The legume-rhizobia relationship in the Mediterranean basin

Howieson J.G., O'Hara G.W., Loi A.

in

Sulas L. (ed.).
Legumes for Mediterranean forage crops, pastures and alternative uses

Zaragoza : CIHEAM
Cahiers Options Méditerranéennes; n. 45

2000
pages 305-314

The legume – rhizobia relationship in the Mediterranean Basin

J.G. Howieson[1,2,3] G.W. O'Hara[1,3] and A. Loi[3]

[1]Centre for *Rhizobium* Studies, Division of Science & Engineering, Murdoch University, Murdoch Drive, Murdoch, Western Australia, 6150, Australia,

[2]Agriculture WA, Baron-Hay Court, South Perth, Western Australia 6151, Australia, and

[3]CLIMA, The University of Western Australia, Nedlands, 6907, Australia.

Summary - To effectively harness the value of symbiotic N-fixation we need to understand G^2 x E, where G refers to the genotypes of both legume and microsymbiont, and E the edaphic environment in which the symbiosis is to function. In the Mediterranean basin there is strong evidence that despite co-evolution of legumes and rhizobia, the relationship is not always optimal. Several research options are presented to improve N-fixation depending on the nature of the limitation to legume or rhizobial performance.

Key words: forage, legume, N-fixation, Mediterranean, *Rhizobium*

Resumé *- Pour renforcer la valeur de la fixation symbiotique de l'azote, il est nécessaire de comprendre le produit G2 x E, où G correspond aux génotypes de la légumineuse et de son associé, et E l'environnement édaphique dans lequel la symbiose doit avoir lieu. Dans le bassin méditerranéen, il est évident que malgré une co-évolution des légumineuses et de leur rhizobium, l'association n'est pas toujours optimale. Plusieurs alternatives de recherche sont proposées afin d'améliorer la fixation de l'azote en fonction du type de contrainte limitant les performances de la légumineuse ou du rhizobium.*

Mots-clés*: légumineuse, fixation d'azote,* Rhizobium

Introduction

The adaptation of agricultural plants to climate is often discussed in terms of G x E –the Genotype x Environment interaction. When dealing with the legume – rhizobia relationship, the interaction becomes one of the second order i.e. G^2 x E, where G refers to the genotypes of **both** the legume and its microsymbiont. To effectively harness the potential of this symbiosis, we must fully understand these multiple interactions.

The capacity to fix N_2 in symbiosis with plants is found in three major groups of microbes: the nodule-forming bacteria (*Allorhizobium, Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium* and *Sinorhizobium,*), actinomycetes (*Frankia*) and cyanobacteria (*Anabaena*, *Nostoc*) (Young, 1996). Nitrogen-fixing legumes contribute the majority of the N_2-fixed in Mediterranean agriculture, in a form that is not readily leached. Industrially manufactured nitrogen is also supplied in roughly equivalent amounts, however every ton of N fertiliser manufactured consumes 1.3 ton of oil equivalents (Kennedy and Cocking, 1997). Thus, the inputs of N into Mediterranean agricultural systems by N-fixation in nodulated legumes are fundamental to sustainable and economic production.

At least some of the legumes of the Mediterranean basin have been comprehensively studied in relation to their environment (e.g. Ehrman and Cocks, 1990; Piano *et al.* 1993). However, probably the most astonishing aspect of the rhizobia of the Mediterranean basin is how little we know about their ecology! Given their relative importance to agriculture in that region, as well as in other world regions that share a similar climate, it is surprising that so few comprehensive studies of the ecology of rhizobia in the Mediterranean basin have been undertaken. Of the many studies of legume ecology that have been reported from the Mediterranean basin, few have focussed upon the symbiosis, whilst even fewer have investigated symbiotic interactions with the environment. The recent publication by Loi *et al.*, (1999) is a notable exception. The aim of this paper is to outline current approaches to understanding and manipulating the legume-rhizobia relationship in Mediterranean environments.

Are Mediterranean forage legumes fixing N optimally?

