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Techniques for the Selection and Development of Elite Inoculant Strains of *Rhizobium leguminosarum* in Southern Australia

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Abstract

The task of matching rhizobial strains to host legumes is not a new one. However, as the price Australian farmers receive for their goods continues to decline relative to the cost of their inputs, more than ever they require optimal performance from their legumes. The suite of legumes in use in southern Australia has changed dramatically during the last decade, and will continue to do so. We report on the development of a successful four phase program for selecting optimal microsymbionts for these legumes such that commercial inocula are available when required. Elite inoculant strains have been selected following screening for nitrogen fixation, edaphic adaptation and performance *in situ* of rhizobial germplasm originally collected from targetted locations in the Mediterranean region.

Keywords: Acidity, infertility, legume, nitrogen fixation, *Rhizobium leguminosarum*

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1. Introduction

Southern Australia has seen a dramatic increase in the diversity of pulse and pasture legume species introduced into its agricultural systems in the last decade. These legumes and their root-nodule bacteria have their evolutionary origins outside Australia. Given the relative infertility of many Australian soils, this provides a serious challenge to the adaptation of these symbioses to the new edaphic environment.

The impetus for the spate of new legume introductions has been the rapidly changing biological, environmental and economic circumstances facing rural producers in Australia (Reeve and Ewing, 1993). Key factors include the development of herbicide resistance in weeds, rising water tables leading to waterlogging and salinity, the declining profitability of rural production and the altered relative profitability of crops in comparison to animal based products (Howieson et al., 2000a).

The wider adoption of legumes, with their concomitant impacts upon soil fertility and disease control, offers a means of improving productivity without increasing production costs (Reeve and Ewing, 1993). To facilitate the wider adoption of legumes, substantial alterations to both pasture and pulse legume ideotypes are required to fit new and emerging farming systems. Scientific research in southern Australia has rapidly accommodated the new requirements of farmers with the release of many new legume cultivars and species of established legume genera (Siddique et al., 1998) and even new genera (Howieson et al., 1995).

The legume introduction programs of southern Australia have been supported in parallel by intensive rhizobial selection for elite inoculant strains. Whilst not a novel concept, in this case the two programs of plant development and strain selection were completely integrated. The rhizobial support has been imperative for the success of the plant introductions because in many cases the new legumes were being targetted for soils whose characteristics were substantially different to those on which the legumes had traditionally been cultivated. It is reported that the southern Australian indigenous root-nodule bacteria rarely form effective nodules on legumes of exotic origin (Lange, 1961).

Rhizobial strain selection in our particular program has focussed on the selection of root-nodule bacteria that offer a broad host-range for nitrogen fixation, and adaptation to the anticipated soil niche of the host legume. An essential trait sought in elite strains for commercial use in Australian agriculture is their ability to fix nitrogen optimally with a broad range of host species because inoculant manufacturers are limited in the number of specific strains they can economically produce. In the pulse legumes, for example, inoculant manufacturers have a pragmatic requirement to service several genera (*Lathyrus, Lens, Pisum* and *Vicia*) with one or two inoculant strains. Within

the pasture legumes, manufacturers service the requirements of ten *Trifolium* spp with only two inoculant strains.

The philosophy behind some of the selection criteria we impose has recently been discussed (Howieson, 1995a,b). This paper reports the methodology of the four phase process we use for strain selection and the results provide examples of the successful selection of elite rhizobial genotypes for both pulse and pasture legumes nodulated by *Rhizobium leguminosarum*. The achievements of this program in the selection of rhizobial inocula potentiated the introduction of new legumes to farming systems in southern Australia e.g. *Trifolium glanduliferum* and *T. purpureum*; and the establishment of pulse crops in new areas on difficult soils.

2. Materials and Methods

The four phases in our strain selection program are: 1. Germplasm acquisition, bacterial isolation and maintenance, 2. Authentication and screening for nitrogen fixation, 3. Assessment of germplasm for edaphic adaptation, and 4. Validation of elite strain performance *in situ*.

