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REGULAR ARTICLE

Selecting improved *Lotus* nodulating rhizobia to expedite the development of new forage species

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Abstract
Aims In the past decades the increasing focus by Australian pasture development programs on the genus *Lotus* has seen the evaluation of many species previously untested in Australia. In field trials, nodulation failure was commonplace. This work was undertaken to select effective symbionts for *Lotus* to ensure further agronomic evaluation of the genus was not compromised. The symbiotic needs of *Lotus ornithopodioides* were a particular focus of the studies.

Methods Glasshouse experiments were undertaken to evaluate symbiotic relationships between 15 *Lotus* spp and 23 strains of nodulating *Mesorhizobium loti*. This was followed by evaluation of elite rhizobial strains for their ability to persist and form nodules under field conditions.

Results Complex symbiotic interactions were recorded between strains of lotus rhizobia and the different species of *Lotus*. Notably, the rhizobia that are currently provided commercially in Australia for the inoculation of *Lotus corniculatus* (strain SU343) and *Lotus uliginosus* (strain CC829) did not form effective symbioses with the promising species *L. ornithopodioides* and *L. maroccanus*. No strain we evaluated was compatible with all the Lotus species, however several strains with a broad host range were identified. WSM1293 and WSM1348 were the most effective strains on *L. ornithopodioides* and *L. peregrinus.*These strains were also moderately effective on *L. corniculatus* (79 and 52% of SU343), less effective on *L. maroccanus* (26 and 49% of SRDI110) but were ineffective on *L. uliginosus.* The latter species overall had very specific rhizobial needs. Both WSM1293 and WSM1348 produced adequate levels of nodulation when inoculated on *L. ornithopodioides,* over two seasons at three field sites.

Conclusions Effective and persistent strains are now available that should allow the un-compromised evaluation of many of the contemporary *Lotus* species in the field. Selecting a strain for use in commercial inoculants will be more problematic, given the very

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large host-strain interactions for nitrogen fixation. Here, the balance of *Lotus* species which are adopted by farmers will have a strong bearing on which rhizobial strains are progressed to commerce.

Keywords *Lotus* spp. · *Mesorhizobium loti* · Rhizobia · Nitrogen fixation · Pasture.

Introduction

Robson (1988) predicted that because of the great variation in management systems, and in edaphic and climatic environments within southern Australia, it was likely that the most appropriate legumes and procedures for maximising benefits from them would vary. Within 10 years of that prediction a second generation of annual pasture legumes had been selected and adopted for Mediterranean environments in Australian agriculture (Howieson et al. 1995, 2000a; Craig et al. 2000). These 'new' species of *Ornithopus, Trifolium* and *Biserrula* complemented the pre-existing sub-clover (*Trifolium subterraneum* L.) and medics (*Medicago* spp.) that had long been recognised as essential to maximising production in ley-farming systems (Cocks et al. 1980; Puckridge and French 1983).

A forage genus that remains to be fully exploited in Australian agriculture is *Lotus*, a diverse group of annual and perennial herbs consisting of more than 180 species (Allen and Allen 1981; Ayres et al. 2006). Only two species of *Lotus* (*Lotus corniculatus* L. and *L. uliginosus* Schukr. (syn *L. pedunculatus*)) are exploited commercially in Australia (Blumenthal et al. 1993), albeit their use is relatively minor (Harris et al. 1993; Hill and Donald 1998) and restricted to higher rainfall and waterlogging environments with rotational grazing practices (Ayres et al. 2006; Real et al. 2008). However, there are several other species of Mediterranean origin that may have the potential to benefit Australian farming systems (Kelman 1993). Most promising is perhaps *L. ornithopodioides* (L.), an annual, reseeding pasture legume with a number of desirable traits. These include a relatively deep root system, prolific seed production, tolerance to insects, excellent pod retention on the stems, and minimal pod shattering compared with other species in the genus (Loi et al. 2002), characters reflecting an optimal ideotype (Howieson et al. 2000a, b). A commercial release of this species is likely (A. Loi, pers. comm. Jan 2011). A second novel species, *L. maroccanus* (Ball.) is a short lived winter active perennial with salt and manganese tolerance (Schachtman and Kelman 1991) that has a putative role in water table control and has previously shown potential in pasture trials in Southern Australia (Hughes et al. 2008). Other *Lotus* species of current interest include *L. creticus* (L.), *L. edulis* (L.), *L. peregrinus* (L.) and *L. tenuis* Waldst. & Kit. (syn. *L. glaber)* (Real et al. 2008; Teakle et al. 2010). There are also two species of *Lotus* that are indigenous and widespread in Australia, *L. australis* Andr. and *L. cruentus* Court (Jessop and Toelken 1986; Harden 1991), however they occur at low frequency, have little agronomic potential (Kelman 1993) and appear not to have left a legacy of rhizobial populations that are able to nodulate their Mediterranean counterparts. The unproductive Mediterranean species including *L. subbiflorus* (syn *L. hispidus*) and *L. augustissimus* are widely naturalised in southern Australia (Hussey et al. 2007) and the nodule bacteria for the latter species has been shown to fix nitrogen with both *L. corniculatus* and *L. uliginosus* (Brockwell et al. 1966). The potential for the nodule bacteria of the indigenous and naturalised *Lotus spp*. to compromise nitrogen fixation from commercial species directly, and to indirectly interact with the inoculants for Mediterranean *Lotus spp*. through lateral gene transfer should not be ignored (Sullivan et al. 1995; Nandasena et al. 2006).

