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Bradyrhizobium sp. (*Lupinus*) in the winter rainfall region of South Africa

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Abstract Widespread cultivation of lupin has resulted in the establishment of effective populations of *Bradyrhizobium* sp. (*Lupinus*) in the winter rainfall region of the Western Cape, South Africa. To determine whether inoculation increased yields of *Lupinus angustifolius* L., field trials were carried out at five sites in this region. Populations ranged from 380 rhizobia g^{-1} in a moderately alkaline (pH 7.6) soil to >5,000 rhizobia g^{-1} in four moderately acid (pH 5.5–5.8) soils. Soil isolates were generally similar to the inoculant strain WU425 in nitrogen-fixing effectiveness but several were significantly less effective. Average effectiveness of isolates from certain soils differed significantly. Although inoculation failed to appreciably increase nodule occupancy by WU425 in acid soils containing high populations of rhizobia, nodule occupancy was increased to 98% in the low population alkaline soil. The latter site was later abandoned because of disease. At the other sites, analysis of seed dry mass and protein content showed that yields were not significantly increased by either inoculation, nitrogen fertiliser (45 kg N ha^{-1}) or molybdenum applications. Analysis of genomic DNA by PCR fingerprinting showed that WU425 (isolated in Western Australia) and serologically related strains from other cultures clustered separately from the soil isolates. Isolates from the four acid soils were genomically diverse, whereas isolates from the alkaline soil formed a homogeneous cluster. Further investigation is required to determine the benefit of inoculation in alkaline soils of the winter rainfall region of the Western Cape.

Keywords *Bradyrhizobium* sp. (*Lupinus*) · *Lupinus angustifolius* · Inoculation · Genomic fingerprinting · Soil rhizobia

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Introduction

Lupinus species were first introduced into the Western Cape winter rainfall region of South Africa at the beginning of the twentieth century, intensive cultivation taking place after 1947 when sweet cultivars were introduced. Sim (1958) considered inoculation of lupins essential in soils of the Western Cape and Nel (1962) demonstrated that inoculation improved nodulation and yields of *Lupinus albus* L. and *Lupinus angustifolius* L. Subsequently, widespread cultivation of lupin resulted in the establishment of effective populations of *Bradyrhizobium* sp. (*Lupinus*) in soils of the Western Cape (Strijdom 1998).

The first locally produced lupin inoculant was introduced in the 1960s and contained a local isolate, strain VK7. Strain VK7 was replaced in the 1970s by the Australian inoculant strain WU425, which is effective on lupin and serradella (*Ornithopus* sp.; Gault et al. 1986; Ballard 1996). A sub-culture of strain WU425, designated VK10, is currently maintained in the South African Rhizobium Collection. Despite the availability of this strain, as many as thirty percent of the farmers in the Western Cape rely on naturally-occurring soil populations for nodulation of their crop (H.J.C. Agenbag, personal communication).

Soil populations of rhizobia present a competitive barrier to nodulation by inoculant strains, the size and effectiveness of such populations being critical to the successful use of inoculants (Weaver and Frederick 1974; Singleton and Tavares 1986; Thies et al. 1992). If the population is large and effective it can be assumed that inoculation is unnecessary, whereas if it is small and poorly effective then inoculation may be beneficial (Brockwell et al. 1995). Soil populations of *Bradyrhizobium* sp. (*Lupinus*) corresponding to both types have been described. A small indigenous population in an Oregon soil contained rhizobia that were less effective than strain WU425 on lupin and serradella (Bottomley et al. 1994), whereas Western Australian soils contained abundant

naturalised rhizobia that were as effective as WU425 when inoculated on serradella (McInnes et al. 1996).

The present study examined the size, effectiveness and diversity of populations of lupin-nodulating rhizobia at five different localities in the Western Cape and assessed whether inoculation was beneficial at these sites. A nitrogen fertiliser treatment was included for comparative purposes and a molybdenum seed treatment. The micronutrient is often routinely applied as beneficial for nitrogen fixation (Graham and Chatel 1983). Genomic diversity of rhizobia in the five Western Cape soils was investigated by means of rep-PCR (Schneider and de Bruijn 1996).

The study showed that inoculation was of insignificant benefit to lupin grown in acid Western Cape soils containing large diverse natural populations of rhizobia. However, inoculation markedly increased nodule occupancy by WU425 in an alkaline soil containing a small homogeneous natural population.

Materials and methods

Biological material

Strains of rhizobia and their origins are listed in Table 1. Soil isolates and their locality are shown in Fig. 4. Lupin seed (*L. angustifolia* cv. Wonga) was a gift from Dr Nick Kotze of Agricol Pty.