Phase 1: Germplasm acquisition, bacterial isolation and maintenance

Information on the edaphic properties of the Mediterranean Basin was sourced from geological atlases or published soil surveys such as Gessa et al. (1978). Legumes were identified by consultation with floral keys of the target regions (e.g. Pignati, 1982; Polunin and Huxley, 1978). Plants were carefully excavated and the soil removed from their root systems. Isolation from fresh nodules was preferred, but microbiological facilities were not always available. In these situations nodules were excised in situ leaving a small amount of root attached, then desiccated over silica gel held in 3–5 ml plastic tubes (Date, 1982). Isolation from nodules was as described by Vincent (1970) with the modifications to growth media described by Howieson et al. (1988). Bacterial colonies that resembled rhizobia (slightly raised, opaque and entire) were selected for purification however if nodules yielded pure rhizobial colonies these were immediately vacuum-dried for long term storage in glass ampoules. Authentication of isolates was carried out as part of the screening process for N-fixation. In our laboratory, we emphasise minimal subculturing of isolates to avoid genetic drift and unwanted selection pressure during growth on artificial media (Labandera and Vincent, 1975). Some of the rhizobial strains collected in this manner and used in the next 3 phases of evaluation are listed, together with their origins, in Table 1.

Table 1. Rhizobial strains used in experiments together with their origins

Rhizobial strain	bv of *Rhizobium leguminosarum*	Country of origin	Host of origin	Experimental phase
TA1	*trifolii*	Australia*	*T. subterraneum*	2,3,4
WU95	*trifolii*	Australia*	*T. subterraneum*	2,3,4
CC4334a	*trifolii*	Australia*	*T. vesiculosum*	2
WSM1328	*trifolii*	Italy	*Trifolium* sp.	2,3,4
WSM409	*trifolii*	Italy	*T. subterraneum*	2,3,4
CC299b	*trifolii*	Australia*	*T. vesiculosum*	2,3,4
NA3039	*trifolii*	Australia*	*T. subterraneum*	3
SU303	*viceae*	Australia*	*V. sativa*	2,3,4
WSM1255	*viceae*	Greece	*V. faba*	2
WSM1274	*viceae*	Greece	*Vicia* sp.	2,3,4
WSM1455	*viceae*	Greece	*V. faba*	2,3,4
WSM1469	*viceae*	Greece	*Vicia* sp.	2
WSM1475	*viceae*	Greece	*V. faba*	2,3
WSM1480	*viceae*	Greece	*V. faba*	2
WSM1481	*viceae*	Greece	*Vicia* sp.	2
WSM1483	*viceae*	Greece	*Vicia* sp.	2,3,4
WSM1488	*viceae*	Greece	*V. faba*	2
WSM1520	*viceae*	Greece	*Lathyrus* sp.	2
WSM1521	*viceae*	Greece	*Lathyrus* sp.	2
WSM1529	*viceae*	Greece	*V. faba*	2,3

Australia* = strains naturalised in Australian soils

Phase 2: Authentication and screening for nitrogen fixation

The second objective in the program was to authenticate the rhizobial isolates and to screen for genetic compatibility between host and microsymbiont for nitrogen fixation. In a naturally lit, controlled temperature glasshouse this screening program emphasised three fundamental aspects:

- the screening environment must be limiting only in plant available nitrogen
- we expected host-strain interactions within species
- we acknowledged the necessity to select strains which will not compromise the field performance of existing important legumes in agriculture in southern Australia.

The sand culture method we employ has previously been described in detail (Howieson et al., 1995). It essentially consists of steamed, coarse river sand

held in free draining pots, with a paper filter system in the base and alkathene beads on the surface. The function of the beads is to minimise evaporative losses and to minimise the washing of airborne rhizobia into the pots. Water and nutrients (autoclaved) are added as required through a capped tube. This system may be utilised for legumes of all seed sizes. It is important, particularly for large seeded legumes (>5 mg), to select seed for uniformity of size and history of production. Likewise, strict attention must be paid to hygiene in the glasshouse to avoid contamination by airborne rhizobia.

Roots were carefully exhumed from the pots, washed free of soil and scored for nodulation based upon the number, size and position of nodules on the root system (Chatel and Parker, 1973). Tops were dried (70°C) then weighed. Authentication was confirmed if isolates nodulated the roots of inoculated plants in experiments where uninoculated plants remained nodule free. Capacity for N-fixation (effectiveness) was determined by comparing yields of inoculated plants with the +N controls as well as with the commercial inoculant strains TA1, WU95 and SU303 (Table 1).