Different species of *Lotus* commonly require specific strains of *Mesorhizobium loti* for effective nitrogen fixation (Brockwell et al. 1966; Gault et al. 1994). It is for this reason that two strains are provided commercially for the inoculation of *Lotus* in Australia. Strain SU343 is recommended for *L. corniculatus*, while strain CC829 is recommended for *L. uliginosus* (Bullard et al. 2005). While these two strains are effective on their respective hosts, they are reported to form ineffective symbioses with many of the other species of *Lotus* (Brockwell et al. 1966; Gault et al. 1994).

The experiments described here were principally undertaken to identify rhizobial strains that were compatible with *L. ornithopodioides*, following reports that nodulation failure of this legume was commonplace in field trials in both Western Australia and in South Australia (Loi et al. 2002). In particular, we were cognisant of the need to provide inoculant

strains that would allow maximum nitrogen fixation from this and other promising *Lotus* species without compromising production of the commercial species *L. corniculatus* and *L. uliginosus*, other forages from the tribe Loteae including *Dorycnium* spp. (Davies et al. 2005) and *Tetragonolobus spp.,* or even unrelated pasture species such as *Biserrula pelecinus* (L.) which are also nodulated by *Mesorhizobium* spp. (Nandasena et al. 2001). Saprophytic competence (Chatel and Parker 1973; Howieson 1995) of promising rhizobial strains was assessed in three soils, since this characteristic is known to be a critical factor in the regeneration and persistence of annual species such as *L. ornithopodioides*.

Materials and methods

Rhizobial and legume germplasm

Rhizobial strains were sourced from genetic resource centres at Murdoch University, Perth, Western Australia (strains with WSM prefix), SARDI, Adelaide, South Australia (strains with SRDI) and CC prefixes; the 'CC' strains formerly curated by J. Brockwell of CSIRO, Canberra, Australian Capital Territory (Table 1).

Legume material (Table 2) was sourced primarily from the Australian legume genetic resource centres in Adelaide, South Australia (SARDI) and Perth, West Australia (Department of Agriculture and Food, WA).

Host range and effectiveness of the *Lotus* rhizobia

A series of glasshouse experiments were undertaken to examine the ability of rhizobial strains to nodulate and fix nitrogen with a diverse range of *Lotus* species.

In Experiments 1a and 1b, 22 strains of rhizobia (including four commercial mesorhizobial inoculants) were compared for their ability to nodulate and fix nitrogen with ten accessions of *L. ornithopodioides.* Experiments 2a, 2b and 2c and Experiment 3 evaluated the more promising strains for nodulation and nitrogen fixation on a wider range of *Lotus* species.

General glasshouse procedures for Experiments 1a–b and 2a–c were as described in Howieson et al. (1995) and modified by Yates et al. (2005). Briefly, plants were grown for 6 weeks under axenic conditions in pots of steamed soil (equal parts of leached yellow sand and river sand) held in a temperature controlled glasshouse and supplied with adequate nutrients, minus N. Experimental design was a split plot factorial, with rhizobial strain as the main treatment and plant genotype as the sub-treatment.

In Experiment 3, the effectiveness of the two commercial *Lotus* strains (SU343 and CC829), and three elite strains identified in Experiments 1and 2 were compared in combination with five species of *Lotus*. Plants were grown in a mix of equal parts of course washed sand and vermiculite, contained in 130 mm diameter pots, which had been sterilised by autoclaving. A pot was planted with five pre-germinated seedlings of a single *Lotus* species and then watered with 300 ml of a nitrogen-free nutrient solution (McKnight 1949) in a laminar flow cabinet. After sowing and watering, the surface of the potting mix in each pot was covered with polypropylene beads. Subsequent water and nutrients were delivered via a sterile watering tube embedded below the surface to maintain the bacterial isolation of each pot. The pots were held on a wire grid to allow free drainage and a sterile paper filter was inserted at the bottom of the pot to minimise airborne contamination. The *Lotus* spp. were subjected to one of 14 treatments. The seedlings were either (i) inoculated 4 day after sowing with 10 ml of a culture estimated to contain $>10^6$ cells/ml of one of the 12 rhizobial strains (YMB Vincent 1970), (ii) not inoculated or (iii) not inoculated but supplied with 50 ml of 15 mM NH_4NO_3 pot^{-1} added at 11, 18, 25, 32, 39, 46 and 53 day after sowing. Each of the treatment combinations was replicated four times and arranged in a randomised block design. Plants were grown in a shaded greenhouse (25/20°C mean day/night temperature), the shoots harvested 56 day after sowing and dried at 60°C for 72 h. Shoot weight was determined and used as a measure of nitrogen fixation.

