The Legume – Rhizobia Symbiosis. Does It Vary for the Tropics Relative to the Mediterranean Basin?

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THE LEGUME – RHIZOBIA SYMBIOSIS. DOES IT VARY FOR THE TROPICS RELATIVE TO THE MEDITERRANEAN BASIN?

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Summary

Symbiotic N fixation from legumes is one of the most important biological processes on the planet. It currently provides the majority of the N requirement in agriculture, yet will have to double if cereal crop production is to meet world demand by 2020 (Kennedy and Cocking 1997). To effectively harness the value of biological N fixation from legumes we need to more fully understand G^2 x E; where G refers to the genotypes of both the legume (G_l) and its microsymbiont (rhizobia; G_r), and E refers to the edaphic environment in which the symbiosis is to function.

In the Mediterranean basin, indigenous legumes are nodulated by specific rhizobial genotypes. Despite co-evolution of the symbionts, their relationship is not always optimal for N fixation. It has been proposed that rhizobial genotypes are differentially adapted to soil conditions and that it is this adaptation, rather than the relationship with their host, that most strongly governs outcomes relating to competition and persistence (Sprent 1994, Howieson 2000). Expressed in terms of the above formula, symbiotic effectiveness (G^2) is unimportant to the persistence and success of rhizobia relative to adaptation of the rhizobia to soil and climate (G_r x E).

In this paper we investigate whether rhizobia which have co-evolved with tropical legumes show similar sub-optimal patterns of N fixation. Tropical legumes differ from Mediterranean legumes in that many nodulate promiscuously, and often effectively, with a broad range of rhizobial genotypes (both rhizobia and bradyrhizobia). This introduces the possibility that impacts of soil and climate on the persistence and success of some tropical rhizobia (G_r x E) are less important for optimal N fixation (G^2) than they are in the Mediterranean region. However, observations on N fixation in promiscuous tropical legumes are mostly based on agricultural species associated with rhizobia from outside the centre of origin of the host legume. We examine N fixation by tropical rhizobia which have co-evolved with their host.

We show that sub-optimal N fixation can be improved using several research options, the choice of which depends on the nature of the limitation to the legume or the performance of the rhizobia. We describe three scenarios that might compromise N fixation: a) where the legume is sown into soil containing a high population of variably effective rhizobia, b) where the rhizobial population is low, and c) where there is no background population of rhizobia capable of nodulation with the legume. Each scenario has presented with it several research strategies for improving symbiotic N fixation. These strategies have application for both Mediterranean and tropical environments.

Key words: ***Bradyrhizobium*,** legume, N-fixation, Mediterranean, *Rhizobium,* Tropics

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 symbiosis, the interaction becomes one of the second order i.e. G^2 x E, where G refers to the genotypes of **both** the legume (G_l) and its microsymbiont (Gr). 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 rhizobia (*Allorhizobium, Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium* and *Sinorhizobium,*), actinomycetes (*Frankia*) and cyanobacteria (*Anabaena*, *Nostoc*) (Young, 1996; Martinez-Romero, 2000). Legumes nodulated by N fixing rhizobia contribute significantly to N input in both Mediterranean and tropical agricultural systems, providing a source of N which is not readily leached (Kennedy and Cocking 1997, Peoples *et al.* 1995). The balance of N is supplied primarily as industrially produced N fertiliser, which is often utilised with less than 50% efficiency by legume crops (Peoples *et al.* 1995). In Mediterranean agriculture, symbiotic N fixation and N fertiliser contribute roughly equivalent amounts to the total N budget, however every ton of N fertiliser manufactured consumes 1.3 ton of oil equivalents (Kennedy and Cocking 1997). In the tropics inputs of N as fertiliser and fixed N vary with agricultural system and country, but N fixation contributes significant amounts of N to both crop and pasture systems (Peoples *et al.* 1995). Thus, legume N fixation is fundamental to sustainable and economic agricultural production in both world climates.