It has recently been proposed that rhizobial genotypes are differentially adapted to soil conditions and may be advantaged both numerically and competitively under conditions that suit them, irrespective of the symbiotic reaction with their host (Howieson 1999). If we follow this statement through to its logical conclusion it infers that symbiotic effectiveness provides a lesser selection pressure upon a rhizobial population than does bacterial adaptation to soil and climate. This is intuitively logical and was, in a sense, implied in a study by Almendras and Bottomley (1987) that illustrated soil P concentration favoured a particular rhizobial strain in nodule occupancy. After all –what advantage does a rhizobial strain derive from being fully effective at N-fixation rather than 75% effective? As long as the host has sufficient N to grow and to compete with its neighbours, the photosynthate supply to the nodules is probably assured and therefore the rhizobia are well nourished. We cannot assume that in any given Mediterranean environment the legume -rhizobia symbiosis is optimised simply because both macro- and micro-symbiont have evolved together in that environment. Is there evidence for this assertion?

Figure 1 shows that there was no yield advantage in *Biserrula pelecinus* L. through inoculation with a rhizobial strain collected from the same region as its host. The genotype of *B. pelecinus* from Greece yielded well with rhizobia from Italy and *vice versa.* In fact, the data indicate that the rhizobia from Italy were generally more effective at fixing N *per se* than those from Greece. This possibility needs to be further investigated.

This result appears to be the rule rather than the exception. Howieson *et al.*, (1999) reported that for another monospecific genus, *Hymenocarpus circinnatus* L. (Savi) the search for rhizobial strains of high effectiveness gave no indication of regional specificity. Eight rhizobial strains isolated from *H. circinnatus* collected in the Cyclades group of Greek Islands nodulated nine host ecotypes from the same region. Surprisingly, only one strain actually fixed nitrogen appreciably. Sulas *et al.*, (1998) improved the yield of *Hedysarum coronarium* L. grown on a traditional soil in Sardinia through inoculation with rhizobia selected for high N-fixation, indicating the soil in that experiment harboured a poorly effective suite of rhizobia. There are numerous examples where background populations of rhizobia restrict N-fixation in the Mediterranean basin (Keating *et al.* 1995; Materon *et al.* 1995; Materon and Danso 1991) and elsewhere (Denton *et al.* 1999).

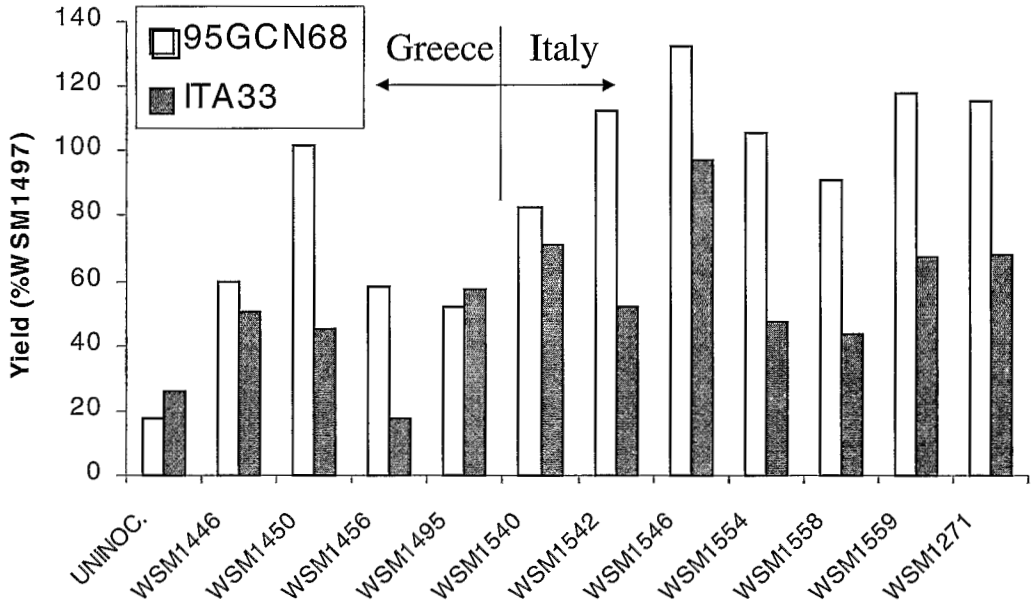

Figure 1. Yield of two genotypes of *Biserrula pelecinus* (95GCN68 from Greece and ITA33 from Italy) when inoculated with rhizobia collected from either Greece or Italy, as arrowed. Data is presented for yield of tops as a proportion of the commercial inoculant strain in Australia, WSM1497 (Loi, Yates and Howieson unpublished data).

