Identification and effectiveness of rhizobial strains that nodulate *Lupinus polyphyllus*

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Abstract

Lupinus polyphyllus plants were heavily nodulated at 10 field sites sampled across the South Island. Twenty-two bacterial isolates from these nodules formed functional nodules on *L. polyphyllus* indicating that rhizobia that nodulate *L. polyphyllus* were present across a wide range of sites in the South Island. Gene sequences identified all 22 isolates and the Group G commercial inoculant as *Bradyrhizobium*. Eleven isolates and the Group G inoculant were tested for their effectiveness on growth of *L. polyphyllus* plants in a high country soil in a glasshouse. All plants nodulated regardless of inoculum treatment but there was variability in effectiveness. This suggests that it may be beneficial to use a rhizobial inoculant for high country soils, but further work is required before a recommendation can be made.

Keywords: nitrogen fixation, perennial lupin, *Lupinus polyphyllus*

Introduction

Wild populations of the perennial legume *Lupinus polyphyllus* have colonised roadsides and riverbeds throughout the South Island of New Zealand. Originally introduced as a horticultural garden species, *L. polyphyllus* and its variants (hybrids of *L. polyphyllus* with several other lupin species; also known as Russell lupins) were valued for their colourful flowers. However, garden escapes and deliberate spreading on bare roadside soils have caused proliferation of the species within the Mackenzie Basin and Arthur's Pass regions. More recently, *L. polyphyllus* has been shown to have potential as a forage plant in extensive high country grasslands (Black *et al.* 2014; Scott 2014).

As with most legumes, *L. polyphyllus* is capable of fixing atmospheric nitrogen (N) via symbiotic bacteria ("rhizobia") in root nodules. This ability can give legumes an advantage under low soil N conditions if other factors are favourable for growth (Andrews *et al.* 2011, 2013). Annual lupin species (e.g. *L. angustifolius*) are generally nodulated by rhizobia strains belonging to the slow-growing, acid-tolerant *Bradyrhizobium* genus (Jarabo-Lorenzo *et al.* 2003; Weir *et al.* 2004). Commercial inoculants that contain *Bradyrhizobium* sp. have been developed for annual lupins, and have also been recommended for use on *L. polyphyllus* (Scott 1989). However, no attempt has been made to select rhizobial strains for use as an inoculum on *L. polyphyllus*, or to identify the range of rhizobia that nodulate our current populations.

The objectives of this study were to 1) determine if *L. polyphyllus* is nodulated over a wide range of sites throughout the South Island of New Zealand, 2) genotypically characterise the rhizobia that nodulate *L. polyphyllus* in the South Island, and 3) assess if there is variability in effectiveness of these rhizobia on growth of *L. polyphyllus* in a high country soil. This final objective will determine whether the use of a commercial inoculant is necessary in high country soil.

Methods

Bacterial isolates

Twenty-two rhizobial isolates were obtained from nodules of different *L. polyphyllus* plants sampled from four sites at Arthur's Pass (AP; 11 isolates), three sites in the Mackenzie Basin (MB; six isolates), two sites in Central Otago (CO; three isolates) and one site near Te Anau (TA; two isolates), South Island, New Zealand in March 2013 (Table 1). All isolates were obtained from wild roadside populations of *L. polyphyllus* except for two isolates that were obtained from an agricultural stand at Sawdon Station, near Lake Tekapo, in the Mackenzie Basin. A further isolate was obtained under sterile conditions from *L. polyphyllus* plants supplied Group G inoculant, recommended for annual lupins (Becker Underwood).

Root nodules were surface sterilised by immersion in 96% ethanol for 5 s and 0.5% sodium hypochlorite for 3 min and then were rinsed with sterile water. Surface-sterilised nodules were crushed in sterile water, and this suspension was streaked onto yeast mannitol agar (YMA) (Vincent 1970) and incubated at 20°C in the dark for 2–4 days. A purified culture was obtained by repetitive subculture. Samples of all cultures were inoculated into a suspension of yeast mannitol broth (YMB) (Vincent 1970) and used for preparation of DNA or inoculum.

Gene sequencing and phylogenetic analyses

DNA was extracted from the bacterial cultures using the standard Gentra PUREGENE Purification Kit (Qiagen) following the protocol for gram-negative bacteria. Two genes were sequenced: the small subunit ribosomal RNA (16S rRNA) and N-acyltransferase nodulation protein A (*nodA*). The 16s rRNA and *nodA* primers used were those described in Weisburg *et al.* (1991) and Chaintreuil *et al.* (2001) respectively. Both primers were manufactured by Integrated DNA Technologies, Auckland, New Zealand.