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), but we know little about their rhizobial ecology. This is surprising considering the relative importance of N fixation to agriculture in the Mediterranean region, as well as in other world regions that share a similar climate (Howieson *et al.* 2000a). 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 comprehensive studies from ICARDA in Syria (Keating *et al.* 1995; Beck 1992) and the recent publication by Loi *et al.* (1999) are notable exceptions.

Considerably more is known about the ecology of rhizobia associated with agriculturally important legumes in the tropics. On the basis of this, tropical legume genera have been divided into three groups according to their symbiotic competencies (G^2), including legumes which i) nodulate promiscuously and effectively ii) nodulate promiscuously and ineffectively and iii) nodulate with a restricted range of rhizobial genotypes (Date 1977, Peoples *et al.* 1989). However, allocation of genera into each group is based largely on studies conducted outside the centre of origin of the host legume. In addition, species within genera often exhibit a range of symbiotic competencies (Date and Halliday 1980).

In this paper, we compare N fixation in co-evolved legume-rhizobia symbioses in studies from both the Mediterranean basin and the tropics. Where N fixation is sub-optimal, we explore whether there are consistent patterns to this across both climates. Finally, we detail research options for improving the effectiveness of the symbioses, depending on the nature of the limitation to the legume or the performance of associated rhizobia.

Major differences between the Tropics and the Mediterranean.

Before we go further into this paper, it is worth noting several features that are useful in distinguishing soils and their root-nodule bacteria from the two regions. Tropical soils are often old, highly weathered and acidic (Uehara 1978) in contrast to the younger, fine-textured and often calcareous soils that abound in the Mediterranean basin. Of course there are acid soils to be found in the latter –the north-west of Sardinia and several Greek Cyclades Islands are good examples. However these acid soils differ considerably from those in the tropics because they are rarely less than pH 5.5 and have high cation exchange complexes (>100meq/100g) often dominated by calcium and magnesium ions.

A second contrast between the two regions is the predominance of fast-growing root nodule bacteria in the Mediterranean relative to the broader spread of both fast- and slow-growing types in the tropics. For example, whereas tropical pulse legume genera may be nodulated by root-nodule bacteria from either the genus *Bradyrhizobium* or *Rhizobium* (and sometimes both forms), *Lupinus* spp. are the only cool season pulses in the Mediterranean nodulated by *Bradyrhizobium* (Howieson 2000). This differentiation was once proposed to be the result of superior adaptation of *Rhizobium* to alkalinity and *Bradyrhizobium* to acidity (Norris 1965). Although this generalisation still holds, acid tolerant species of *Rhizobium* from the tropics (Vargas and Graham 1988) and *Bradyrhizobium* from alkaline soils in Mediterranean climates have since been reported (Marsudi *et al.* 1999).

A generalisation that appears to remain intact is that the more promiscuous root-nodule bacteria and legumes are found mostly in the tropical parts of the world. Promiscuity in nodulation refers to bacteria that have the capacity to infect many legume species, or legumes that are capable of infection by many bacterial genera or species. The molecular basis of symbiotic promiscuity has been revealed through studies on biochemical signalling between the symbionts. A full description is given by Perret *et al.* (2000). Although *Acacia* spp. in mediterranean Australia appear promiscuous in their ability to nodulate with both fast- and slow-growing bacteria (Lawrie, 1983, Barnet *et al.* 1985; Marsudi *et al.* 1999; Yates *et al.* 1999), this attribute probably relates to their tropical ancestry. Many of the monospecific legume genera of the Mediterranean such as *Biserrula* and *Hymenocarpus* are very specific in their rhizobial requirements i.e. non-promiscuous (Nandasena *et al.* 2000; Howieson 2000). It may be, however, that we have not looked widely enough at the interactions between the root-nodule bacteria isolated from such narrow genera and the common (but less intensively studied) legume flora of that region, particularly woody trees and shrubs.

Given that there appears to be substantial differences between nodulation patterns between Mediterranean and tropical legumes, it will be interesting to determine whether effectiveness traits in co-evolved symbioses are related to nodulation by a) bradyrhizobia versus rhizobia or b) promiscuity attributes of the host legume.