Table 1 Bacterial strains used in this study

Strain	Host plant	Source[a]
Bradyrhizobium elkanii USDA76	Soybean	USDA
Bradyrhizobium japonicum USDA6	Soybean	USDA
Bradyrhizobium sp. (*Lupinus*)		
WU425	Lupin	WSM
WSM425 (= WU425)	Lupin	Urbana
Lup025	Lupin	Urbana
VK7	Lupin	SARC
VK10 (= WU425)	Lupin	SARC
Mesorhizobium plurifarium LMG11892	*Acacia senegal*	LMG
Rhizobium etli CFN42	Bean	CIFN
Sinorhizobium fredii LMG6217	Soybean	LMG
Sinorhizobium meliloti LMG6133	Lucerne	LMG

[a] *USDA* U.S. Department of Agriculture, Beltsville, Maryland; *WSM Rhizobium* genebank, Murdoch University, Western Australia; *Urbana* Urbana laboratories, Missouri; *SARC* South African Rhizobium Collection, Pretoria, South Africa; *LMG* Laboratorium voor Microbiologie, Universiteit Gent, Belgium; *CIFN* Centro de Investigacion sobre Fijacion de Nitrageno, Universidad Nacional Autonoma de Mexico, Mexico

Greenhouse experiments

Soil populations of rhizobia were estimated by a most probable number (MPN) plant infection technique, using plants grown in sterile Leonard jars containing sand and N-free Hoagland solution as previously described (Bloem and Law 2001). Isolates were compared with strain VK10 for nitrogen-fixing effectiveness on lupin grown for 8 weeks in Leonard jars. Four replicates with three plants in each jar were used in a completely randomised experimental design.

Field trials

Field trials were carried out during June-November 2000 at localities in the western Swartland (Hopefield, Langewens, Eendekuil) and southern Rûens (Roodebloem, Tygerhoek) regions of the Western Cape. While lupins are well-established in the Swartland, they were introduced more recently into the Rûens region. Soils were fertilised and prepared for planting by the Western Cape Department of Agriculture. Random soil samples were taken at planting for chemical analysis and rhizobial populations estimated using the MPN technique. Nitrogen-fixing effectiveness of soil populations was determined by the whole-soil method of Brockwell et al. (1988), and isolates were obtained from nodulated plants. Soils were analysed by the ARC-Industrial Crops Institute for exchangeable cations (Na, K, Ca, Mg) and available P. Properties of the soils are shown in Table 2.

The field experiments were carried out under dry-land conditions and were laid out in a randomised factorial complete block design with four replications. Uninoculated and inoculated treatments were included as well as an uninoculated nitrogen treatment in which lime ammonium nitrate equivalent to 45 kg N ha^{-1} was applied alongside seed furrows. In addition, lupin seed was treated with either 2% methyl cellulose sticker containing 50 ppm sodium molybdate, or sticker without molybdate. Peat inoculant containing *Bradyrhizobium* sp. (*Lupinus*) strain VK10 was applied to sticker-coated seed at a rate of about 10^6 colony forming units per seed. Each treatment plot was 3 m long and contained five rows with a 0.75 m inter-row spacing. Individual plots were separated by a 1-m space. Seed was planted 10 cm apart in moist soils, except in Tygerhoek, where conditions were dry. Depending on plant growth, plants were sampled 4 and/or 6 weeks after planting by removing ten plants from an outer row of a plot for shoot dry mass and root nodule occupancy analysis. Only five plants were removed at Roodebloem because of poor growth and survival. At the end of the season, all plants in the inner three rows were harvested. Seed dry mass was determined and total-N estimated by the ARC-Institute for Soil, Climate and Water using the Dumas dry oxidation method. Protein content was calculated by multiplying percent N by 6.25.

Nodule occupancy

Nodules were removed from roots of 4-week-old plants at Hopefield and Eendekuil where growth was faster. Nodules from uninoculated and inoculated treatments were surface-sterilised and used to isolate rhizobia for serological identification. At 6 weeks, nodules were removed from plants at all five sites and stored desiccated over silica gel at 4°C.

Table 2 Properties of soils (*MPN* most probable number)

Soil	MPN cells g^{-1}	Sand %	Silt %	Clay %	pH (H_2O)	Na ppm	P ppm	K ppm	Ca ppm	Mg ppm
Hopefield	1.9×10^4	94	0	6	5.5	8	35	49	161	35
Langewens	9.8×10^4	80	10	11	5.4	10	85	199	364	81
Eendekuil	5.5×10^4	86	2	12	5.8	10	47	103	54	10
Roodebloem	3.8×10^2	67	14	20	7.6	10	31	79	2,835	92
Tygerhoek	6.5×10^3	60	21	20	5.5	52	38	253	580	221

Purified antibodies were used to detect strain VK10 in nodules by an indirect ELISA method (Kishinevsky and Moaz 1983). Nodules were crushed with plastic rods directly in microtiter plate wells, whereas rhizobial cell suspensions were boiled for 30 min before addition to wells. Initial analysis of isolates indicated that the VK10 antibody preparation reacted specifically and did not require removal of cross-reactive antibodies.

Statistical analyses

Variance analysis was performed using the Genstat 5 (3.2) programme (NAG, United Kingdom). Percentage nodule occupancy values were transformed using arcsin$\sqrt{\%}$ for variance analysis and back-transformed for presentation of data.

