Plant and Soil **147**: 151–158, 1992.

PLSO 9451

Selection of Portuguese *Rhizobium leguminosarum* bv. *trifolii* strains for production of legume inoculants

I. Screening for effectiveness in laboratory conditions

E.M. FERREIRA and J.F. MARQUES
Estação Florestal Nacional, Tapada das Necessidades, Rua do Borja 2, P-1300 Lisboa, Portugal and Estação Agronómica Nacional, Quinta do Marquês, P-2780 Oeiras, Portugal

Received 20 December 1991. Revised July 1992

Key words: growth cabinet, legume inoculants, native strains, nitrogen fixation, *Rhizobium leguminosarum* bv. *trifolii*, *Trifolium subterraneum*, symbiotic effectiveness

Abstract

From several native clover species, growing in six different soil types, 170 *Rhizobium leguminosarum* biovar *trifolii* strains were isolated, covering the central and southern regions of Portugal. The effectiveness of the strains varied from ineffective to highly effective on *T. subterraneum* cv. Clare and on *T. fragiferum* cv. Palestine, with a predominance of medium and high effectiveness on both host plants. The effectiveness was not influenced by provenence (soil or plant), except for the strains from the rankers soils and for the strains isolated from *T. pratense*, that were ineffective or medium effective on *T. subterraneum*.

Selected strains were evaluated for effectiveness on *T. subterraneum* cv. Clare, using the commercial strain TA1 as reference. Several of the isolated strains were more effective than TA1, indicating that local strains may be used to produce better inoculants.

Introduction

The inoculation of legume seeds with *Rhizobium*, one of the most successful cases of the exploitation of soil microbiology, is a frequent agricultural practice and an environmentally safer source of nitrogen, than chemical nitrogen fertilizers.

Portugal, a country with an abundance of native clovers, with centuries of co-evolution of the legume-*Rhizobium* association, should have a high genetic variation among rhizobial populations (Brockwell, 1981; Brockwell and Katznelson, 1976; Lie, 1981; Lie et al., 1987; Mytton and Livesey, 1983). Under such conditions a large diversity of *Rhizobium* strains could be present and the frequency of occurrence of effective strains could also be high. Under these circumstances, the establishment of introduced strains may not be successful (Brockwell, 1981).

Currently the Australian strains TA1, WA67 and CC2480a have been used as inoculants for subterranean clover in Portugal. Since these strains are aliens, they may be less successful in coping with the biological and abiotic factors in the new surroundings (Brockwell, 1981; Dudman and Brockwell, 1968). The strains most competitive in nodule formation and persistent in a particular field environment are often those isolated from similar environments (Chatel and Parker, 1973; Chatel and Greenwood, 1973; Chatel et al., 1973).

The purpose of this research was to test the hypothesis that locally isolated rhizobial strains are more effective than the imported strains. Strains of *Rhizobium leguminosarum* biovar *tri-*

folii were isolated from several different species of native clovers growing in a wide range of different soils in Portugal. This is the first investigation of symbiotic effectiveness diversity of rhizobial strains in Portugal.

A collection was also made of several other species of *Rhizobium*.

Materials and methods

Isolation of Rhizobium *strains from native clovers*

The *Rhizobium* strains tested were isolates from several native clovers (*Trifolium angustifolium* L., *Trifolium campestre* Schreb., *Trifolium cherleri* L., *Trifolium pratense* L., *Trifolium subterraneum* L., *Trifolium* sp. and *Trifolium tomentosum* L., respectively designated, Ta, Tc, Tch, Tp, Ts, TR and Tt) occurring in a variety of soils in the south and central regions of Portugal.

The samples came from 87 different sites covering 6 soil types (lithosols, luvisols, podzols, cambisols, regosols + podzols and rankers). At each site, a random plant showing good growth in a native pasture under typical conditions of the region was chosen. The whole plant was uprooted, packed into a plastic bag and brought to the laboratory. From each plant 2 or 3 large nodules were taken.

Rhizobium isolates were obtained as described by Vincent (1970). Growth characteristics on solid and liquid media, and data on the origin and main characteristics of each isolate were recorded.

The isolates were labeled by consecutive numbering, followed by two letters identifying the host plant and by a number indicating the number given to the nodule taken for analysis, e.g. 123Ts2, where 123 is the number of sample, Ts is *Trifolium subterraneum* and 2 is the second nodule chosen.

Authentication of isolates and measurement of symbiotic effectiveness

Preliminary screening
The ability of isolates to nodulate test host legumes and their capacity to fix atmospheric nitrogen under bacteriologically controlled conditions, was used as a preliminary evaluation of symbiotic effectiveness.

The host plants in these studies were two Australian commercial species of clover: *Trifolium subterraneum* spp. *brachycalycinum* cv. Clare, an annual plant, and *Trifolium fragiferum* cv. Palestine, a perennial. These clovers are widely used in Portugal showing a good performance in field pastures under different soil conditions. Since these selected strains were tested on these commercial clovers, the effects of host-bacteria interaction should have been minimized (Date and Norris, 1979; Gibson, 1980; Mytton, 1976; Mytton and Livesey, 1983; Mytton et al., 1984; Nurhayati et al. 1988) when used for production of legume inoculants.