Phase 3: Assessment of germplasm for edaphic adaptation

For many symbioses, the greatest challenge in developing inoculants is to transfer a consistent nodulation pattern from the region of evolution of the legume to the new agricultural environment. Our selection process evaluated the strains of root-nodule bacteria that had displayed optimal nitrogen fixing capacity in phase 2 for their relative ability to survive in, and to colonise, target soils when in the free-living, saprophytic state. The methodology embraced was the "cross-row" technique (Howieson and Ewing, 1986). Briefly, strains were introduced to the soil as inocula at a site of appropriate chemical and physical characteristics, and free of the rhizobial species of interest. For clover rhizobia, cross row experiments were conducted in soils of pH 4.1 at Kojonup (33° 50' S, 117° 10' E) and pH 4.2 at Merredin, Western Australia (31° 28' S, 118° 18' E). Both soils are deep sands of 5% and 10% clay content respectively. Rhizobial strains were introduced into the soils as peat inocula on *T. subterraneum*. Strain NA3039 was included in the experiments as it belongs to a group of strains with reported acid soil tolerance (Table 1, Gemmel and Roughley, 1993).

In seeking elite strains for several pulse legumes, cross-row experiments were conducted at Meckering (31° 38' S, 117° 1' E) (pH 4.2 with 5% clay) and also at Kojonup. In both experiments the test host for introduction of the rhizobia was *Vicia faba*. Soils with a sandy texture (5–10% clay) expedite recovery of roots for examination of the nodules and also place substantial stress on inoculant survival.

Plots were sown as 2 m lines of inoculated legume seed separated by 1 m buffers and fertilised with all necessary macro- and micro- nutrients except N. Plants grew through the winter during which production parameters were scored and were then allowed to senesce naturally during the dry summer. In the following autumn the individual rhizobial strains were traced for their survival and movement away from the line of introduction to the soil using a nodulation bio-asssay. In this assay, uninoculated (surface sterilised) seed was sown across the original line in a perpendicular pattern at two or three points. Individual plants were excavated 10–12 weeks after sowing and their nodulation pattern recorded. Experimental design was as randomised blocks, or adapted to take advantage of spatial analysis techniques (Cullis and Gleeson, 1991).

Phase 4: Validation of strain performance in situ

The rhizobial strains identified in the previous phases as being superior in their capacity to colonise acid soils, to nodulate and to fix N were ultimately assessed for their suitability as inoculants in "rotation experiments". These experiments were conducted at sites that placed stress upon the establishment and survival of the symbiotic relationship. For evaluation of the pasture legume rhizobia, the site selected was a sandy loam soil (17% clay, pH 4.5) at Northam (31° 39' S, 116° 40' E) carrying a background population of rhizobia capable of nodulating and fixing nitrogen on the clover species of interest. For the pulse legume rhizobia an experiment was initiated at Mingenew in the northern wheatbelt of Western Australia (29° 11' S, 115° 27' E) on a loam soil (23% clay, pH 5.0). This site was free of background rhizobia capable of nodulating *L. culinaris*. In both experiments inoculated seed was sown with a precision seeder in 5.5 m × 20 m plots. The treatments were replicated four times in blocks and experiments typically continued for three seasons.

In the first season an *in situ* assessment of nitrogen fixation may be gathered by measuring peak biomass and nitrogen accumulation using the N^{15} natural abundance technique (Unkovich et al., 1994). Nodule scores, plant biomass accumulation and seed yield were also measured. In the second season a "break" crop such as a cereal was sown to reduce soil nitrogen status and to expose the inoculant strains to the pressure of survival as a soil saprophyte. For the pulse legumes, in the third season plots were split for +/– inoculation treatments that allowed a direct comparison of nodulation achieved by soil-borne rhizobia versus freshly applied rhizobia. Pasture species were allowed to naturally regenerate and their nodulation and productivity examined. Nodule occupancy was validated using PCR RAPDs (Richardson et al., 1995) where required.

3. Results

Phase 1: Germplasm acquisition, bacterial isolation and maintenance

The anticipated target niche for the legume under evaluation influenced the locality from which we sourced our rhizobial germplasm. We were predominantly interested in adapting legume symbioses to acid, infertile soils in a Mediterranean-type environment and hence regions with these edaphic characteristics were sought. Mildly acidic soils derived from granite parent materials were readily found in Sardinia and Morocco (Howieson and Loi, 1994) and the Cyclades group of Greek Islands (Nutt et al., 1996).