DNA purification and PCR analysis

Whole cell DNA was extracted from cells using the method of Wilson (1989). Purified DNA was quantified using a Dyna Quant 200 fluorometer (Hoefer, California) and Hoescht H 33258 intercalating dye (Polysciences, Pennsylvania). DNA concentration was adjusted to 25–50 ng μl^{-1} for each strain.

PCR was performed in 10-µl volumes containing PCR buffer (10 mM Tris-HCl, pH 9.0 at 25°C; 50 mM KCl; 0.1% Triton X-100), 3.5 mM $MgCl_2$; 150 µM dNTPs; 1.0 µM primer; DMSO 2.5%; BSA 160 µg; mercaptoethanol 10 mM; Taq polymerase 0.15 units per reaction volume; 25–50 ng template μl^{-1}. Primers used in PCR amplifications included repetitive BOXA1R, ERIC and REP sequences (Rademaker and de Bruijn 1997) and the M13 phage core primer sequence according to Vassart et al. (1987). PCR reaction volumes were overlaid with 12 µl sterile mineral oil and PCR amplifications performed in a Hybaid Omni Gene thermocycler (Teddington, United Kingdom) according to Rademaker and de Bruijn (1997) for rep-PCR primers, and Vassart et al. (1987) for the M13 phage primer.

All PCR products were separated in 1.8% agarose containing TBE buffer (0.089 M Tris, 0.089 M boric acid, 20 mM EDTA) at 80 constant volt. Gels were stained with ethidium bromide and gel images captured with the aid of CCD-photography (Ultraviolet Products, Cambridge, United Kingdom). Digitised images were stored as TIFF files. Rep-PCR patterns were normalised and cluster analysis was performed with the aid of GelCompar II software version 2.5 (Applied Maths, Kortrijk, Belgium). Dendrograms were constructed based on whole curves using Pearson's coefficient and the UPGMA type of dendrogram as recommended by Rademaker and de Bruijn (1997).

Results

Soils

Soils at the various localities were sandy to sandy clay in texture (Table 2). The four moderately acid soils (pH 5.5–5.8) each contained large populations of rhizobia (>5,000 cells g^{-1}), whereas the moderately alkaline Roodebloem soil (pH 7.6) contained only 380 cells g^{-1} (Table 2).

Whole-soil inoculation of lupin elicited an effective response to rhizobial populations in each of the soils (Table 3). Higher yields were obtained with soils diluted 10^{-1} than with 10^{-2} dilutions but generally plants of both dilutions were well-nodulated and their growth was similar or superior to that of VK10-inoculated plants. Lowest yields were with dilutions of Roodebloem soil (Table 3).

Serological analysis of strains and isolates

Positive ELISA reactions were obtained when antiserum prepared against strains VK7 and VK10, respectively, was tested against the heterologous strain. Both antisera also reacted positively with strains WU425, WSM425 and Lup025. Immune diffusion reactions of the respective strains were identical when cell suspensions were tested against VK7 antiserum (Fig. 1). Using VK10 antiserum, identical major precipitin bands were formed by all the strains, but a minor precipitin band was absent in the reaction with VK7 and Lup025 (Fig. 1).

Further serological analysis by ELISA of 56 soil isolates showed that only one (1S4) reacted with VK10 antibodies. Immune diffusion analysis showed that 1S4 formed a non-identical precipitin band to that of VK10 (data not shown).

Table 3 Response of lupin to inoculation with strain VK10 and dilutions of soil from five localities in the Western Cape. *Values followed by the same letter* do not differ significantly. LSD (Tukey, $P = 0.05$) $= 0.71$

Soil/strain	Soil dilution	Shoot dry mass[a] (g)
Eendekuil	10^{-1}	2.55a
Hopefield	10^{-1}	2.21ab
Eendekuil	10^{-2}	2.00abc
Hopefield	10^{-2}	1.98abcd
Langewens	10^{-1}	1.92abcd
Langewens	10^{-2}	1.82bcde
Tygerhoek	10^{-1}	1.74bcde
VK10		1.58bcdef
Tygerhoek	10^{-2}	1.41cdef
Roodebloem	10^{-1}	1.34cdef
Roodebloem	10^{-2}	1.16ef
Uninoculated		0.94f

[a] Mean of three replicates, three plants per replicate

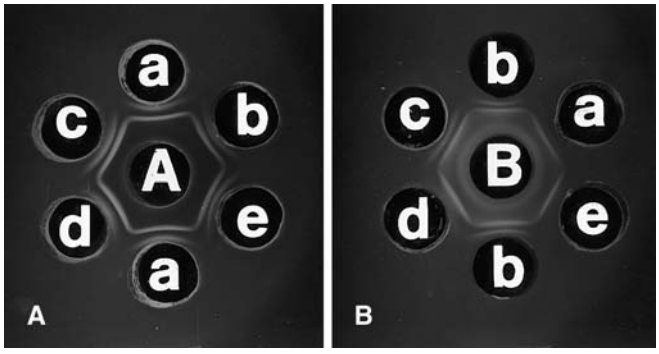

Fig. 1A, B Immune diffusion analysis of five strains of *Bradyrhizobium* sp. (*Lupinus*). Central wells contained antibodies to VK7 (**A**) and VK10 (**B**). Outer wells contained cell suspensions of VK7 (*a*), VK10 (*b*), WU425 (*c*), WSM425 (*d*) and Lup025 (*e*)