Are legumes in Agriculture fixing N optimally?

It has been proposed that rhizobial genotypes are differentially adapted to soil conditions and that it is this adaptation, rather than the relationship with their host, that most strongly governs outcomes relating to competition and persistence (Sprent 1994, Howieson 2000). Expressed in terms of the genotype-environment formula, we contend symbiotic effectiveness (G^2) is unimportant to the persistence and nodulation success of rhizobia relative to adaptation of the rhizobia to soil and climate (G_r x E). Less important again is G_l x E. 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.

An alternative view is that soil populations of rhizobia are likely to become enriched with strain types which are effective on the resident host legume through the superior growth of effectively nodulated plants and subsequent release of rhizobia from nodules (Vincent 1974). That is, G^2 becomes more important to rhizobial population development than (G_r x E). Some legume species may facilitate this process through their capacity to select effective rhizobial strains from mixed soil populations (Robinson 1969).

Is there evidence from effectiveness studies of co-evolved legume symbioses in the Mediterranean or tropics to support either of these proposals?

Evidence from the Mediterranean

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 result appears to be the rule rather than the exception. For another monospecific genus, *Hymenocarpus circinnatus* L. (Savi), which occurs throughout the Mediterranean basin, the search for rhizobial strains of high effectiveness indicated there was a low probability of predicting an optimal symbiotic relationship between legume host and rhizobia (G^2) based upon regional co-location of the germplasm. 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 (Howieson 2000). There are numerous other 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.* 2000). Thus patterns of effectiveness in Mediterranean symbioses lend support to the proposal that adaptation of rhizobia to soil and climate (G_r x E) more strongly govern outcomes of rhizobial persistence and competition for nodule occupancy than the N fixing capacity of the legume-rhizobia genotypes (G^2).

The above examples indicate that poorly effective symbioses are often established by relatively non-promiscuous co-evolved symbionts in the Mediterranean, where the microsymbiont is fast-growng (rhizobia). However, a curious contrast in a Mediterranean environment is seen with the annual herbaceous species *Ornithopus compressus* in symbiosis with the slow-growing *Bradyrhizobium* sp. (*Lupinus*). Whether in the Mediterranean basin or in south-west Australia, this particular symbiosis appears to be non-promiscuous and generally effective (McInnes *et al.* 1996).

Evidence from the Tropics

In contrast to the Mediterranean studies, we have evidence that some legume symbioses in the tropics are highly effective (Date 1977; Peoples *et al.* 1989). This introduces the possibility that G_r x E may be less important in determining nodule occupancy for tropical symbioses than in the Mediterranean region. However, tropical legumes in the promiscuous effective group such as

Arachis hypogaea, Vigna mungo, *V. radiata* and *V. unguiculata* have been shown to respond significantly to inoculation in over 50% of field trials in an international study (Singleton *et al.* 1992). Some of the legume species in this study responded to inoculation in their centre of origin. This may indicate that co-evolved tropical symbioses show sub-optimal patterns of N fixation, as found in the Mediterranean studies. Singleton *et al.* (1992) point out that there can be local heterogeneity of reponse to inoculation within the centre of origin of tropical legume species.

In Table 1 we have presented results from agar slope effectiveness tests on isolates of the tropical legumes *Stylosanthes seabrana* nom. nud. (formerly *S.* sp. aff. *scabra* – Date *et al.* 1996) and *S. macrocephala* collected from their centres of origin in the states of Bahia, Minas Gerias or Goias in Brazil (R.A. Date unpublished data). Of the isolates that nodulated *S. seabrana* and *S. macrocephala* (Table 1), 87% and 83% (respectively) gave over 80% growth relative to control plants receiving nitrogen. This indicates effective N fixation by the majority of isolates tested.

Similarly, sand-jar effectiveness tests with 28 rhizobial isolates from *Leucaena leucocephala* originating from the centre of origin for this species in Mexico showed that the majority of isolates (24) were highly effective (80%+ growth relative to +N controls) on *L. leucocephala* cv. Cunningham (CB *Rhizobium/Bradyrhizobium* strain collection database, CSIRO Tropical Agriculture, Brisbane Australia; Mullen *et al.* 1998).