All PCR amplifications were performed using the FastStart™ Taq DNA Polymerase kit (Roche Applied Science, Auckland) optimised for annealing temperature and primer concentrations. The PCR products were resolved via gel electrophoresis (1% agarose gel in 1× Tris-acetate-EDTA buffer) followed by staining with ethidium bromide and viewing under UV light. PCR products were sequenced by the Biological Protection Research Centre Sequencing Facility, Lincoln University. DNA sequence data were obtained via Sequence Scanner v 1.0 software (©Applied Biosystems) and were edited and assembled using DNAMAN Version 6 (©Lynnon Biosoft Corporation).

DNA sequences were aligned and Maximum Likelihood trees constructed with 500 bootstrap replications with partial deletion and 80% coverage cut off using MEGA6 software (Tamura *et al.* 2007). Only bootstrap values ≥50% are shown for each tree. Type strains of the most closely related *Bradyrhizobium* spp. on the GenBank sequence database (www.ncbi.nlm.nih.gov/genbank) were included in the 16s rRNA and *nodA* trees. *Ensifer meliloti* was used as an out-group on both trees.

Table 1 Bacterial isolates were obtained from *Lupinus polyphyllus* at four sites at Arthur's Pass (11 isolates: AP1–11), three sites in the Mackenzie Basin (six isolates: MB1–6), two sites in Central Otago (three isolates: CO1–3), and one site near Te Anau (two isolates: TA1–2) in March 2013. Plants were sampled from wild populations except at one agricultural site at Sawdon Station, Lake Tekapo (MB5–6).

Isolate	Location	Location co-ordinates
AP1–4, 11	Arthur's Pass	S42°57.720′, E171°34.521′
AP7–10	Arthur's Pass	S43°01.049′, E171°35.624′
AP5	Arthur's Pass	S43°01.268′, E171°35.844′
AP6	Arthur's Pass	S43°16.613′, E171°42.453′
MB1–2	Mackenzie Basin	S44°04.838′, E170°31.945′
MB3–4	Mackenzie Basin	S44°03.901′, E170°21.801′
MB5–6	Mackenzie Basin	S44°03.925′, E170°29.357′
CO1–2	Central Otago	S44°30.872′, E169°49.712′
CO3	Central Otago	S44°53.561′, E168°59.600′
TA1–2	Te Anau	S45°08.185′, E167°55.802′

Nodulation and N_2 fixation

All isolates were tested for nodulation and N_2 fixation under sterile laboratory conditions. Seeds of *L. polyphyllus* were scarified then surface sterilised in 0.5% sodium hypochlorite for 15 min, rinsed in deionised water and then germinated on moist germination paper at room temperature in the dark. After germination, seedlings were transferred to polyethylene terephthalate jars (one seedling per jar) containing vermiculite and were supplied with a complete nutrient medium (pH 6.0) as described previously (Tan *et al.* 2012). Plants were grown in a controlled environment cabinet and exposed to a 16-h photoperiod (400 μmol photons/m^2/s) at a constant 25°C. At planting, seedlings were inoculated with 5 ml of the appropriate rhizobial strain grown to log phase, ca. 1×10^8 cfu/ml. Uninoculated plants supplied with YMB only were used as controls. There were three replicate jars per treatment. At 40–50 days after inoculation, plants were tested for nitrogenase activity using the acetylene reduction assay (Cummings *et al.* 2009).

Rhizobial effectiveness

Eleven isolates, representative of all 22 strains obtained from *L. polyphyllus*, were tested along with the commercial Group G inoculant for effectiveness on growth of plants in high country soil in a glasshouse. Germinated *L. polyphyllus* seeds were planted into 1.5 litre pots (two seedlings per pot) containing topsoil from a site at Glenmore Station, Lake Tekapo, with no known history of *L. polyphyllus* or other lupin crops (pH 5.5, Olsen P 14 mg/litre, K 0.69 me/100 g, Mg 0.83 me/100 g, and Al 2.5 mg/kg). Plants were inoculated with the appropriate strain at planting and after 24 days. Five replicate pots were used for each strain and an uninoculated control. Pots were watered with tap water when necessary. When seedlings had 2–3 leaves, they were thinned to one per pot. Plants were harvested 79 days after sowing, and symbiotic effectiveness assessed by analysis of variance of dry weights using GenStat 16 software.