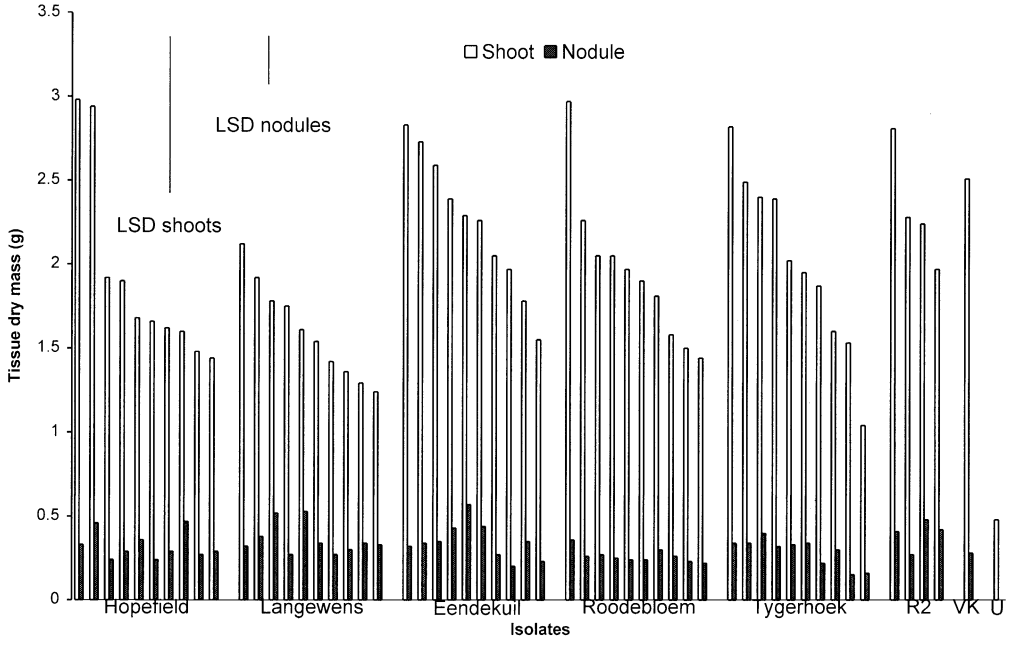

Fig. 2 Dry mass of shoots and nodules of lupin after inoculation with strain VK10 and *Bradyrhizobia* isolated from soils at Hopefield, Langewens, Eendekuil, Roodebloem, Rooodebloem 2 (*R2*) and Tygerhoek in the Western Cape. Associated shoot and nodule bars each represent an individual isolate, except for: *VK* strain VK10, *U* uninoculated control

Table 4 Effectiveness ratios (isolate shoot dry mass:VK10 shoot dry mass) and nodule efficiencies (ratio shoot dry mass:nodule dry mass) of isolates from five Western Cape soils. *Values followed by the same letter* do not differ significantly. LSD (Tukey, P =0.05) =0.23 (Effectiveness ratio) and 1.9 (Nodule efficiency)

Locality	Effectiveness ratio[a]	Nodule efficiency[a]
Eendekuil	0.89±0.05a	6.8±0.6a
Tygerhoek	0.80±0.07ab	7.1±0.5a
Roodebloem	0.78±0.08ab	7.4±0.3a
Hopefield	0.77±0.07ab	6.1±0.5ab
Langewens	0.64±0.04b	4.4±0.4b

[a] Each value is the average (±SE) of means for each of 10 isolates

Symbiotic properties of field isolates

At least ten non-VK10 isolates from each soil were analysed for nitrogen-fixing effectiveness. As rep-PCR analysis showed high genomic similarity between the Roodebloem isolates, the effectiveness study included four isolates from a nearby locality (Roodebloem 2) where the soil pH was 6.4.

Under greenhouse conditions, 37 (68%) of the isolates did not differ significantly in effectiveness from VK10 (Fig. 2). Only 8 (17%) elicited higher shoot dry mass yields than VK10 (Fig. 2). Several from Hopefield (1), Langewens (4), Roodebloem (1), and Tygerhoek (1) were significantly less effective than VK10 (Fig. 2). Effectiveness of isolates from within individual soils varied appreciably; only those from Langewens did not show significant differences. The average effectiveness ratio (isolate shoot dry mass ratio: VK10 shoot dry mass ratio) of isolates from each soil was less than 0.9 (Table 4), that of Eendekuil (0.89) being significantly greater than Langewens (0.64). Intermediate values were obtained for isolates from Tygerhoek, Roodebloem and Hopefield (Table 4). The ratio shoot mass:nodule mass (nodule efficiency) was used as a measure of efficiency of nodules in stimulating plant growth through nitrogen fixation. Analysis showed that nodule efficiencies of isolates from Eendekuil, Tygerhoek and Roodebloem were significantly greater than isolates from Langewens, an intermediate value being obtained for Hopefield (Table 4).