Improving N-fixation

There is considerable scope for improving N-fixation in forage legumes within the Mediterranean basin, as there is where Mediterranean legumes have been introduced worldwide. How then can this optimisation be best achieved? It is in understanding G^2 x E that progress will be made and we have developed a set of protocols for this purpose. Firstly, however, we must understand the *status quo* with respect to the symbiosis as it applies to our legume of interest. Figure 2 outlines three relatively common scenarios leading to research options that arise when investigating legume nodulation and N-fixation:

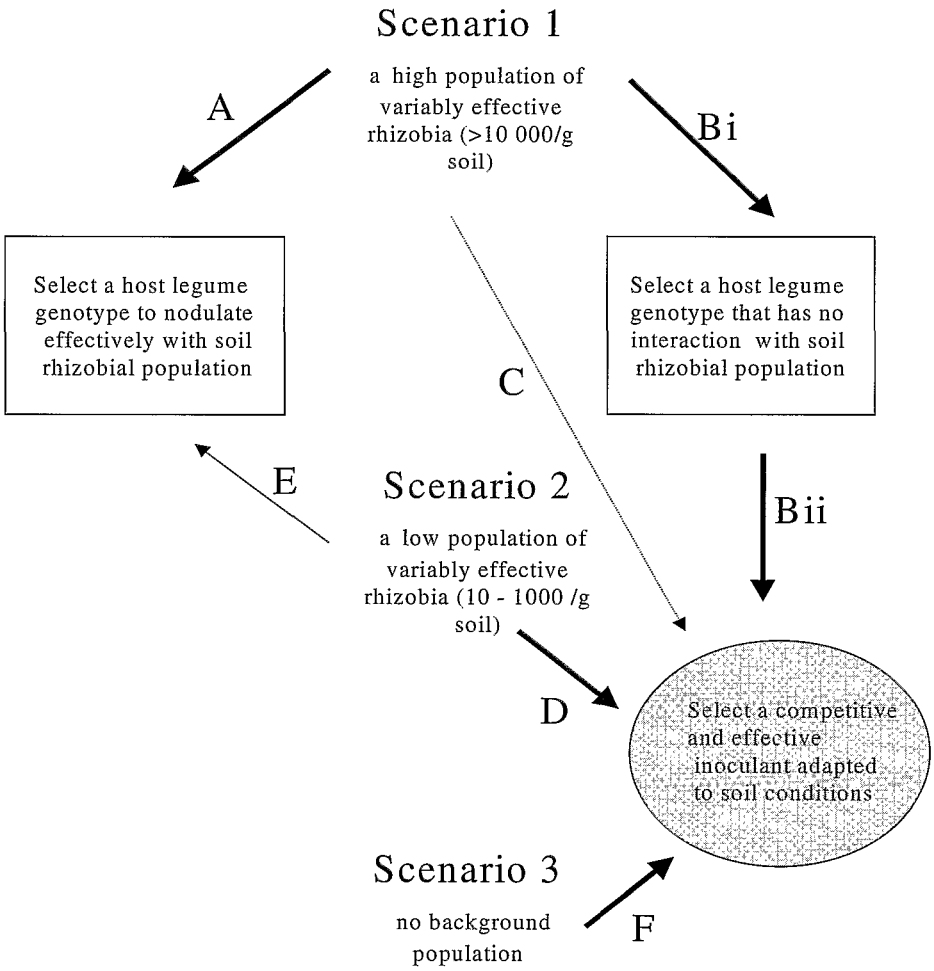

Figure 2. A schematic representation of strategies to improve N-fixation in legumes through selection of either the host or rhizobial genotype.