Results and Discussion

At all 10 field sites (Table 1), all *L. polyphyllus* plants sampled were heavily nodulated. These nodules were pink inside and assumed to be functional. Twenty-two bacterial isolates obtained from these nodules were shown to form functional nodules on *L. polyphyllus* on inoculation in sterile laboratory conditions (acetylene reduction activity). These results indicate that rhizobia that nodulate *L. polyphyllus* are present across a wide range of sites in the South Island, including

the agricultural stand of *L. polyphyllus* at Sawdon Station, Lake Tekapo, which was established using uninoculated seed (Black *et al.* 2014).

The 16s rRNA and *nodA* gene sequences identified all 22 isolates and the Group G inoculant as *Bradyrhizobium* spp. One isolate (AP11) showed substantially different 16s rRNA and *nodA* sequences from the others and was not included in the phylogenetic trees (Figure 1). The 16s rRNA sequence for this strain showed 97.5% similarity to that of *B. japonicum* isolated from the crop plant *Glycine max* (soya bean) in Japan (Kaneko *et al.* 2011). Its *nodA* sequence showed 72% similarity to that of *B. cytisi* isolated from *Cytisus villosus* (hairy broom) in the Moroccan Rif (Chahboune *et al.* 2011). The origin of this isolate is unclear.

The other 21 isolates from *L. polyphyllus* clustered together for both 16s rRNA (Figure 1A) and *nodA* (Figure 1B) gene sequences. The 16s rRNA sequences separated into four groups. Groups 1 and 2 (11 isolates) were most closely related to *B. canariense* (99.58%–99.75% similarity) which was isolated from *Chamaecytisus palmensis* (tree lucerne) in La Laguna (Tenerife), Spain (Vinuesa *et al.* 2005) and to the Group G inoculant (99.00%–99.17% similarity). Groups 3 and 4 were most closely related to *B. japonicum* (99.57%–99.83% similarity). The *nodA* sequences separated into two major groupings. Two of the strains aligned closest to the isolate from the Group G inoculum, but 19 of the strains clustered together closest to, but clearly separate from (92.89%–96.67% similarity), *B. cytisi* isolated from *C. villosus*.