Field persistence of Lotus rhizobia

Experiments 4, 5 and 6 examined the field persistence of 13 promising rhizobial strains at Mannum (34°54'S, 139°18'E; sandy loam, pH_{Ca} 6.3), at Wynarka (35°07'S, 139°43'E; sandy loam, pH_{Ca} 7.2) both in the Murray-Mallee region of South Australia, and two strains at Karridale (34°08'S, 115°10'E; sandy loam, pH_{Ca} 5.9) in the south—west of Western Australia. A cross-row technique (Howieson and

Table 1 Description of rhizobial strains used in the experiments

Strain of rhizobia	Method of collection	Host of isolate	Country of origin
WSM1284	Nodule (*in situ*)[a]	*Biserrula pelecinus*	Greece
WSM1497	Nodule (*in situ*)	*Biserrula pelecinus*	Greece
CC1192	Nodule (*in situ*)	*Cicer arietinum*	Israel
SU343	Nodule (*in situ*)	*L. corniculatus*	USA
WSM1294	Nodule (*in situ*)	*L. corniculatus*	Morocco
WSM1296	Nodule (*in situ*)	*L. corniculatus*	Morocco
WSM1297	Nodule (*in situ*)	*L. corniculatus*	Italy
WSM1307	Nodule (*in situ*)	*L. corniculatus*	Morocco
WSM1348	Nodule (*in situ*)	*Lotus sp.*	Greece
CC821	Nodule (*in situ*)	*L. maroccanus*	Australia
SRDI144	Soil trapping (GH[b])	*L. maroccanus*	Morocco
SRDI110	Soil trapping (GH)	*L. ornithopodioides*	Morocco
SRDI137	Soil trapping (GH)	*L. ornithopodioides*	Morocco
SRDI140	Soil trapping (GH)	*L. ornithopodioides*	Morocco
SRDI210	Soil trapping (GH)	*L. ornithopodioides*	Greece
SRDI225	Soil trapping (GH)	*L. ornithopodioides*	Greece
WSM653	Nodule (*in situ*)	*L. ornithopodioides*	Sardinia
WSM1292	Nodule (*in situ*)	*L. ornithopodioides*	Greece
WSM2290	Nodule (*in situ*)	*L. ornithopodioides*	South Africa
WSM2316	Nodule (*in situ*)	*L. ornithopodioides*	Greece
WSM2317	Nodule (*in situ*)	*L. ornithopodioides*	Greece
WSM1646	Nodule (*in situ*)	*L. purshianus*	USA
WSM1648	Nodule (*in situ*)	*L. purshianus*	USA
CC829	Nodule (*in situ*)	*L. uliginosus*	USA
WSM805	Nodule (*in situ*)	*Lotus sp.*	Greece
WSM819	Nodule (*in situ*)	*Lotus sp.*	Greece
WSM1414	Nodule (*in situ*)	*L. uliginosus*	Australia
CC801a	Nodule (*in situ*)	*Lotus edulis*	Algeria
WSM725	Nodule (*in situ*)	*Lotus* sp.	unknown
WSM1293	Nodule (*in situ*)	*Lotus sp.*	Greece
CC820	Nodule (*in situ*)	*Tetragonolobus palaestinus*	Jordan

[a] nodule preserved and strain isolated in laboratory

[b] *GH* glasshouse in Australia

Ewing 1986) over a 2-year period was established to assess strain persistence and colonisation in experiments 4 and 5, whilst a small plot trial was established with a precision seeder for experiment 6, sown in 2009 and monitored for nodulation into 2010.

Preparing inoculants for field experiments

The 13 strains of rhizobia used as the source of inoculant for the field experiments were cultured as previously described (Howieson et al. 1995) and allowed to mature in sterilised peat with the resultant cell density estimated on spread plates of yeast mannitol agar (YMA,Vincent 1970; Somasegaran and Hoben 1985). Each of the inoculants used contained $>1\times10^9$ colony forming units g^{-1} peat (the Australian industry standard), with the possible exception of strain WSM2316 for which a reliable population estimate was not obtained. Rhizobia were applied to the seed at 2-fold the rate recommended commercially (equivalent of 500 g peat inoculant applied to 25 kg of seed), and coated with Plastaid (Goliath Portland Cement Company) pelleting compound (experiments 4, 5) or lime (experiment 6). An uninoculated control treatment was also sown for comparison. Each treatment was replicated four times and arranged in a randomised block design.