Field trials

Best growth conditions were at the Hopefield and Eendekuil sites and plants were sampled at 4 and 6 weeks as well as at final harvest. At 6 weeks, plants at Hopefield were in flower while those at Eendekuil were about to flower. Growth at Langewens and Tygerhoek was slower and plants were sampled only at 6 weeks and at harvest. Plants at the latter sites were still in the vegetative growth stage at 6 weeks. At Roodebloem, nodule occupancy was analysed at 6 weeks, but the site later had to be abandoned because of disease. At Hopefield, Eendekuil and Tygerhoek, analysis of plant shoots harvested at 28 days and/or 42 days showed no response to inoculation, nitrogen or molybdenum seed treatments (data not shown).

As a result of previous inoculation, strain VK10 was detected in a substantial number of nodules formed on uninoculated plant treatments at Langewens (23.5%), Hopefield (25.9%) and Eendekuil (55.6%; Table 5). Although there was no record of previous inoculation with VK10 in either of the Rûens soils, a small number of uninoculated plant nodules from Tygerhoek (2.1%) and Roodebloem (5.9%) reacted with VK10 antibodies (Table 5).

At Hopefield and Langewens, nodule occupancy by VK10 was higher in inoculated treatments but differences were statistically significant only for Hopefield plants

Table 5 Nodule occupancy by strain VK10 following inoculation with molybdenum-treated lupin seed at five localities in the Western Cape

Locality		% Nodule occupancy by VK10[a]					
		28 days			42 days		
		Mo+	Mo–	Mean	Mo+	Mo–	Mean
Hopefield	Inoculated	22.5	28.2	25.7[b]	32.8	36.5	34.1
	Uninoculated	11.2	11.8	11.5[b]	28.6	23.2	25.9
	Mean	18.0	11.9		30.6	29.2	
Langewens	Inoculated				29.8	38.6	34.1
	Uninoculated				24.5	22.5	23.5
	Mean				27.1	30.3	
Eendekuil	Inoculated	5.9[c]	26.8[c]	14.8	29.7	44.4	36.9
	Uninoculated	15.4[c]	18.1[c]	16.7	49.8	61.1	55.6
	Mean	10.1	22.3		10.1	22.3	
Roodebloem	Inoculated				98.6	99.3	99.0
	Uninoculated				7.9	4.0	5.9
	Mean				75.9	88.1	
Tygerhoek	Inoculated				4.3	8.4	6.2
	Uninoculated				0.3	5.4	2.1
	Mean				1.7	7.1	

[a] Values for Hopefield at 28 days were each from 62–78 nodules and were analysed by the χ^2 method. Other values are each the mean of four replicates and variance analysis was used. Fifty nodules were typed per replicate except for uninoculated treatments from Roodebloem and Tygerhoek where some replicates had fewer nodules

[b] Significant difference between these values (χ^2=14.6, *df* =1)

[c] Treatment interaction, LSD (Tukey, P =0.05) =3.6

harvested at 28 days (Table 5). Nodule occupancy by VK10 tended to be lower in inoculated plants treated with sodium molybdate at planting, but values were statistically significant only in 28-day-old plants at Eendekuil (Table 5). The latter result is difficult to interpret, however, as VK10 nodule occupancy in inoculated molybdenum-treated plants was also significantly less than in the uninoculated treatments (Table 5).

Almost all nodules of inoculated plants at Roodebloem were occupied by VK10, irrespective of molybdenum treatment (Table 5). At Tygerhoek, however, maximum nodule occupancy by VK10 in inoculated plants was only 8.4% (Table 5).

Variance analysis of seed dry mass and protein content showed that yields were not significantly (P =0.05) increased by either inoculation, nitrogen fertiliser or molybdenum applications (data not shown). Average seed yields (tonnes ha^{-1}) at harvest were Hopefield 1.95, Eendekuil 0.87, Langewens 0.74 and Tygerhoek 0.46.

Rep-PCR analysis of genomic DNA

Genomic DNA from the lupin isolates as well as from several different rhizobial species were subjected to PCR reactions using REP, ERIC, BOX and M13 phage primers. The reaction products were separated by agarose gel electrophoresis yielding DNA band patterns 2 kb to 100 bp in size (data not shown). Cluster analysis of the patterns showed that the serologically related strains VK7, VK10, WU425, WSM425 and Lup025 could be differentiated from one another, although patterns and grouping of isolates differed according to the primer used. For example, the strains all had high (>75%) similarity using BOX and ERIC primers, whereas appreciable divergence was observed between VK10 and the other four strains using M13 phage primer, and between the VK10, WSM425, WU425 and VK7, Lup025 clusters using REP primers (Fig. 3). When data from all four primers was combined into a single UPGMA dendrogram, WU425 and derived strains WSM425 and VK10 diverged from Lup025 and VK 7 (Fig. 4).