Scenario 1 where the soil contains a high population of variably effective rhizobia that cause reduced N-fixation

Scenario 2 where the soil contains a low population of variably effective rhizobia that produce poor nodulation and reduced N-fixation

Scenario 3 where the soil does not contain rhizobia capable of nodulating with the host legume of interest and hence inoculation is required.

For each scenario there are a number of research options to improve legume nodulation, with the most likely option to succeed denoted by increased line density in Figure 2. Some examples where the research options A-F have been successful in Australian research are given in Table 1.

Of course, other scenarios exist. For example, where there is a high population of effective rhizobia and no response to inoculation (the case with many pulse legumes in tropical Asia) or where the legume of interest is grown predominantly with fertiliser N and inoculation is ineffectual (e.g. *Phaseolus vulgaris* in north-eastern Australia). However, where inoculation is

required and having decided upon the approach, there are a number of techniques available to select adapted rhizobial strains.

Table 1. Recent examples where applying the research pathways A-F in Fig. 2 have been successful in developing improved symbiotic N-fixation in Australia.

Pathway	Legume	Reference	Comments
A	*Medicago littoralis* *Trifolium michelianum*	Ballard 2000, this issue	cv. Pildappa well adapted to the medic rhizobial population in alkaline soils
Bi, Bii	*Lotus ornithopodiodes,* *Biserrula pelecinus*	Ballard, Howieson, Loi unpubl., Howieson *et al.* 1995	legumes which avoid interaction with a poorly effective medic rhizobial population on soils pH 6-9.
C	*Ornithopus* spp	McInnes (unpubl.)	Good nodule occupancy in the year of sowing on acid soils.
D	*Trifolium* spp	Howieson *et al.* 1999 Watkin 1999	A broad host range strain WSM409 selected for new trifoliums and sub-clover to replace WU95.
D	*Vicia, Pisum, Lens*	Howieson *et al.* 1999	Selection of adapted inoculants for moderately acid soils
D	*Medicago polymorpha*	Howieson and Ewing 1986	An acid tolerant inoculant was selected to colonise soils pH 4.5-6
D, E	*Medicago murex*	Howieson and Ewing 1989	In combination with E, a symbiotically competent legume was selected for the soils of pH 4-5.
F	*Cicer, Hymenocarpus,* *Scorpiurus, Biserrula,* *Hedysarum etc.*		The current scenario with many introduced legumes in Australia

Techniques for selecting and evaluating rhizobia to match strains with both legume hosts and soil conditions

The genetic structure of rhizobial populations can now be investigated with molecular methodologies (Vinuesa *et al.* 1998). This, for the first time, empowers rhizobial ecologists to follow strain population dynamics, categorise population biodiversity and investigate genetic diversification. In doing so, ecologists can refine characteristics that are required for commercial, inoculant quality, strains of rhizobia.

The attributes needed in inoculant-quality rhizobial strains are:

1. high N_2-fixation with the intended host species without compromising production from related species;
2. adaptation to the edaphic environment targeted for the host;

3. genetic stability in culture, storage and soil;
4. satisfactory growth and survival in inoculant manufacturing procedures, and
5. competitiveness with indigenous soil rhizobia.

1. Selecting effective rhizobial strains with broad host-range characteristics

To screen for genetic compatibility for N_2-fixation between host and microsymbiont our program uses a naturally lit, controlled temperature glasshouse rather than growth chambers or pouches. We emphasise three fundamental aspects:

- the screening environment must be limiting only in plant available N
- we expect host-strain interactions within species for N_2-fixation
- we acknowledge the necessity to select strains that will not compromise production from existing important legumes grown in the target region

We screen symbioses for nodulation and N_2-fixation in sand culture (Howieson *et al.*, 1995) rather than agar or vermiculite because it has proven impracticable to optimise growth culture conditions for each "new" legume studied. The method 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 beads minimise evaporative losses and contamination from airborne rhizobia. Autoclaved water or plant nutrient solution are added as required through a capped tube. This system can be utilised for legumes of all seed sizes. It is important, with large seeded legumes (>5 mg) particularly, to select seed for uniform size and history of production. Strict attention must be paid to hygiene in the glasshouse to avoid contamination by rhizobia. Effectiveness of N_2-fixation is determined by comparing yields and %N of inoculated plants with nitrogen-fed and uninoculated controls.