Table 2 Description of *Lotus* germplasm used in the experiments

Lotus spp.	Accession/cultivar	Origin of species	Growth	Expt.
L. ornithopodioides L.	97JH39	Greece	Annual	1a
L. ornithopodioides L.	98SNO-16	Greece	Annual	1b
L. ornithopodioides L.	98LOI20	Italy	Annual	1a
L. ornithopodioides L.	BR128	Medit Basin, N Europe[a]	Annual	1b
L. ornithopodioides L.	BR298	Medit Basin, N Europe[a]	Annual	1b
L. ornithopodioides L.	BR072	Medit Basin, N Europe[a]	Annual	1a
L. ornithopodioides L.	PI308038	Czech	Annual	1a
L. ornithopodioides L.	SA33845	Tunisia	Annual	5
L. ornithopodioides L.	SA33846	Spain	Annual	3, 4, 5
L. ornithopodioides L.	SA5016	Tunisia	Annual	4
L. ornithopodioides L.	SA5017	Tunisia	Annual	1a
L. ornithopodioides L.	SA5020	Israel	Annual	1b
L. ornithopodioides L.	SA834	Tunisia	Annual	1b
L. ornithopodioides L.	2000ITA7.1.C	Italy	Annual	6
L. arenarius Brot.	54974	Mediterranean basin[a]	Perennial	2c
L. corniculatus L.	cv. Grasslands Goldie	Spain	Perennial	2b, 3
L. corniculatus L.	cv. San Gabriel	Mediterranean basin[a]	Perennial	6
L. creticus L.	S1012	Mediterranean basin[a]	Perennial	2c
L. cytisoides L.	not known	Mediterranean basin[a]	Perennial	2c
L. discolor E. Mayer	not known	East Africa	Perennial	2c
L. edulis L.	SA22716	Mediterranean basin[a]	Perennial	2c
L. maroccanus Ball	SA12953	Mediterranean basin[a]	Perennial	2c, 3
L. mearnsii (Britton) Greene	27463	Africa	Perennial	2c
L. peregrinus L.	SA13753	Medit Basin, N Europe[a]	Perennial	2c
L. peregrinus L.	SA5021	Medit Basin, N Europe[a]	Perennial	3
L. purshianus (Benth.)	610	Medit Basin, N Europe[a]	Annual	2c
L. subbiflorus Lagasca	Q18438 (P15304)	Medit Basin, N Europe[a]	Perennial	2c
L. subbiflorus Lagasca	cv. Rincon	Medit Basin, N Europe[a]	Annual	2c
L. tenuis Waldst. & Kit.	S2791	N Africa, Europe, Asia[a]	Perennial	2c
L. uliginosus Schukr.	cv. Grasslands Maku	Medit Basin, N Europe[a]	Perennial	2a, 3

[a] collection details not available

In experiments 4 and 5, in the year of establishment, 2.5 m rows containing seed coated with individual strains of rhizobia were established in the soil in 2000 and 2001, at Mannum and Wynarka respectively. The genotypes of seed were *L. ornithopodioides* accessions SA5016 and SA33846 respectively. Following senescence of the legume at the end of the growing season, the rhizobia were allowed to over-summer without disturbance. After opening rains the following season, uninoculated seeds of *L. ornithopodioides* (accession SA33845 at Mannum; SA33846 at Wynarka) were sown perpendicularly across the lines of inoculum (forming sub-plots) and the plants subsequently sampled 11 and 16 weeks after planting (at Mannum and Wynarka respectively) at four distances (0–2, >2–12, >12–22 and >22 to 32 cm) from the initial line of inoculum. From each sampling region, 20 plants were selected at random and all nodules removed. Nodules were dried at 60°C for 48 h and then weighed.

In the small plot experiment (6), sown with a precision seeder, *L. ornithopodioides* and *L. corniculatus* (2000ITA7.1.C and San Gabriel respectively, 7 kg ha^{-1}) plots were sown (1.2×10 m) into two banks each containing two replicates (four in total) arranged in a randomised block design but separated into species. Approximately 15 h before sowing seeds were inoculated separately with SU343 or the promising strain WSM1293 at the recommended inoculation rate (250 g

inoculant peat per 25 kg seed) and established in plots with uninoculated controls (sown first) in June 2009. A most probable number estimate (MPN, Brockwell et al. 1982) of background nodulating bacteria was undertaken prior to sowing. Plots were sub-sampled in October 2009 for shoot dry weight and nodulation assessment, and regenerating plants were again sampled in July 2010 for assessment of nodule occupancy using PCR fingerprint profiling (Yates et al. 2005).

Data interpretation and analysis

For experiments 1a–b and 2a–c symbioses were considered effective (E) where plant weight exceeded 75% of the +N treatment. Where plant weight was less than 20%, the symbiosis was deemed ineffective (I), and in between these parameters the symbiosis was considered partially effective (P).

For Experiment 3, the data were subjected to Analysis of Variance without transformation. Values for the +N treatment were excluded from the analysis and are not reported. For Experiments 4 and 5, the data were subjected to Analysis of Variance using a split plot model (sampling distances as the sub plots). The data were not transformed prior to analysis. Only four of the five replicates in Experiment 4 were analysed due to poor plant establishment in one field replicate. Standard errors were generated on data contained in Experiment 6 through Genstat 12® (Release 8.1, Lawes Agricultural Trust, Rothamsted Experimental Station)

Results

Rhizobial strain reaction with *L. ornithopodioides* (Experiments 1a–b)

The commercial *Mesorhizobium* inoculants for *L. corniculatus* (SU343), *B. pelecinus* (WSM1497), and *Cicer arietinum* (CC1192) failed to form nodules on any of the ten accessions of *L. ornithopodioides* (Table 3). The commercial inoculant for *L. uliginosus* (CC829) formed rudimentary nodules on four of the ten accessions only. Similarly, strains isolated from *L. purshianus* nodules (WSM1646 and WSM1648) collected from the USA were generally not infective on *L. ornithopodioides*. Contrastingly, there were ten strains, including WSM1284 that was originally isolated from *B. pelecinus,* that were both infective and able to fix nitrogen in symbiosis with *L. ornithopodioides*. Of these, strains WSM1293, WSM1348 and WSM805 resulted in most accessions of *L. ornithopodioides* having shoot dry weights greater than 75% of the nitrogen-fed control plants (Table 3).