In Fig. 4, type strains of *Sinorhizobium fredii* (LMG6217), *Sinorhizobium meliloti* (LMG6133), *Mesorhizobium plurifarium* (LMG11892), and *Bradyrhizobium elkanii* (USDA76) grouped separately from one another (similarity <50%) as well as from the isolates of *Bradyrhizobium* sp. (*Lupinus*) from Western Cape soils. The latter consisted of two major groups, the larger of which contained 61% of the isolates as well as the cluster composed of the five related inoculant strains (Fig. 4). It also contained *Rhizobium etli* strain CFN42. The smaller group contained the remaining lupin nodule isolates together with *Bradyrhizobium japonicum* strain USDA6 (Fig. 4).

The rep-PCR dendrogram showed that isolates from the four acid soils were genomically diverse, whereas isolates from the alkaline Roodebloem soil formed a homogeneous group. Ten of the 11 isolates in the latter group formed a cluster with similarity >60%, of which 7 isolates had similarity >90% (Fig. 4). However, diversity between the four isolates from the nearby Roodebloem 2 soil was greater than that of the isolates from the Roodebloem soil (Fig. 4). Tygerhoek had five isolates that grouped together with similarity level ≥70%. In most other instances, similarity ≥70% within a soil was limited

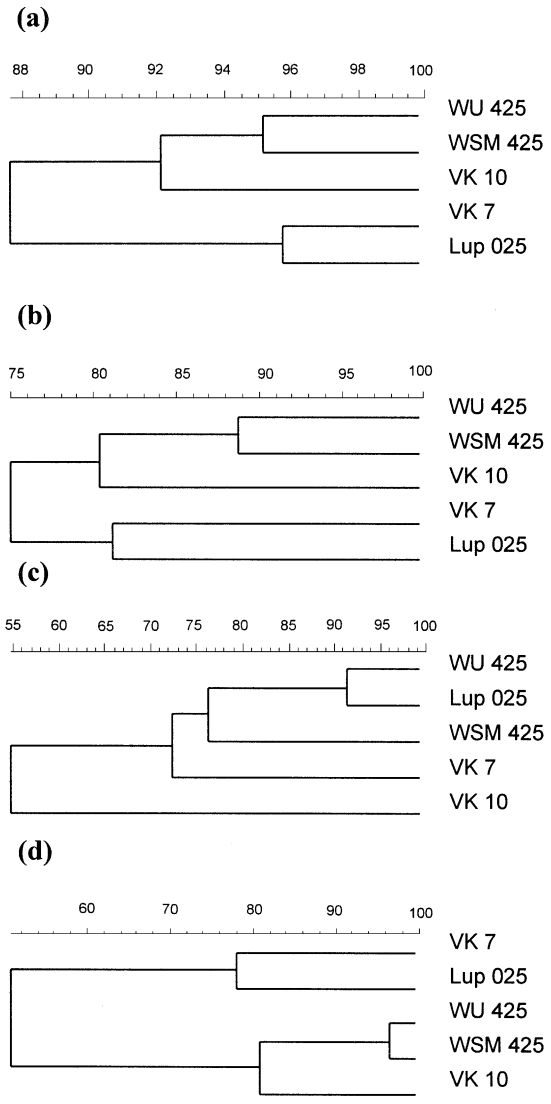

Fig. 3a–d UPGMA-dendrograms derived from PCR fingerprints of *Bradyrhizobium* sp. (*Lupinus*) strains serologically related to VK10, generated using BOXA1R (**a**), ERIC (**b**), M13 phage (**c**) and REP (**d**) primers. *Scale* shows percentage similarity

to pairs of isolates and was not appreciably higher than that observed between isolates from different localities. For example, isolates 3S9 and 3S17 from Eendekuil clustered with the five related isolates from distant Tygerhoek (Fig. 4).