Thus overall, the DNA sequence data indicate that

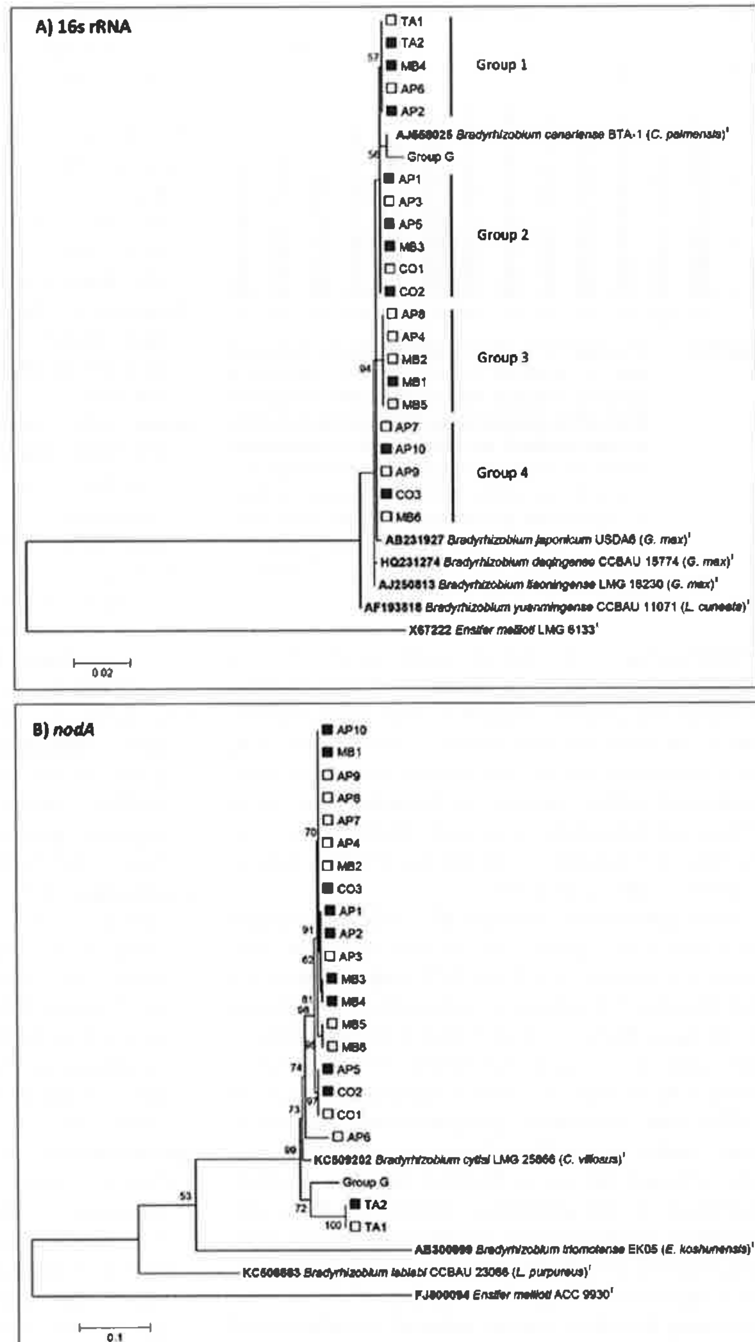

Figure 1 Phylogenetic genetic trees of 16s rRNA (A) and *nodA* (B) gene sequences of 21 bacterial isolates from *Lupinus polyphyllus* sampled in soils at Arthur's Pass (AP1–10), Mackenzie Basin (MB1–6), Central Otago (CO1–3) and Te Anau (TA1–2) in New Zealand, commercial Group G inoculant currently recommended for use on annual lupins, and selected type strains (T) of *Bradyrhizobium* spp. GenBank accession numbers are in bold and host legume species are in parentheses. *Ensifer meliloti* was used as an out-group. Selected isolates (■) were tested in a glasshouse experiment. Numbers on branches indicate bootstrap % from 500 replicates (shown only when ≥50%).

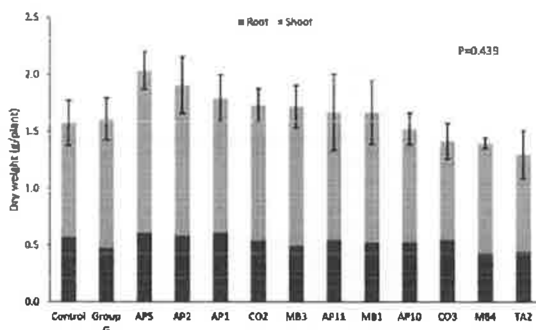

Figure 2 Dry weight of *Lupinus polyphyllus* plants inoculated with 11 bacterial isolates from *L. polyphyllus* sampled in soils at Arthur's Pass (AP), Mackenzie Basin (MB), Central Otago (CO) and Te Anau (TA) in New Zealand, an isolate from a commercial Group G inoculant currently recommended for use on annual lupins, and an uninoculated control, in high country soil collected from an agricultural site at Glenmore Station, Lake Tekapo. Standard errors of means and the level of significance for total plant dry weight are given.

bradyrhizobia with distinct *nodA* genes are of widespread occurrence in the South Island of New Zealand. The possible sources of these bradyrhizobia are: 1) an inoculant used in New Zealand in the past, 2) a strain from outside New Zealand that has become established with *L. polyphyllus* throughout the South Island, and 3) naturally occurring bradyrhizobia in New Zealand that nodulate *L. polyphyllus*. Further work is required to clarify this point.

In the glasshouse experiment, all *L. polyphyllus* plants were nodulated regardless of inoculum treatment. This result is in agreement with the field study and indicates that rhizobia that nodulate *L. polyphyllus* were present in the agricultural soil tested from Glenmore Station, Lake Tekapo, with no known history of *L. polyphyllus* or other lupin crops. It is also in agreement with Scott (1989), who stated that *L. polyphyllus* will nodulate in high country soils without inoculum, although it was also indicated that use of rhizobial inoculum could be beneficial. In the glasshouse experiment, there was variability in total plant dry weight across the inoculum treatments (Figure 2). In particular, growth with strain AP5 appeared to be greater than that of the control, indicating that there may be potential for selection of an elite strain for use as a rhizobial inoculum. However, this experiment needs to be repeated and carried out for a longer time period before recommendations could be made with regard to use of inoculum on *L. polyphyllus* in high country soils.

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