In contrast, sand-jar effectiveness tests with *Desmanthus* spp. showed variable effectiveness response patterns. *Rhizobium* isolates from *D. acuminatus*, *D. fruticosus* and *D. virgatus* (obtained from Argentina and Brazil) ranged from effective (80%+ growth relative to +N controls) to ineffective (<35% growth relative to +N controls) on accessions of their homologous host. Similarly, more than 100 *Desmanthus* isolates collected from a region in Argentina contained only 53% of isolates which could be considered effective on an accession of *Desmanthus* collected from the same region (species not identified) (CB *Rhizobium/Bradyrhizobium* strain collection database, CSIRO Tropical Agriculture, Brisbane, Australia and R.A. Date personal communication). *Bradyrhizobium* associated with *Parasponia andersonii*, a non-legume species native to New Guinea (Trinick and Hadobas 1990), also showed sub-optimal effectiveness patterns with considerable variation in shoot and nodule dry matter production and N accumulation with the host of origin.

These studies indicate that rhizobial populations which have co-evolved with tropical legume hosts may be either highly effective on their homologous host or show sub-optimal effectiveness, as found in the Mediterranean basin. This is independent of the rhizobial genus, as hosts in each category were nodulated by *Rhizobium* (*L. leucocephala* and *Desmanthus* spp. isolates) and *Bradyrhizobium* (*Stylosanthes* spp. and *P. andersonii* isolates). In addition, there was no relationship between effectiveness patterns of co-evolved tropical legume symbioses and recognition of hosts as being promiscuous and effective (*L. leucocephala* and *Desmanthus* spp.) or more stringent in their strain requirements (*S. seabrana, S. macrocephala* and *P. andersonii*).

Therefore rhizobial persistence and competition for nodule occupancy may be determined by G_r x E as well as by G^2 in co-evolved tropical symbioses, depending on the symbiosis examined.

It is interesting to note the reaction of the tropical legume *Phaseolus vulgaris* when sown into soils in the Mediterranean basin. *Phaseolus vulgaris* grown in the Mediterranean is nodulated by up to five different species of *Rhizobium* (*Rhizobium leguminosarum* bv. *phaseoli, R. tropici,* *R. etli, R giardini* and *R. gallicum;* Van Berkum *et al.* 1996). However, in keeping with its performance in the tropics, the nodulation of this species is usually poorly effective.

At the outset of this paper, we had hoped to draw some conclusions relating efficiency of N fixation in the tropics relative to the Mediterranean, or at least of bradyrhizobial symbioses relative to rhizobial symbioses. As the symbiotic genes of *Rhizobium* are plasmid bound and prone to transfer we had hoped to show that symbioses involving *Rhizobium* were more unpredictable in N fixation than those involving *Bradyrhizobium* (whose symbiotic genes are chromosomal). We find, however, that poor symbiotic N fixation may be present in both climatic regions and with either rhizobial genus. With this as background, the next section illustrates that improvement in N fixation may be a man-managed activity.

Improving N fixation in Mediterranean and tropical Agriculture

There is considerable scope for improving N fixation (G^2) in sub-optimal Mediterranean and tropical legume symbioses. How 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.

For each scenario there are a number of research options to improve G^2, with the most likely option to succeed denoted by increased line density.

Scenario 1 -where the soil contains a high population of variably effective rhizobia that cause reduced G^2.

Scenario 2 -where the soil contains a low population of variably effective rhizobia that cause reduced G^2.

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

Some examples where the research options A-F have been successful in Australian research with Mediterranean legumes are given in Table 2. Examples of successful application of these research options to tropical legumes are given in Table 3.

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.

Optimising G^2 x E: 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 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:

A) optimal G^2: high N_2-fixation with the intended host species without compromising production from related species;
B) optimal G_r x E: adaptation to the edaphic environment targeted for the host;
C) maintenance of G^2: genetic stability in culture, storage and soil;
D) satisfactory growth and survival in inoculant manufacturing procedures, and
E) competitiveness with indigenous soil rhizobia.