Target legumes were identified and their nodules collected by excavating plants and excising the nodules leaving a small amount of root attached. If the plastic tubes remained airtight, nodules stored over silica gel yielded rhizobia with a success rate of over 75% when isolation was attempted. Upon purification strains were stored in the culture collection at the Centre for *Rhizobium* Studies (prefixed WSM) which currently contains over 3000 accessions of root-nodule bacteria.

Phase 2: Authentication and screening for nitrogen fixation

It emerged as an imperative in our program to screen symbioses for nodulation and nitrogen fixation in sand culture, rather than in agar or vermiculite in glass tubes. This was because in dealing with many "new" legumes it became impracticable to optimise artificial growth systems for each individual species. Some species of *Medicago* and *Biserrula pelecinus*, for example, will not grow and nodulate satisfactorily in glass tubes or in hydroponic solutions, but will do so readily in sand culture (Howieson et al., 2000b).

In assessing the rhizobial requirements of two species of clover recently introduced to Australia (*T. purpureum* and *T. glanduliferum*) we found the standard inoculant strains WU95 and TA1 were poorly effective at fixing nitrogen (Fig. 1a). Several alternative strains doubled the yield of *T. purpureum* (e.g WSM409 and WSM1328) whilst also being highly effective at N-fixation with *T. glanduliferum* and *T. subterraneum*.

Our research into suitable strains for the pulse legumes was initiated for a different reason. Although the commercial inoculant strain SU303 was effective on a broad range of pulses, farmers had reported nodulation failure in *V. faba* and *L. culinaris* when cultivated on acid soils. SU303 has previously been shown to persist poorly on such soils (Carter et al., 1995; Howieson, 1995b). We were particularly focussed on *L. culinaris* because of the urgent need to improve the vigour and productivity of this species. Large host-strain

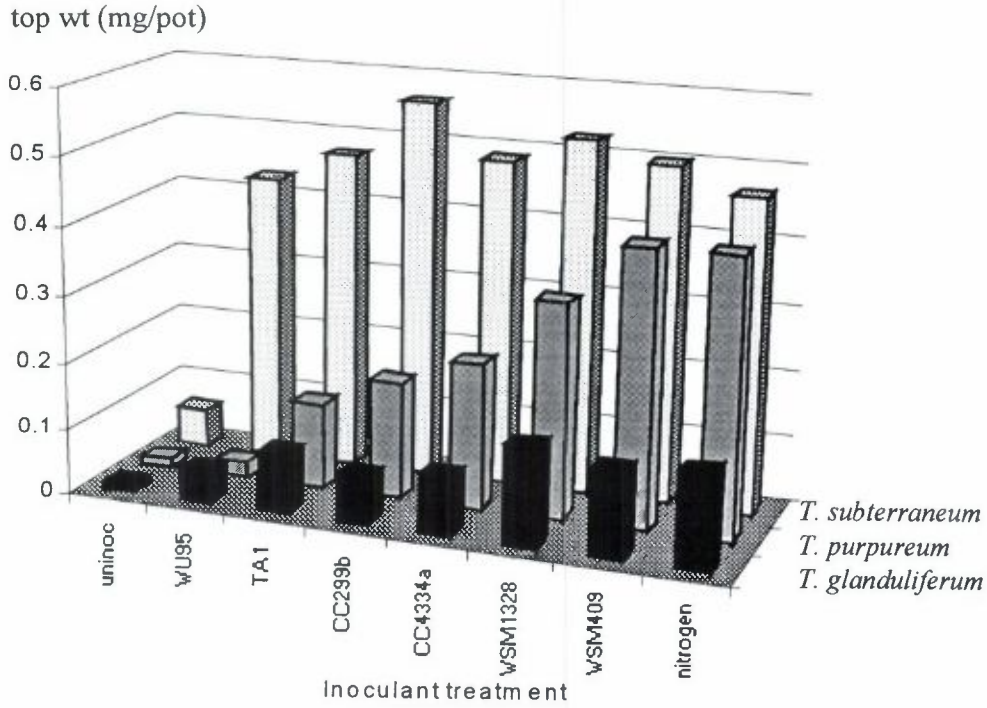

Figure 1a. The yield (top dry weight, mg/pot) achieved by three species of *Trifolium* six weeks after separate inoculation with six different strains of *Rhizobium leguminosarum* bv *trifolii*.

interactions were evident when considering nitrogen fixation on *P. sativum*, *L. culinaris* and *V. faba* (Fig. 1b). Some of the most effective strains on *P. sativum* (e.g. WSM1521) were relatively poor at N-fixation with *V. faba* and *L. culinaris* (Fig. 1b). Nonetheless, it was possible to select strains such as WSM1455, WSM1483 and WSM1480 that were capable of elevated N-fixation with all three pulse legumes.