Strain reaction on multiple *Lotus spp*. (Experiments 2a–c and Experiment 3)

No single strain was able to form an effective symbiosis with all the 15 *Lotus* species evaluated (Table 4). Strains WSM1293 and WSM1348 produced almost identical symbiotic profiles, forming effective associations with the same five *Lotus* species. Strain SU343 was only able to form effective symbioses with *L. mearnsii*, *L. subbiflorus*, and *L. corniculatus*. Strain CC829 also had a narrow host range and was unique in its ability to form effective symbioses with *L. purshianus* and *L. uliginosus*.

Similarly, with a wider set of strains in Experiment 3, CC829 was the only strain able to form an effective symbiosis (45 mg) with *L. uliginosus* cv. Maku (Table 5). Strains WSM1293 and WSM1348 produced the most effective symbioses with both *L. ornithopodioides* (91 and 89 mg respectively) and *L. peregrinus* (69 and 58 mg respectively), but were less effective than strain SU343 with *L. corniculatus* (48 mg). SRDI110, which was trapped from Moroccan soil with seed of *L. ornithopodioides,* was outstanding with *L. maroccanus* (110 mg) but relatively poor in association with its trap host. In fact, the strains trapped from Moroccan and Greek soils by *L. ornithopodioides* were generally less effective than strains isolated from nodules collected *in situ* from this host.

Field experiments (Experiments 4, 5 and 6)

All field sites were highly responsive to rhizobial inoculation. The MPN at Karridale prior to sowing returned an estimation of <10 cells g^{-1} soil capable of nodulating *L. ornithopodioides* despite the presence of naturalised *L. subbiflorus,* and $>10^3$ cells g^{-1} soil for *L. corniculatus* (data not shown). No nodules were detected in the uninoculated plots of *L. ornithopodioides* at Mannum whilst only low levels were detected in the uninoculated plots at Wynarka and Karridale (Tables 6 and 7). In contrast, 97% of

Table 3 Summary of the nodulation and symbiotic effectiveness[a] of 22 strains of rhizobia with ten accessions of *Lotus ornithopodioides* (Experiments 1a and 1b)

Accession of *Lotus ornithopodioides*										
Strain	BR072	BR128	BR298	PI308038	SA834	SA5017	SA5020	97JH39	98LOI20	985N0-16
CC829[b]	X	I	X	X	X	X	I	I	I	X
CC1192[b]	X	X	X	X	X	X	X	X	X	X
SU343[b]	X	X	X	X	X	X	X	X	X	X
WSM1497[b]	X	X	X	X	X	X	X	X	X	X
WSM653	E	P	P	E	P	E	E	E	P	E
WSM725	I	I	I	I	P	I	P	I	I	P
WSM805	E	E	P	E	E	E	E	E	P	E
WSM819	P	P	I	P	E	P	P	P	P	E
WSM1292	I	I	P	I	P	I	P	I	I	P
WSM1293	E	E	E	E	E	E	E	E	P	E
WSM1294	P	I	P	P	E	P	E	P	P	P
WSM1296	I	P	I	I	P	I	P	I	I	P
WSM1297	I	P	I	I	P	I	P	I	I	P
WSM1307	P	P	P	P	P	E	E	E	P	E
WSM1348	E	E	P	E	E	E	E	E	P	P
WSM1414	I	I	I	I	P	I	P	I	I	P
WSM1646	I	P	P	I	P	I	P	I	I	I
WSM1648	I	P	I	I	I	I	P	P	I	P
WSM2290	I	P	P	I	I	I	P	P	I	P
WSM2316	P	P	I	P	P	P	E	E	I	E
WSM2317	P	E	P	P	E	P	P	E	P	E
WSM1284	E	I	P	E	E	E	E	E	P	E

[a] The capacity for N_2-fixation (effectiveness) was determined by comparing yields of inoculated plants with +N controls and then separating the strains into four groups; effective = >75% of +N control (E), partially effective = >20% but <75% of +N control (P), ineffective = <20% of +N control (I) or no nodulation (X). [b] Current Australian commercial inoculant strains (CC829 = *L. uliginosus* (Group D), CC1192 = *Cicer arietinum* (Group D), SU343 = *L. corniculatus*, WSM1497 = *Biserrula pelecinus*. Note: *L. ornithopodioides* accessions 97JH39, BR072, PI308038, SA5017 and 98LOI20 tested in Experiment 1a. *L. ornithopodioides* accessions SA834, SA5020, BR128, 98SN0-16 and BR298 tested in Experiment 1b

uninoculated *L. corniculatus* plants achieved nodulation at Karridale.