2. Screening for edaphic adaptation

For many symbioses, the greatest challenge is to develop a consistent nodulation pattern for the legume in the agricultural environment. Our selection process, therefore, focusses on effective root-nodule bacteria and then differentiates between them on the basis of their relative ability to survive in, and to colonise, target soils. The methodology embraced has been termed the "cross-row" technique as originally described by Howieson and Ewing (1986). Briefly, strains are introduced to the soil as inocula at a site of appropriate chemical and physical characteristics, and generally free of the rhizobial species of interest. The pH of the site should be in the range targeted for the host-legume, particularly if this is likely to be a constraint upon rhizobial survival. Soils with a sandy texture (5-10% clay) expedite recovery of roots for examination of the nodules and also place increased stress upon inoculant survival.

In the "cross-row" bio-assay the plots are sown as 2m lines of inoculated legume seed separated by 1m buffers and fertilised with all necessary macro- and micro- nutrients except N. Plants are allowed to grow through a full season during which top dry weight and N-fixation can be assessed. If the target soils are low in available N, the biomass production of the tops is an excellent indicator of symbiotic performance. This can provide valuable information given that pre-selection in stage 1 was based upon N-fixation under favourable conditions. If the soil contains appreciable N, then the N^{15} natural abundance method (Unkovich and Pate, 1998) can reliably indicate strain symbiotic performance. It is possible that strains differ in their relative

abilities to survive on the seed or in the legume rhizosphere in difficult soils, hence data on *in situ* performance are essential for the selection of elite strains.

Following a (usually dry) summer in the soil, the individual strains are traced for their survival and movement away from the line of introduction to the soil using a nodulation bio-assay In this assay, uninoculated, surface sterilised seeds are sown across the original line at two or three points.

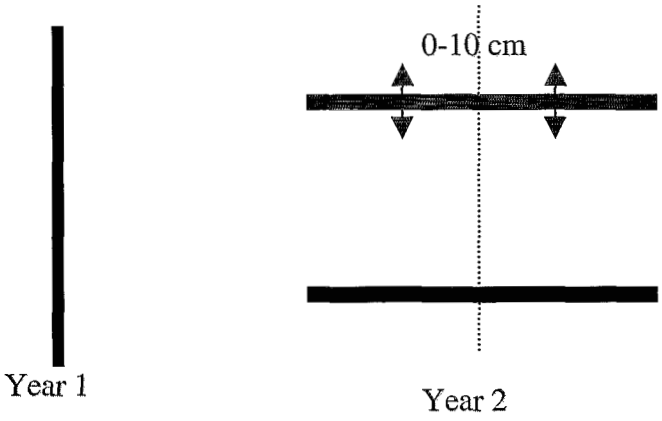

Figure 3. A schematic diagram of the "cross-row" technique. Bold lines represent seed sown inoculated (year 1) or uninoculated (year 2).

Individual plants are excavated 10-12 weeks after sowing and the nodulation pattern recorded. Experimental design can be as randomised blocks, or adjusted to take advantage of spatial analysis techniques (Cullis and Gleeson 1991).

3. Genetic stability in culture, storage and soil

Unfortunately, rhizobia are sometimes stored on rich media that can precipitate genetic changes that affect symbiotic performance. Whilst it is understandable that microbiologists must use such media to culture rhizobia, prolonged exposure to such media should be minimised because the rapidity of change is surprising. For example, in *Rhizobium leguminosarum* bv. *viceae* WSM937 non-nodulating variants arose in 30% of colonies after only 10 sub-cultures (Helen Mifka, pers. comm.). It is desirable to store rhizobia at –80C^{o} in glycerol or as vacuum-dried cultures (Vincent 1970).