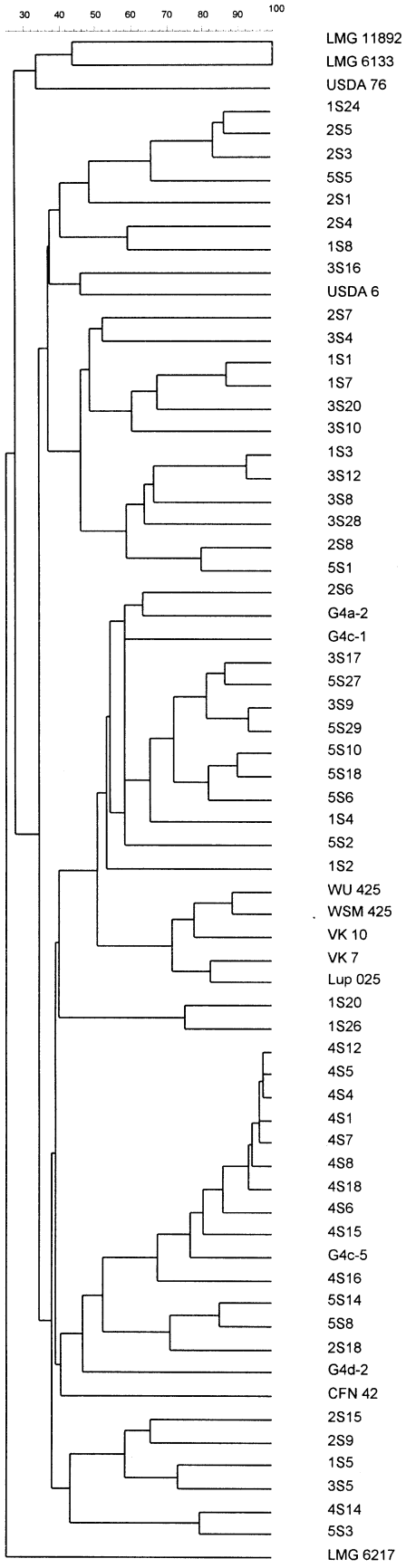

Fig. 4 UPGMA-dendrogram derived from combined PCR fingerprints of rhizobial strains and *Bradyrhizobium* sp. (*Lupinus*) isolates, generated using BOXA1R, ERIC, M13 phage and REP primers. Strains are described in Table 1. Locality of isolates is indicated by prefix *1S* Hopefield, *2S* Langewens, *3S* Eendekuil, *4S* Roodebloem, *5S* Tygerhoek, *G4* Roodebloem 2. *Scale* shows percentage similarity

Discussion

The size, effectiveness and diversity of populations of lupin rhizobia found in soils of the Western Cape resembled those described in soils of Western Australia by McInnes et al. (1996). Although all five Western Cape soils contained effective populations of lupin rhizobia, it is noteworthy that the smallest population (380 cells g^{-1}) was in the Roodebloem soil which had a relatively high pH of 7.6. In contrast, moderately acid soils with pH 5.4–5.8 had larger populations (≥6,500 cells g^{-1}). Sensitivity of lupin rhizobia to high pH was previously demonstrated by a survey in France which showed that soils with pH <6 had over 100 cells g^{-1} whereas those with pH >7 had less than 1 cell g^{-1} (Amarger et al. 1984). Lower numbers of lupin rhizobia in alkaline soils appears to be associated with poorer root growth and reduced nodulation by lupin at pH levels above 6.0 (Tang et al. 1993; Tang and Robson 1993, 1995).

Brockwell et al. (1988) suggested that the whole-soil inoculation method could provide a useful guide to the need for inoculation in the field. In the present study, whole-soil inoculation showed that rhizobial populations in Western Cape soils had levels of nitrogen-fixing effectiveness comparable to the inoculant strain VK10 (Table 3). Effectiveness ranked in the order Eendekuil ≥ Hopefield ≥ Langewens ≥ Tygerhoek ≥ Roodebloem. Roodebloem ranked last because of reduced nodulation due to the small population size (Table 3). The three soils in the Swartland were previously inoculated with VK10 and nodulation data showed that each contained an appreciable proportion (23–55%) of this strain (Table 5). Analysis of non-VK10 isolates from Eendekuil indicated that they had significantly higher nitrogen-fixing effectiveness and nodule efficiency ratios than those from Langewens, whereas isolates from Hopefield, Tygerhoek and Roodebloem were similar (Table 4).

Although most soil isolates did not differ in effectiveness from VK10, a few were significantly less effective (Fig. 1). It is noteworthy that the average effectiveness ratio (isolate:VK10) of each set of soil isolates ranged from 0.89 at Hopefield to 0.64 at Langewens (Table 4). Although this suggests that nitrogen fixation in a soil such as Langewens could be improved by increasing VK10 nodule occupancy, at least 66% of the nodules would have to be formed by the inoculant strain (Weaver and Frederick 1974; Singleton and Tavares 1986; Thies et al. 1992). This is difficult to achieve and in the present study conventional seed inoculation failed to significantly increase nodule occupancy by VK10 in soils containing high populations of rhizobia (Table 5). It is therefore not surprising that inoculation did not increase lupin yields in these soils (data not shown).

Although inoculation was not previously practised at Roodebloem and Tygerhoek (H.J.C. Agenbag, personal communication), a small proportion (<6%) of the nodules from uninoculated plants at each site reacted with VK10 antibodies (Table 5). Although cross-reaction with naturally-occurring strains may have occurred at each site, a more likely possibility is that VK10 was introduced on contaminated seed (Perez-Ramirez et al. 1998). Despite the low levels of VK10 at Tygerhoek, inoculation failed to improve VK10 nodule occupancy, which remained below 10% as a result of competition from the large natural population. In contrast, nodule occupancy by VK10 was increased to 98% in the low population soil at Roodebloem (Table 5). Unfortunately, disease prevented harvesting of plants at this site to demonstrate a yield response.