In the cross-row trials, the mean weight of nodules per plant (across all sampling distances) formed on *L. ornithopodioides* varied significantly according to which strain of rhizobia had been established in the soil the previous year (Table 6). At Mannum, strain WSM1348 resulted in more nodule weight (5.5 mg) than the other strains and five times more than the poorest strains (CC801a and WSM2316). At Wynarka, strain WSM1348 produced 6.6 mg of nodules, just less than the four top ranked strains, which were statistically similar (SRDI210, WSM1293, WSM805 and SRDI225) producing between 7.9 and 9.8 mg of nodules plant^{-1}. These four strains resulted in substantial nodulation, even in the outer sampling regions, with mean nodule weights of 6.9 mg (0–2 cm), 9.9 mg (>2–12 cm), 9.7 (>12–22 cm) and 7.8 mg (>22–32 cm). By comparison, the poorest strains at this site (strains CC821, SRDI140 and SRDI137 both trapped from Moroccan soil) produced <3 mg of nodule dry weight in the >22–32 cm sampling region.

At Karridale (experiment 6) background nodulation of *L. corniculatus* in the uninoculated plots, although substantial, was ineffective (Table 7). These nodules were small (4.3 mg plant^{-1}) and white and later identified by PCR RAPD to be associated with the naturalised *L. subbiflorus* (data not shown). The

Table 4 Summary of the nodulation and symbiotic effectiveness[a] of four strains of rhizobia (two Australian commercial strains and two highly effective strains on *L. ornithopodioides*) when inoculated onto 15 species of *Lotus* (Experiments 2a, 2b and 2c)

Lotus spp.	Accession/Cultivar	CC829[b]	SU343[b]	WSM1293	WSM1348
L. arenarius	54974	I	I	**E**	**E**
L. creticus	S1012	I	I	P	P
L. cytisoides		N	I	P	N
L. maroccanus	SA12953	X	X	**E**	**E**
L. ornithopodioides	SA5020	I	X	**E**	**E**
L. peregrinus	SA13753	I	I	P	P
L. corniculatus	cv. Grasslands Goldie	I	**E**	P	P
L. edulis	SA22716	X	P	**E**	**E**
L. mearnsii	27463	I	**E**	**E**	**E**
L. discolor		I	X	X	X
L. subbiflorus	Q18438 (P15304)	I	**E**	I	I
L. subbiflorus	cv. Rincon	X	P	I	I
L. uliginosus	cv. Grasslands Maku	**E**	I	I	I
L. purshianus	610	**E**	P	X	X
L. tenuis	S2791	I	P	I	I

[a] as previously defined, N = untested. [b] Current Australian commercial inoculant strains (CC829 = *L. uliginosus* and SU343 = *L. corniculatus*)

nodule mass of *L. corniculatus* in plots inoculated with WSM1293 and SU343 was greater than for the uninoculated plots (12 mg, 13 mg plant^{-1} respectively) with 100% of plants nodulating (Table 7). The nodulation of *L. corniculatus* by WSM1293 produced a top dry weight equivalent to SU343 (Table 7).

The nodule mass produced upon *L. ornithopodioides* was greatest with WSM1293 (52 mg plant^{-1}, cf 18 mg plant^{-1} SU343, Table 7), with commensurate differences in top dry weight of the plants (0.7 g plant^{-1} cf. 0.4 g plant^{-1}). The uninoculated control achieved only sparse nodulation (less than 1 mg nodule tissue plant^{-1}) and plants were pale and unthrifty.

The uninoculated plots of *L. ornithopodioides,* and also those treated with SU343, had increasing nodulation over time in the first year (1% to 18%, week 9 to 19; SU343 16% to 79%, Table 7) indicating progressive colonisation of these plots by nodulating rhizobia. Analysis of nodule occupancy by PCR in these plots in the second year (2010) showed them to

Table 5 Effect of inoculation treatment on the shoot dry weight (mg plant^{-1}) of five species of *Lotus* (Experiment 3). Interaction LSD (P=0.05) for comparing all values is 19

Inoculation treatment	*L. uliginosus* cv. Maku	*L. corniculatus* cv. Goldie	*L. ornithopodioides* SA33846	*L. peregrinus* SA5021	*L. maroccanus* SA12953
Uninoc.	5	7	11	6	3
CC821	6	15	12	25	36
CC829	45	10	8	6	5
SU343	9	48	9	6	3
SRDI110	3	23	45	8	101
SRDI137	3	26	55	10	61
SRDI140	6	26	35	32	5
SRDI144	5	24	33	6	66
SRDI210	5	25	65	5	31
SRDI225	3	8	48	51	7
WSM805	3	21	68	57	52
WSM1293	3	38	91	70	26
WSM1348	3	25	89	58	49

Table 6 Effect of inoculation treatment on the weight of nodules on the roots of *Lotus ornithopodioides* sampled at four distances from lines of inoculant established the previous year in the field at Mannum (Experiment 4) and Wynarka (Experiment 5) in South Australia. The LSD (P=0.05) for comparing the effect of inoculation treatment on nodule weight (mean of all distances) is 1.7 for Mannum and 2.5 for Wynarka