Genetic stability in soils is a much more difficult character to evaluate. *Mesorhizobium loti* appear to have a predisposition to genetic diversification through transfer of a "symbiosis island" to other bacteria (Sullivan and Ronson 1998). How widespread this is amongst other rhizobial species is a matter for conjecture, however the reports of substantial genetic diversity in Australian populations of *Sinorhizobium meliloti, R. l. trifolii* and *Bradyrhizobium* sp (Lupinus) are anecdotal evidence that genetic exchange occurs for these rhizobial species. Given our awareness of this phenomenon, it may soon be possible to identify the genetic factor(s) pre-disposing strains to gene transfer and to construct probes to screen strains for this characteristic.

4. Satisfactory growth and survival in inoculant manufacturing procedures

There is emerging evidence that the manufacturing environment can affect the adaptation of inoculants to the soil environment. For example, in the fermentation process, if the rhizobial culture is exposed to a moderately acid pH, then it may initiate a series of physiological and biochemical responses that pre-dispose it to better handle an acid soil environment. This is termed the adaptive acid tolerance response (ATR) and was recently described in rhizobia by O'Hara and Glenn (1994). Not all strains possess the capacity to express an ATR: the acid tolerant *R. trifolii* strain WSM409 does, whereas the acid sensitive strain TA1 does not (Watkin 1999).

5. Competitiveness with indigenous soil rhizobia

Of particular relevance to the Mediterranean basin is how we can improve N-fixation through the introduction of competitive strains of rhizobia. A modern approach to study competition is to label strains with a molecular marker such as *GUS* (Wilson, 1995) and visualise the outcome of competition experiments. If strains have been selected on the basis of their adaptation to the environment, there is a strong chance they can be competitive with indigenous and less effective genotypes.

If the laboratory does not have access to molecular marker technology, then PCR RAPDs offers a means to readily discriminate between rhizobial strains after they are cultured. It appears important to use a range of directed, or semi-directed, primers for this purpose (Vinuesa *et al.* 1998).

Conclusions

There is evidence that legumes do not always fix N optimally in the Mediterranean environment. We should not assume that because of geographic co-evolution, the legume and its microsymbiont are perfectly matched to fix N. There is not, necessarily, strong selection pressure on rhizobia for optimal N-fixation. In view of this, we have discussed several different approaches to improving symbiotic N-fixation through developing an understanding of G^2 x E and then applying some protocols for elite rhizobial strain selection.

References

Almendras, A.S., Bottomley, P.J. (1987). Influence of lime and phosphate on nodulation of soil-grown *Trifolium subterranmeum* L. by indigenous *Rhizobium trifolii. Appl. Env. Micro.* 53, 2090-2097.

Cullis, B. R., Gleeson, A.C. (1991). Spatial analysis of field experiments and extension to two dimensions. *Biometrics* 47, 1449-1460.

Denton, M.D., Coventry, D.R., Bellotti, W.D., Howieson, J.G. (1999). Distribution, abundance and symbiotic effectiveness of *Rhizobium leguminosarum* bv. *trifolii* from alkaline pasture soils in South Australia. *Aust. J. Agric. Res.*, (in press).

Ehrman, T., Cocks, P.S. (1990). Ecogeography of annual legumes in Syria: distribution patterns. *J. Appl. Ecol.* 27, 578-591.

Howieson, J.G., Ewing, M.A. (1986). Acid tolerance in the *Rhizobium meliloti – Medicago* symbiosis. *Aust. J. Agric. Res.* 37, 55-64.

Howieson, J.G., Ewing, M.A. (1989). Annual species of *Medicago* differ greatly in their ability to nodulate on acid soils. *Aust. J. Agric. Res.* 40, 843-50.

Howieson, J.G., Loi, A., Carr, S.J. (1995). *Biserrula pelecinus L.* -a legume pasture species with potential for acid, duplex soils which is nodulated by unique root-nodule bacteria. *Aust. J. Agric. Res.* 46, 997-1007.