For both the pasture and pulse legumes, the rhizobial strains which exhibited enhanced N-fixation during authentication were taken through to the next phase of testing; screening for adaptation to the edaphic environment.

Phase 3: Assessment of germplasm for edaphic adaptation

The highly weathered, granite derived soils common to south-western Australia have presented a difficulty for long term nodulation in many legume species (Parker, 1962). The primary constraint has been identified as the poor survival of the microsymbiont induced by the combined stresses of acidity and

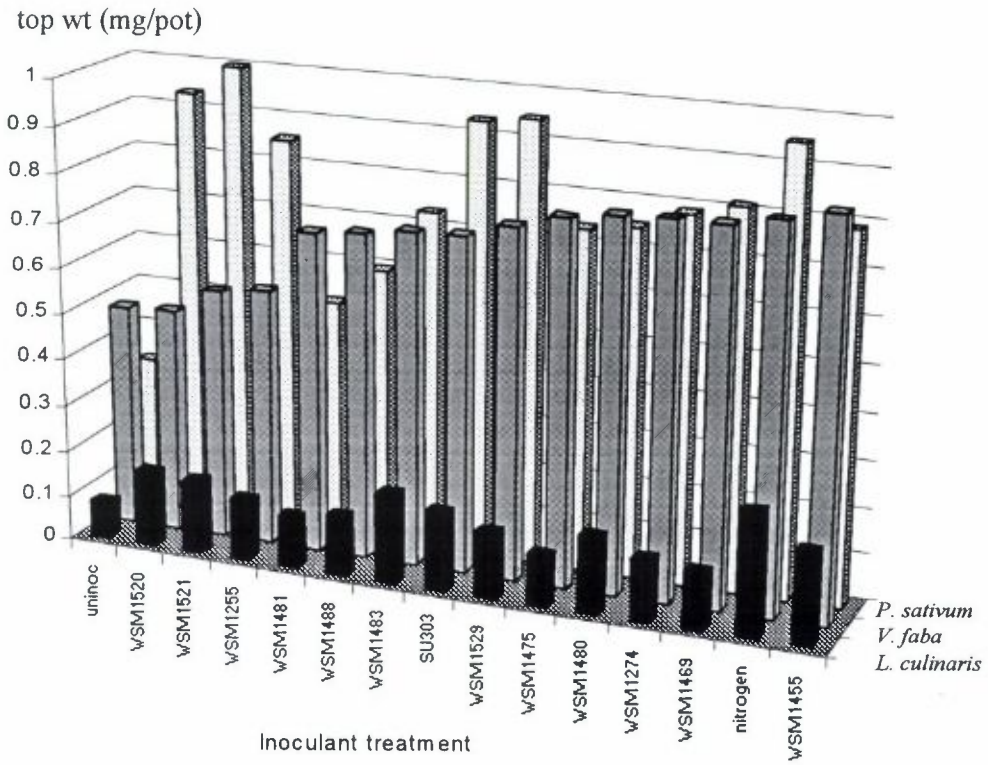

Figure 1b. The yield (top dry weight, mg/pot) achieved by three pulse legumes six weeks after separate inoculation with 13 strains of *Rhizobium leguminosarum* bv. *viceae*.