Inoculation treatment	Nodule dry weight (mg dry matter/plant)									
	0–2 cm		>2–12 cm		>12–22 cm		>22–32 cm		All distances (mean)	
	Mannum	Wynarka	Mannum	Wynarka	Mannum	Wynarka	Mannum	Wynarka	Mannum	Wynarka
Uninoc.	0.0	2.1	0.0	2.1	0.0	0.3	0.0	0.0	0.0	1.1
WSM1348	7.8	6.1	8.1	8.0	5.3	8.0	1.0	4.1	5.5	6.6
WSM1293	5.4	8.3	5.5	10.1	2.1	9.3	0.7	6.1	3.4	8.5
[a]CC801a	2.8	–	1.1	–	0.0	–	0.2	–	1.0	–
[a]CC820	4.8	–	4.4	–	2.8	–	0.3	–	3.1	–
WSM2316	1.5	–	1.2	–	0.0	–	0.1	–	0.7	–
WSM2317	5.0	–	4.9	–	2.5	–	0.3	–	3.2	–
CC821	–	5.1	–	8.1	–	1.9	–	2.9	–	4.5
SRDI137	–	4.5	–	7.5	–	3.3	–	2.0	–	4.3
SRDI140	–	3.9	–	6.0	–	5.7	–	2.3	–	4.5
SRD110	–	5.0	–	8.2	–	6.4	–	3.8	–	5.9
SRDI225	–	5.6	–	8.8	–	9.3	–	8.0	–	7.9
WSM805	–	6.2	–	8.5	–	8.3	–	9.3	–	8.1
SRDI210	–	7.5	–	12.0	–	12.0	–	7.7	–	9.8

[a] Strains CC801a and CC820 were included at Mannum because they had previously been shown to form effective symbioses with *L. ornithopodioides* as well as *L. halophilus* and *L. edulis*. Strain CC820 is also known to form effective symbioses with *Tetragonolobus purpureus* and *T. palaestinus* (N. Charman and R.A. Ballard, unpublished data)

be dominantly nodulated by WSM1293 (100%). SU343 was not detected in these plots. The uninoculated plots of *L. corniculatus* achieved 97% nodulation in 2009; as indicated by the MPN the background rhizobia were capable of nodulating this host. Analysis of these plots in 2010 showed them to be dominated by WSM1293 (67%). None of the nodules formed on *L. subbiflorus* in and around the experiment in 2010 were identified as containing either SU343 or WSM1293.

Discussion

These studies highlight the diverse and often specific symbiotic interactions that occur between *Lotus* nodulating rhizobia and some of the many species that comprise the genus *Lotus*. This host-strain specificity at the species level has been previously described (Brockwell et al. 1966; Safronova et al. 2004), and despite beginning this research with a wider diversity of rhizobia than previous studies, we did not detect a strain that could be considered as having a sufficiently broad host range to fix nitrogen maximally with both the traditional commercial species of Lotus (*L. corniculatus, L. uliginosus*), and the emerging species. A similar scenario exists for *Trifolium spp.* (Howieson et al. 2005) but not the *Medicago* genus, where recent research has provided commercial manufacturers with broad host range strains (Howieson et al. 2000b; Ballard et al. 2004; Garau et al. 2005). The studies also emphasise that the rhizobia currently provided in Australia for the inoculation of *L. corniculatus* (strain SU343) and *L. uliginosus* (strain CC829), apart from having a narrow host range, are unable to satisfy the symbiotic needs of several promising species (principally *L. ornithopodioides* and *L. maroccanus*) being evaluated by contemporary Australian pasture development programs, and do not reliably colonise infertile soils. It is enigmatic that one of the most highly traded and valuable legume genera (Howieson et al. 2008) has not managed a stronger

Table 7 Mean total nodule score plant^{-1}, mean nodule rating plant^{-1}, percentage of plants nodulated, mean nodule dry weight plant^{-1} (mg), mean dry weight plant^{-1} (g) and total herbage production (mean D/W kg ha^{-1}) at 9, 19 and 27 weeks after sowing generated by *Lotus ornithopodioides* (2000ITA7.1.C) and *L. corniculatus* (cv. San Gabriel) at Boathaugh farm, Augusta, WA 2009 (Experiment 6)

Measurement	Plant growth (wks)	Plot inoculant treatment		
		Uninoculated	WSM1293	SU343
Lotus ornithopodioides				
Mean total nodules plant^{-1}	9	0.25±0.2	135±12.6	7±3.8
% plants nodulated	9	1 (n=80)	100 (n=80)	16 (n=80)
Mean nodule rating plant^{-1}	19	0.21±0.1	6.50±0.23	2.13±0.08
% plants nodulated	19	18 (n=119)	100 (n=102)	79 (n=94)
D/W plant^{-1}	19	0.258±0.038	0.708±0.105	0.410±0.066
Nodule mass plant^{-1} (mg)	19	1.073±0.6	52.597±2.3	17.302±2.3
Mean D/W kg ha^{-1}	27	890±45	6450±75	3590±55
Lotus corniculatus				
Mean nodule rating plant^{-1}	19	2.67±0.3	3.69±0.5	4.24±0.5
% plants nodulated	19	97 (n=81)	100 (n=65)	100 (n=68)
D/W plant^{-1}	19	0.0356±0.001	0.102±0.017	0.128±0.016
Nodule mass plant^{-1} (mg)	19	4.355±0.3	12.05±1.7	13.0825±1.7
Mean D/W kg ha^{-1}	27	690±30	1590±40	3450±20

foothold in Australia, an observation perhaps explained by these results.