A. *Screening for* G^2: *Selecting effective rhizobial strains with broad host-range characteristics*

To screen for genetic compatibility for N fixation between host and rhizobia ($G^{2)}$ our programs use naturally lit, controlled temperature glasshouses 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 fixation
- we acknowledge the necessity to select strains that will not compromise production from existing important legumes grown in the target region

We normally screen symbioses for nodulation and N fixation in sand culture (Howieson *et al.* 1995, Norris and Date 1976) rather than agar or vermiculite because it has proven impracticable to optimise growth culture conditions for each "new" legume studied. The sand culture method of Howieson *et al.* 1995 used to screen Mediterranean legumes consists of steamed or autoclaved 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. Tropical legumes are screened using a modified Leonard jar system (Norris and Date 1976). These systems 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 fixation is determined by comparing yields and %N of inoculated plants with nitrogen-fed and uninoculated controls. G^2 can then be ranked against a known standard and compared between experiments.

B. *Screening for* G_r *x E (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 selecting effective rhizobia for thew target legume (i.e high G^2) and then differentiates between them on the basis of G_r x E. One methodology for testing survival and colonisation by rhizobia in target soils is the "cross-row" technique, 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. However, if legumes are targeted for clay soils (eg. the black cracking clays of southern Queensland) then it may be necessary to take cores over root systems and recover roots and nodules through a process of soaking and sieving. Clay soils may also contain high levels of soil N which mask inoculation responses. In this case strain selection is best performed on soils where N levels have been depleted by non-legume crop or pasture species.

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 dry season 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.

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).

C. *Maintaining G^2: 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. These species were not inhabitants of Australian soils before the 19th Century, hence the diversity must have arisen somehow. The development of diversity within the bradyrhizobial population of south-western Australia has been well documented. Intensive studies in both 1930 (Adams and Riches 1930) and then 30 years later (Lange and Parker 1960) found that the *Ornithopus* nodulating population of root nodule bacteria was very narrow. However by 1996, that population had become very diverse (McInnes *et al.* 1996). 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.

D. *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 survival in 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 *et al.* 2000).

E. *Competitiveness with indigenous soil rhizobia*

Introduction of rhizobial inoculant strains into populations of variably effective rhizobia (strategies C and D, Fig. 2) requires selection of strains which are competitive for nodule occupancy. A modern approach to studying 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 G_r x E, 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 the polymerase chain reaction (PCR) 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 Mediterranean and tropical agricultural environments. That is, G^2 is often sub-optimal. We should not assume that because of geographic co-evolution, the legume and its microsymbiont are perfectly matched to fix N. The strongest selection pressure on rhizobia may not be 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.

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Table 1 - Effectiveness of bradyrhizobia collected from the centre of origin of *Stylosanthes* spp. in Brazil when inoculated on *S. seabrana* and *S. macrocephala.*

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Rating = % growth relative to +N controls	Effectiveness of *S. seabrana* isolates on *S. seabrana* (number of isolates)	Effectiveness of *S. macrocephala* isolates on *S. macrocephala* (number of isolates)
100%+	152	81
80-100%	131	100
50-80%	39	24
<50%	3	14

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

Pathway	Legume	Reference	Comments
A	*Medicago littoralis* *Trifolium michelianum*	Ballard *et al.* 2000	cv. Pildappa well adapted to the medic rhizobial population in alkaline soils
Bi, Bii	*Lotus ornithopodiodes, Biserrula pelecinus*	Ballard, Howieson, Loi unpublished data, Howieson *et al.* 1995	legumes which avoid interaction with a poorly effective medic rhizobial population on soils pH 6-9.
C	*Ornithopus* spp	McInnes (unpublished data)	Good nodule occupancy in the year of sowing on acid soils.
D	*Trifolium* spp	Watkin *et al.* 1999, Howieson 1999, Howieson *et al.*2000c	A broad host range strain WSM409 selected for new trifoliums and sub-clover to replace WU95.
D	*Vicia, Pisum, Lens*	Howieson *et al.* 2000c	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.*	Howieson unpublished data	The current scenario with many introduced legumes in Australia

Table 3 - Examples where applying the research pathways A-F in Fig 2 have been successful in developing improved symbiotic N fixation with tropical crop and forage legumes.