Table 2. Soil pH values that represent the threshold for growth and survival of root-nodule bacterial species in acid soils of Western Australia containing 5–10% clay (Howieson, unpublished data)

Species of root-nodule bacteria	pH that limits survival (0.01 M $CaCl_2$)
R. leguminosarum bv *trifolii*	4.1
R. leguminosarum bv *viceae*	4.2
Rhizobium meliloti	4.5
Mesorhizobium sp. (*Biserrula*)*	4.0
Bradyrhizobium sp. (*Lupinus*)	3.5
Mesorhizobium cicer	4.1

*Yet to be taxonomically defined

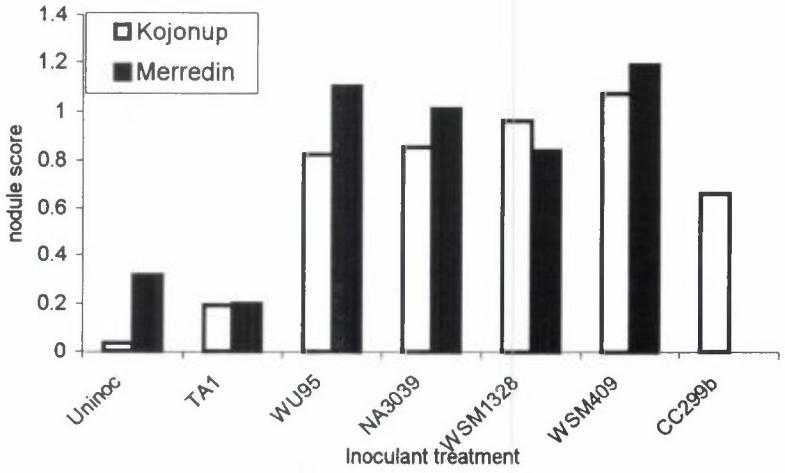

Figure 2a. Nodulation (nodule score per plant) achieved by uninoculated *T. subterranean* sown 0–10 cm distant from the placement of rhizobial strains 12 months previously at two experimental sites (lsd P<0.05 Kojonup 0.33, Merredin 0.38).

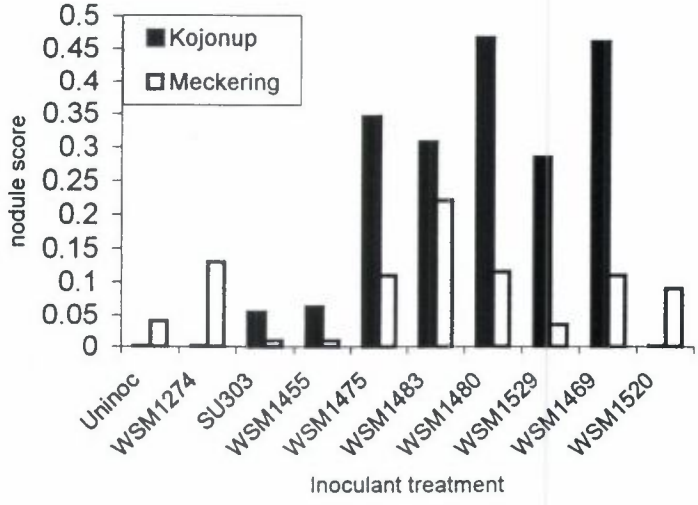

Figure 2b. Nodulation (nodule score per plant) achieved by uninoculated *V. faba* sown 0–10 cm distant from the placement of rhizobial strains 12 months previously at two experimental sites (lsd P<0.05 Kojonup 0.30, Meckering 0.16).

desiccation, in soils of low cation exchange capacity and chemical infertility (Robson, 1969; Chatel and Parker, 1973). Table 2 lists the minimum pH for survival of common Mediterranean root-nodule bacteria in sandy soils of

Western Australia compiled on the basis of our "cross-row" experiments.

In the clover experiments strains differed in their ability to colonise the region of soil within 10 cm of the original point of inoculation (Fig. 2a). There was also a remarkable similarity in strain performance at the two experimental sites. Control strain TA1 persisted very poorly in the acid soils, whereas strains WSM409, WSM1328 and NA3039 persisted as well as a second control strain, WU95 ($P<0.05$b). NA3039 could not be separated from any strain except TA1 ($P<0.05$) under these conditions. Strain WSM409 produced a greater nodule score than CC299b at Kojonup ($P<0.05$).