Nonetheless, these studies have identified a suite of effective strains which can now be used for the inoculation of some hitherto unexploited *Lotus* species. Pasture evaluators have been provided with the outstanding strains WSM1293 and WSM1348 for *L. ornithopodioides* and strain SRDI110 for *L. maroccanus* over the last decade, thus removing symbiotic impediments to the evaluation of these species.

This study has also exposed the high level of strain specificity in the weedy naturalised species *L. subbiflorus* and the promising salt tolerant species *L. tenuis* which, like *L. uliginosus*, achieved nitrogen fixation only with one experimental strain. However, some cross nodulation was achieved. *L. purshianus* formed an effective symbiosis with CC829 and SU343, and *L. corniculatus*, *L. edulis* and *L. mearnsii* achieved effective and partially effective symbioses with SU343, WSM1293 and WSM1348.

For the strains that were effective at nitrogen fixation with *L. ornithopodioides,* their performance could be further separated on their ability to reliably produce nodules under field conditions. The ability to colonise the soil, and to persist through seasons subsequent to the year of inoculation is a fundamental requirement for inoculant quality strains developed for self-regenerating annual legumes (Chatel and Parker 1973; Howieson 1995). At Mannum, strain WSM1348 produced five times the nodule mass of strain CC801a, while at Wynarka there was a two-fold difference in nodule mass, the best strains being SRDI210 and WSM1293. Background nodule bacteria associated with indigenous *L. subbiflorus* at Karridale, whilst incapable of nodulation of *L. ornithopodioides,* were able to nodulate *L. corniculatus.* However despite this, nodules of *L. corniculatus* were dominantly occupied by WSM1293 by the second year of the experiment in uninoculated plots. This indicates either a high level of competitiveness by WSM1293 in this niche against the background *L. subbiflorus* nodulating bacteria, or selection for effective nodulation as described Yates et al. (2005, 2008). Generally speaking, the persistence of the best strains was outstanding, with significant levels of nodulation occurring even in the 22–32 cm sampling region in South Australia. Where strains persist poorly, such as is the case with *Sinorhizobium meliloti* in very acid soils, nodules are rarely found past the 12 cm sampling region (Howieson and Ewing 1986; Ballard et al. 2004). While the

persistence of the *Lotus* rhizobia in the sandy neutral soils (pH_{Ca} 6.3 and 7.2) is encouraging, their evaluation in the more acidic soil was important as *Lotus* spp. are often promoted as having moderate tolerance of acidity (Schachtman and Kelman 1991), albeit in experiments conducted asymbiotically. There have been infrequent reports of nodulation problems in acidic environments, as well as large plant growth responses to lime application in *Lotus* (Wedderburn 1986; Lowther et al. 1987; Patrick and Lowther 1992) so it cannot be assumed that all genetic material related to this symbiosis is acid tolerant.

Effective strains are now available for several of the exotic *Lotus* species being evaluated in Australian pasture programs. Although many other factors could be considered in selecting the strains that will be used in commercial inoculants (see Brockwell et al. 1995), several of the strains in this study look to be promising candidates based on their effectiveness and soil persistence. An important character not yet assessed, however, is genetic stability. The *Mesorhizobium* have emerged as a genus with a propensity for transmission of a symbiosis island to resident soil bacteria (Sullivan et al. 1995; Nandasena et al. 2006), facilitated by a set of excision and integration genes (Nandasena et al. 2009). For promising strains such as WSM1293 we should exploit the genome sequence information now available to identify whether a similar set of transmission enabling genes are present. WSM1284, originally isolated from *B. pelecinus* and previously shown to have a broad host range (Nandasena et al. 2001), nodulated and fixed nitrogen well with *L. ornithopodioides,* suggesting it may well have been the recipient (and potentially a donor) of symbiotic genes. If the majority of background nodulating bacteria for *L. subbiflorus* in Australian soils are *Bradyrhizobium,* as reported for this legume in Uruguay (Irisarri et al. 1996), the potential for significant interaction is lessened. However this requires a separate study.

Ultimately, the balance of *Lotus* species which are adopted commercially and the systems they are adapted to will have a strong bearing on which rhizobial strains are progressed to commerce, given the very large symbiotic interactions between host species and rhizobial strain that we have observed. *L. ornithopodioides* is expected to be adopted in medium rainfall wheatbelt zones (350–500 mm annual rainfall, Loi et al. 2002) and *L. tenuis* in defined saline regions (Teakle et al. 2010) whereas the two resident commercial species *L. uliginosus* and *L. corniculatus* appear adapted only to wet regions with permanent pastures, hence the potential conflict between these symbioses might be limited in Australia by their geographic and edaphic separation.

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