Pathway	Legume	Reference	Comments
A,E	*Gycine max* (promiscuous)	Mpepereki *et al.* (2000)	In Africa
Bi, Bii	*Glycine max* (specific)	Mpepereki *et al.* (2000)	In Africa
C	*Arachis hypogaea*	H.V.A Bushby and R.A. Date personal communication	In Australia
	Calliandra calothyrsus	R.A. Date personal communication	In Australia
D	*Desmanthus* spp.	Date 1991	In Australia
	Vigna spp.	Bushby 1988	In Australia
	Trifolium semipilosum	Jones and Date (1975)	In Australia
F	*G. max* (specific)	Bushby *et al.* (1983)	No background population for commercial cultivars with specific strain requirements in Australia
	Stylosanthes seabrana	Date *et al.* (1996)	Mostly no background bradyrhizobia in Australia
	Lotononis bainesii	Diatloff (1977)	In Australia

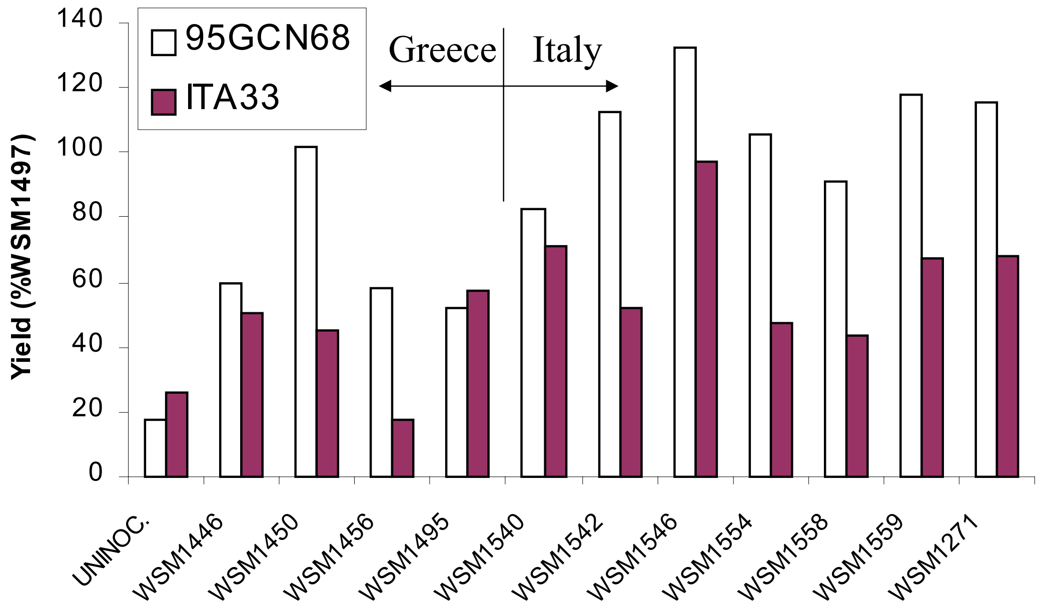

Figure 1- Yield of two genotypes of *Biserrula pelecinus* (95GCN68 from Greece and ITA33 from Italy) when inoculated with rhizobia collected from either Greece or Italy, as arrowed. Data are presented for yield of tops as a proportion of the commercial inoculant strain in Australia, WSM1497 (Howieson *et al.* 2000b).