Howieson, J.G. (1999). The host-rhizobia relationship. In: S.J. Bennett, P.S. Cocks (Eds.) *Genetic Resources of Mediterranean Pasture and Forage Legumes,* Kluwer Academic Publishers, Netherlands, pp. 96-106.

Howieson, J.G., O'Hara, G.W. and Carr, S.J. (1999). Changing roles for legumes in Mediterranean agriculture: Developments from an Australian perspective. *Field Crops Res.* (in press).

Keating, J.D.H., Materon, L.A., Beck, D.P., Yurtsever, N., Karug, K. and Altuntas, S. (1995). The role of rhizobial biodiversity in legume crop productivity in the West Asian highlands. I. Rationale, methods and overview. *Exp. Agric.* 31, 473-483.

Kennedy, I.R., Cocking, E.C. (1997). Biological NitrogeN-fixation : The Global Challenge & Future Needs, A position paper. The Rockefeller Foundation Bellagio Conference Centre, Lake Como, Italy, April 8-12, 1997. ISBN 1-86451-364-7.

Loi, A., Cocks, P.S., Howieson, J.G., Carr, S.J. (1999). Genetic variation in populations of two Mediterranean annual pasture legumes and associated rhizobia. *Aus. J. Agric. Res.* 50, 305-315.

Materon, L.A. and Danso, S.K.A. (1991). NitrogeN-fixation in two annual *Medicago* legumes, as affected by inoculation and seed density. *Field Crops Res.* 26, 253-262.

Materon, L.A., Keating, J.D.H., Beck, D.P., Yurtsever, N., Karug, K. and Altuntas, S. (1995). The role of rhizobial biodiversity in legume crop productivity in the West Asian highlands. III *Rhizobium meliloti. Exp. Agric.* 31, 493-99.

O'Hara, G.W., Glenn, A.R. (1994). The adaptive acid-tolerance response in root nodule bacteria and *Escherichia coli. Archives Microbiol.* 161, 286-292.

Piano, E., Spanu, F., Pecetti, L. (1993). Structure and variation of subterranean clover populations from Sicily, Italy. *Euphytica* 68, 43-51.

Sulas, L., Re, G.A., Loi, A., Howieson, J.G. (1997). The selection of optimal root-nodule bacteria inoculants increases forage yield of *Hedysarum coronarium* (sulla). *Proc. 17th General Meeting of the European Grassland Federation.* Hungary, May 18-21, 1997.

Sullivan, J.T., Ronson C.W. (1998). Evolution of rhizobia by acquisition of a 500-kb symbiosis island that integrates into a phe-tRNA gene. *Proc. Natl Acad. Sci. USA,* 95, 5145-5149.

Unkovich, M.J., Pate, J.S. (1998). Symbiotic effectiveness and tolerance to early season nitrate in indigenous populations of subterranean clover rhizobia from S.W. Australian pastures. *Soil Biol. Biochem.* 30, 1435-1443.

Vinuesa, P., Rademaker J.L.W., de Bruin, F.J., Werner, D. (1998). Genotypic characterisation of *Bradyrhizobium* strains nodulating endemic woody legumes of the Canary Islands by PCR-restriction fragment length polymorphism analysis of genes encoding 16S rRNA (16SrDNA) and 16S-23S rDNA intergenic spacers, repetitive extragenic palindromic PCR genomic fingerprinting, and partial 16S rDNA sequencing. *Appl. Env. Microbiol.* 64, 2096-2104.

Vincent, J.M., (1970). A manual for the practical study of root-nodule bacteria. IBP Handbook 15, Blackwell Scientific Publications, Oxford.

Watkin, E.L.J., (1999). Integrating field and physiological studies to understand acid tolerance in *Rhizobium leguminosarum* bv. *trifolii*. Ph D Thesis. Murdoch University, Perth.

Wilson, K.J. (1995). Molecular techniques for the study of rhizobial ecology in the field. *Soil Biol. Biochem.* 27, 501-514.
Young, J.P.W. (1996). Phylogeny and taxonomy of rhizobia. *Plant Soil* 186, 45-52.