Fig. 2b illustrates the fate in two sandy soils of a range of strains previously identified as optimal for nitrogen fixation with *L. culinaris* and *V. faba*. Strains WSM1475, WSM1480 and WSM1483 produced a greater nodulation score ($P<0.05$) than the control strain SU303 at both sites in plants sampled within 10 cm of the point of first year inoculation. Of these three strains WSM1475 is relatively poor at N-fixation with *L. culinaris* (Fig. 1b). Of the other strains identified with improved N-fixation potential, WSM1274 colonised the acid sand quite well at Meckering but did not persist at Kojonup, whereas WSM1455 appeared to be saprophytically challenged at both sites. As the deficiency in the control strain relates to its saprophytic abilities in infertile soils, the performance of both WSM1483 and WSM1480 was encouraging.

Phase 4: Validation of strain performance in situ

Fig. 3a illustrates the *in situ* performance in large plot experiments of rhizobial strains for the pulse legumes. Nodule number on *L. culinaris* closely reflected plant biomass accumulation on the acid soil. Inoculation with WSM1455 or WSM1483 more than doubled nodule number at ten weeks, relative to the control strain SU303 ($P<0.05$). This greater nodule number was translated into an increase in top yield of approximately 100% at 13 weeks after sowing for both strains. Grain yield was also increased by inoculation with the improved strains ($P<0.05$).

For the clover rhizobia experiments, inoculation with the improved strains increased the yield of *T. purpureum* by at least 30% (WSM1328 cf. TA1, Fig. 3b) and of *T. glanduliferum* by 20% (WSM409 cf. WU95) in cuts taken of above ground biomass 90 days after sowing. This was despite the competition for nodulation by background rhizobia at the site. Assessment of nodule occupancy by inoculant strains using PCR revealed all were present in >90% of nodules in the spring of the year of sowing (data not shown).

The assessment of rhizobial survival in the rotation experiments is continuing with a view to following the fate of the introduced strains.

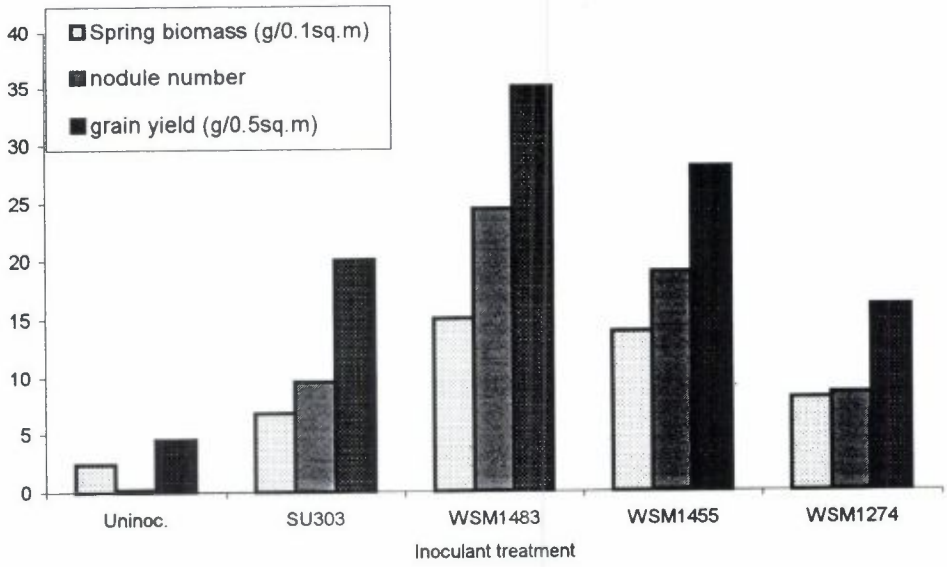

Figure 3a. Spring biomass, nodule number per plant and seed yield of *L. culinaris* recorded in the first year of a rotation experiment on an acidic loam (pH 5.0), when inoculated separately with four strains of rhizobia (lsd P<0.05 nodule number 2.74, biomass 2.1, seed yield 14).

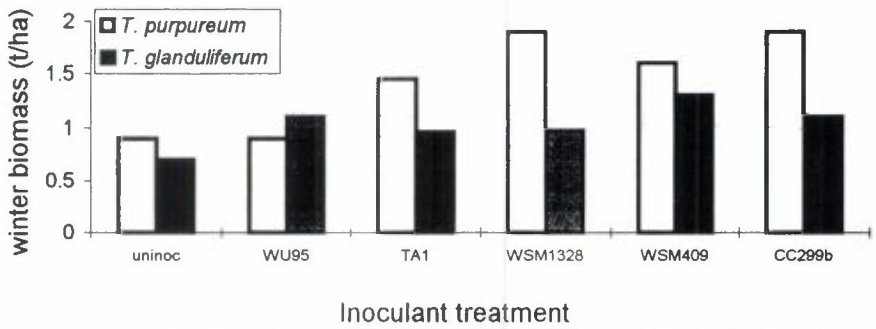

Figure 3b. Winter production from two clovers inoculated separately with improved strains of rhizobia and grown in a loam soil containing background rhizobia capable of nodulation and N-fixation (lsd P<0.05 0.23).