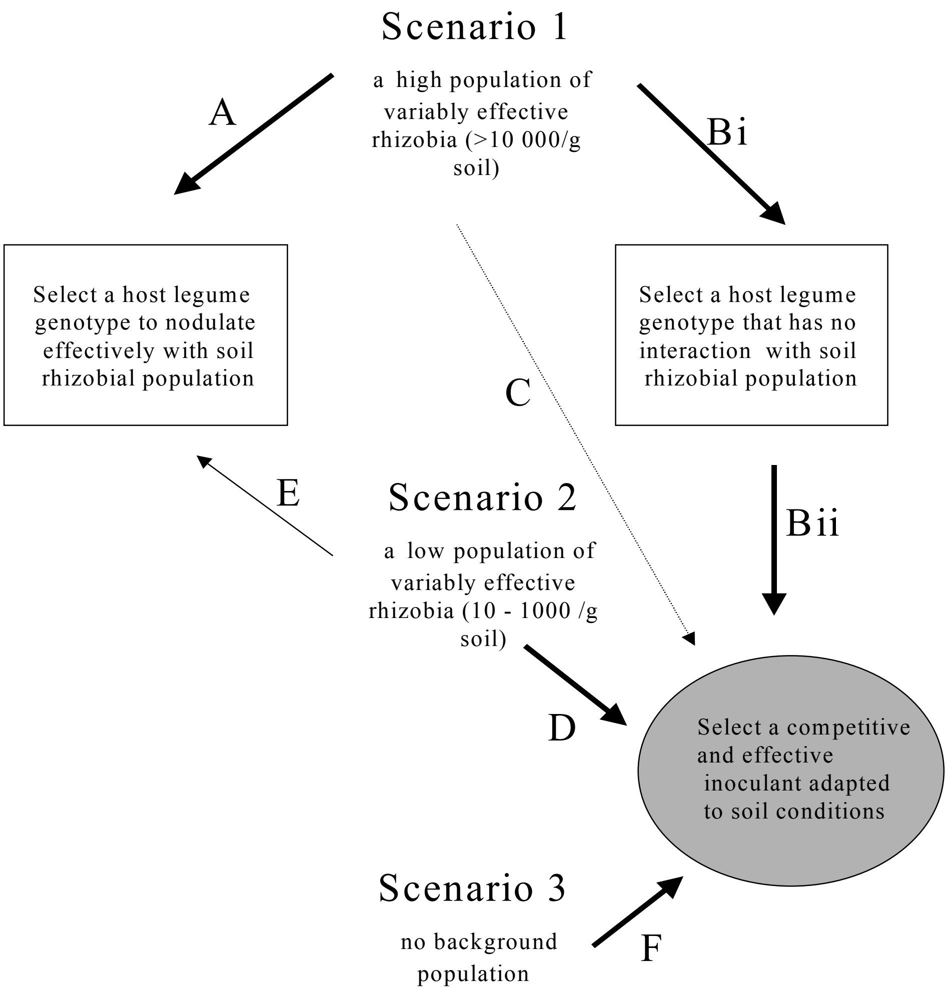

Figure 2 - A schematic representation of strategies to improve N fixation in legumes through selection of either the host or rhizobial genotype (from Howieson *et al*. 2000b).

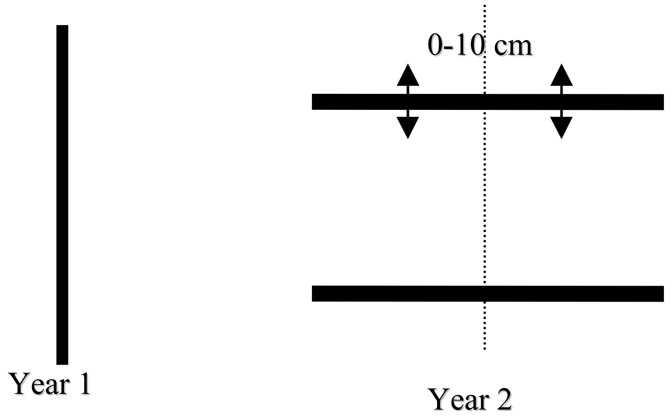

Figure 3 - A schematic diagram of the "cross-row" technique. Bold lines represent seed sown inoculated (year 1) or uninoculated (year 2).