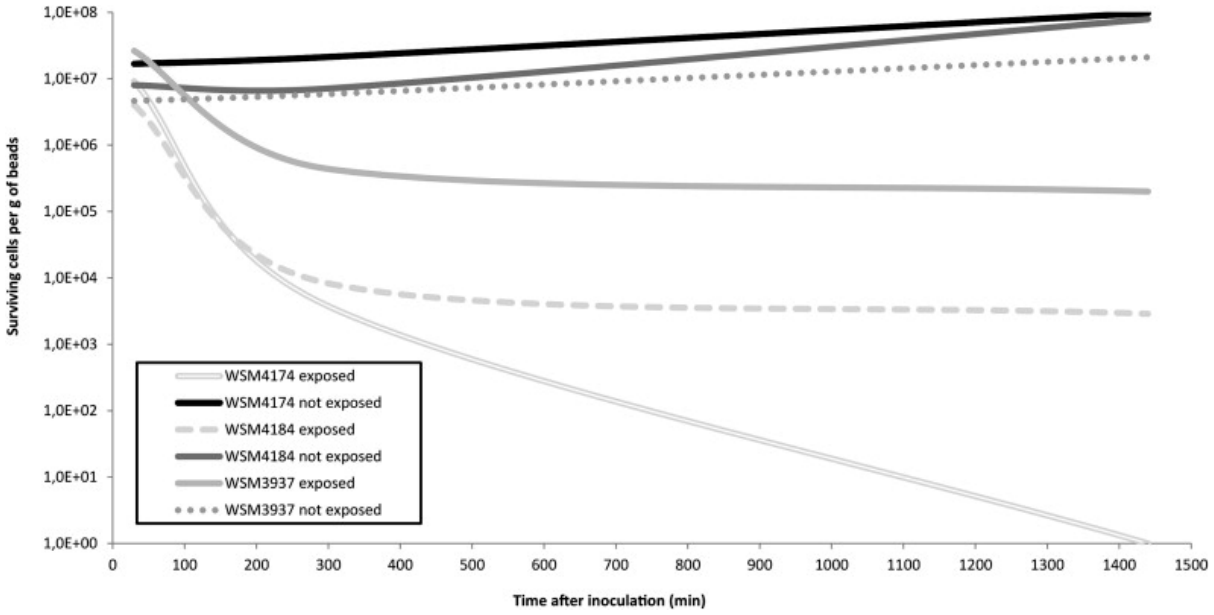

Fig. 5. Pictures of: (a) *Lebeckia ambigua* (in yellow bloom) providing a component of sheep pasture located at Nieuwoudtville, South Africa (right of picture has been grazed). (b) A mature *L. ambigua* plant. (c) Crotaloid, indeterminate, N_2-fixing nodules *L. ambigua*when inoculated with strain WSM4176. (d) *L. ambigua* plant growth in an effective test displaying left to right; N- (no inoculation control), 12 (inoculation with WSM4174) and N+ (nitrogen-supplied control, no inoculation). (e) Transverse section of a young nodule, with the infected zone in the outer cortex developing laterally. Bar 200 μ m. (f) Densely packed symbiosomes in the infected cells. Bar 50 μ m.