4. Discussion

In the preliminary examination of a range of new legumes introduced to southern Australia it was established that their N-fixation patterns were sub-

optimal when inoculated with commercially available rhizobia. We report here upon procedures and methodologies to select optimal inoculants for some of these legumes.

The screening process began with the examination of a broad genetic base of root-nodule bacteria collected from soils in the Mediterranean basin which, as closely as possible, reflected the edaphic characteristics of the target soils in southern Australia. We have traditionally confined our exploration of rhizobial genetic resources to the Mediterranean Basin, a region that reflects the climate of parts of southern Australia (Bennett et al., 1998). Because of this climatic homology the legume itself seems to transfer quite readily to southern Australia. However, when intending to introduce the legume microsymbiont to new regions, it has been equally (if not more) important to be aware of soil chemical and physical characteristics because these can impact directly upon rhizobial survival (Robson, 1969).

Inoculant survival is critical to gain maximum production from pasture legumes as these regenerate naturally and are only sown (and hence inoculated) once in perhaps 20 years. Inoculant rhizobia must therefore have considerable saprophytic abilities. Similar attributes are beneficial in pulse rhizobia, because in second sowings farmers would prefer to coat seed with fungicides that are incompatible with rhizobial inoculation and rely on soil borne rhizobia to provide nodulation. It is difficult to acquire strains with an adaptation to acid soils for these soils are relatively rare in the Mediterranean region. Nonetheless, there are small pockets of moderately acid soils in several localities whose chemical and physical characteristics reflect those of the acid soils of south-western Australia (Howieson, 1995b).

The number of suitable rhizobial strains rapidly decreased as they were screened for parameters considered important when selecting inoculant strains for conditions in southern Australia. In the glasshouse we examined strain capacity to fix nitrogen optimally with quite a wide range of host legumes. This eliminated a high proportion of otherwise useful strains. The "cross-row" technique, which explores the saprophytic properties of strains, gave reproducible rankings that differed only slightly across sites and seasons. The outcome of the cross-row screening technique was an exclusive set of strains with commercial potential and which could then be field evaluated.

For the pulse legumes, inoculation with either WSM1483 or WSM1455 increased yield and nodulation in *L. culinaris* by approximately 100%. The probable explanation for the vastly superior symbiotic performance of WSM1483 relative to commercial strain SU303 lies in its better adaptation to acid soil conditions in combination with its greater capacity for N-fixation with the host legume. The latter attribute applies also to strain WSM1455, however its apparent inability to persist in acid, sandy soils may compromise its suitability as an inoculant strain. Data to support this contention will be

available in the final year of the rotation experiment. For the pasture legume rhizobia, the yield advantages of strains such as WSM409 and WSM1328, combined with their adequate soil persistence, ideally suit them as inocula for the new suite of clovers introduced to southern Australia during this period. Their broad host range ensures that if they become numerically dominant amongst the suite of rhizobial strains already resident in the field they will not compromise the productivity of the important pasture legume *T. subterraneum*. The cross-row data, which provides an indication of acid soil tolerance, gave encouragement that replacing WU95 and TA1 as inoculants for *Trifolium* spp would not compromise second year nodulation in acidic, infertile soils.

The strains of rhizobia developed with commercial potential in this research program must be screened for genetic stability, their "manufacturability" and broader edaphic adaptation before they can be recommended as inoculants across southern Australia. Given that optimal legume performance is pivotal to agricultural productivity in southern Australia, the investment in rhizobial research as detailed in this manuscript is a necessary means of ensuring continued profitabilty of agricultural enterprises. As new initiatives in legume usage emerge, such as the current challenge of adapting perennial species for wheatbelt usage, rhizobial research will remain a manifestly vital component of the development process.

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