Although sodium molybdate is toxic to rhizobia (Graham and Chatel 1983), incorporation of this micronutrient generally caused only minor, statistically insignificant, reductions in nodule occupancy by VK10 in inoculated treatments (Table 5). Sodium molybdate application did not have a beneficial effect on yields, suggesting that molybdenum deficiency was not a significant factor (data not shown). Nitrogen fertiliser applied at the level recommended for grain crops in the Western Cape did not significantly increase lupin yields, indicating that the supply of fixed nitrogen was adequate at each site (data not shown). Ayisi et al. (1992) similarly found that nitrogen treatments did not increase yields of *L. albus* over inoculated treatments in a loamy sand soil.

During tests to determine the specificity of VK10 antiserum used for nodule identifications, the SARC culture VK10 (= WU425) and the previous inoculant strain, VK7, were found to be almost identical serologically (Fig. 1). Subsequent reactions with a fresh culture of WU425 from Murdoch University confirmed this relationship. It is noteworthy that the strains were all serologically related to Lup025, originally isolated from south-eastern United States probably in the 1930s or 1940s (T. Wacek, personal communication). In contrast, WU425 was isolated in Western Australia (Ballard 1996) while VK7 was isolated in 1965 at Stellenbosch in the Western Cape.

The five strains were further compared using rep-PCR fingerprinting, a method designed to distinguish rhizobia at the sub-species and strain level (Schneider and de Bruijn 1996), and capable of differentiating strains of *B. japonicum* that are serologically indistinguishable (Judd et al. 1993). Mathis et al. (1997) were able to discriminate separately maintained cultures of the same *B. japonicum* strain using a related PCR technique. Similarly, the serologically related lupin strains could be differentiated from one another by rep-PCR, although groupings varied with different primers (Fig. 3). This variation was encountered by Judd et al. (1993) and Nick et al. (1999) who resorted to combined dendrograms. This approach was used to generate the dendrogram shown in Fig. 4, in which the five WU425-related strains/cultures cluster together.

The genetic relatedness between strains WU425, Lup025 and VK7 was interesting, considering that each was isolated on a different continent. Their nitrogen-fixing effectiveness on *L. angustifolius* was also similar (data not shown), thus the reason for replacing VK7 with WU425 is not clear. Presumably the strains were assumed

to be different and the decision was based on the previous track-record of WU425 (Ballard 1996). The observation that cultures VK10 and WSM425 could be differentiated by rep-PCR from the parent strain WU425, emphasises the value of molecular fingerprinting techniques in benchmarking strains and monitoring genetic variation (Thies et al. 2001).

Barrera et al. (1997) analysed isolates of *Bradyrhizobium* sp. (*Lupinus*) indigenous to Mexican soils and found that their 16S rRNA sequences were highly related to the *B. japonicum* type strain USDA6 but not to the *B. elkanii* strain USDA76. The rep-PCR results similarly showed that isolates of lupin rhizobia from the Western Cape were more closely related to USDA6. In contrast, *B. elkanii* strain USDA76 grouped apart from the isolates as did arbitrarily chosen species of *Sinorhizobium* and *Mesorhizobium* (Fig. 4). The *R. etli* type strain CFN42 grouped with lupin rhizobia, however, emphasising that rep-PCR must be used with caution at the species level (Nick et al. 1999). The five inoculant strains described above, differed serologically from other *Bradyrhizobium* sp. (*Lupinus*) isolated from Western Cape soils, and formed a unique cluster on rep-PCR analysis (Fig. 4).

The genomic diversity encountered amongst lupin rhizobia in the Western Cape resembled that of *Bradyrhizobium* sp. (*Lupinus*) populations in Western Australia (McInnes et al. 1996). Both groups originated from naturalised rhizobia, yet appeared to have as great a diversity as that found in populations associated with lupin species indigenous to the United States and Mexico (Bottomley et al. 1994; Barrera et al. 1997). Isolates from the four acid soils were genomically diverse, whereas isolates from the alkaline Roodebloem soil formed a homogeneous group. As the four isolates from the nearby Roodebloem 2 soil with intermediate pH 6.4 had greater genomic diversity, further study of the effect of soil pH on this property would be of interest.

Inoculation is usually essential when a crop is newly planted, but not if sufficient effective rhizobia are already present in the soil (Brockwell et al. 1995). The present study demonstrated that inoculation was of little benefit in the four moderately acid Western Cape soils with large soil populations, and would be of value only as a precautionary measure. However, nodulation results indicated that the situation in the alkaline Roodebloem soil differed, as this soil contained a small homogeneous population that was unable to compete against inoculated VK10 (Table 5). Australian researchers have shown that the lupin symbiosis is sensitive to alkaline conditions and that inoculation improved nodulation and nitrogen fixation in alkaline soils (Tang and Robson 1995). A similar situation appeared to exist at Roodebloem and further investigation is required to determine the extent to which inoculation may benefit lupin yields in alkaline soils of the Western Cape.

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