Seeds of *T. subterraneum*, cv. Clare and *T. fragiferum* cv. Palestine, were rinsed with ethanol, surface-sterilized in acidified mercuric chloride (Vincent, 1970) for 5 minutes, washed with 6 changes of sterile water and placed in sterile petri dishes containing water agar to germinate. Two-day old seedlings, with straight radicles about 5 mm long, were aseptically transferred to large cotton wool plugged tubes (300 mm by 32 mm) containing washed river sand (120 g), moistened with 20 ml of N-free Jensen's medium (Jensen, 1942). After three days growth, 5 replicate seedlings of *T. subterraneum* and *T. fragiferum*, were inoculated with a 1 mL suspension (10^8 cells) of each isolate. Appropriate uninoculated and nitrogen controls (5 mM KNO_3) were included (Vincent, 1970).

Plants were grown for an average of 7 weeks in a controlled environment growth cabinet (18°C/12 hours of light period, 15°C/12 hours of dark period) with a mean photosynthetic photon flux density of 250 μmol m^{-2} s^{-1} at plant height (outside the tubes).

The mean dry weight of shoots (X) was used to calculate an index of effectiveness (E) defined as:

$$E_j = \frac{X_j - X_{TO}}{X_{TN} - X_{TO}} \times 100$$

where j is the inoculated test strain, TO is the uninoculated control and TN the nitrogen control (Marques Pinto, pers. com.). The terms ineffective (I), medium (ME) and highly (HE) effective strains were adopted when E < 25, 25–74 or ≥75, respectively.

The strains were grouped by indices of effectiveness according to their effectiveness response on *T. subterraneum* and *T. fragiferum* in order to determine if groupings corresponded to different origins, soil and host (Tables 1 and 2).

Second screening

Due to the high number of isolates (170) collected and screened during two consecutive years in 9 experiments, it was decided to carry out a second screening involving selected strains to be evaluated at the same time.

For this screening we chose from each of the earlier experiments the most effective strains on *T. subterraneum* and *T. fragiferum* and some others with special features (differential effectiveness on different species of clover, diversity of origin – soil and plant) in order to obtain a diversity of germplasm, and 41 strains were selected. The plants were grown as described above for a period of 45 days with 10 replicate seedlings of each clover cultivar. The results are listed in Table 3.

As the selected strains, if found suitable, are intended to replace the Australian strains, we used the strain TA1 as a reference, since it was the best under these conditions. Relative effectiveness against strain TA1 was calculated by the following expression (Bergersen et al., 1971; Gibson 1980, 1987; Gibson et al., 1975).

$$\text{relat. effectiveness} = \frac{\text{Dry wt., inoculated test strain}}{\text{Dry wt., inoculated standard strain}} \times 100$$

Statistical analysis

Statistical analysis was carried out using STATGRAPHICS version: 4.0 package, and the mean separation procedure of LSD.

Results

Preliminary screening

All inoculated plants formed nodules, but varied in symbiotic effectiveness from ineffective to highly effective.

Effectiveness of strains according to soil and host plant of origin

The 170 isolates, grouped within each soil type and host plant by indices of effectiveness on *Trifolium subterraneum* cv. Clare and *Trifolium fragiferum* cv. Palestine are shown in Tables 1 and 2, respectively.

We found wide differences in effectiveness among the 170 strains isolated from native clovers, ranging from ineffective to highly effective (Tables 1 and 2), with a predominance of strains of medium and high effectiveness (66 and 72 respectively with *T. subterraneum* and 64 and 81 respectively with *T. fragiferum*). Of the 170

Table 1. Number of indigenous *R. leguminosarum* bv. *trifolii* strains for groups of effectiveness (I, ME and HE) on *T. subterraneum* cv. Clare and *T. fragiferum* cv. Palestine, according to soil or origin

Soil		Indices of effectiveness								
	Clare	I	I	I	ME	ME	ME	HE	HE	HE
	Palestine	I	ME	HE	I	ME	HE	I	ME	HE
Lithosols		5	4	6	1	16	7	0	4	30
Luvisols		4	1	3	1	15	4	0	7	19
Podzols		0	1	0	2	6	1	1	0	5
Cambisols		1	1	0	3	3	0	0	1	2
Regosols + Podzols		1	1	1	1	3	0	1	0	2
Rankers		3	0	0	1	1	1	0	0	0
Total		14	8	10	9	44	13	2	12	58

Table 2. Number of indigenous *R. leguminosarum* bv. *trifolii* strains for groups of effectiveness (I, ME and HE) on *T. subterraneum* cv. Clare and *T. fragiferum* cv. Palestine, according to host plant of origin

Plant of origin		Indices of effectiveness								
	Clare	I	I	I	ME	ME	ME	HE	HE	HE
	Palestine	I	ME	HE	I	ME	HE	I	ME	HE
T. subterraneum		10	3	7	2	37	9	1	7	41
T. pratense		4	2	1	5	2	1	0	0	0
T. angustifolium		0	0	0	0	2	2	0	2	9
T. spp.		0	2	1	0	3	1	0	2	5
T. campestre		0	0	0	2	0	0	1	0	1
T. tomentosum		0	1	0	0	0	0	0	0	2
T. cherleri		0	0	1	0	0	0	0	1	0
Total		14	8	10	9	44	13	2	12	58

strains, 54 showed different indices of effectiveness according to test plant and 116 showed similar indices for both test plants, 14, 44 and 58 being ineffective, medium and highly effective, respectively.

A wide range of strain effectiveness on both test plants was found in all the soils and host plants (Tables 1 and 2). However, none of the strains from the rankers soils (6) or from *T. pratense* (15) were highly effective on *T. subterraneum*.

The strains isolated from *T. subterraneum*, grouped in Table 2 seem to possess greater effectiveness with *T. fragiferum* than with *T. subterraneum*. We obtained 10 (3 + 7) ineffective strains on *T. subterraneum* which are medium or highly effective on *T. fragiferum* and only 3 (2 + 1) with opposite behaviour. We also found 9 strains with medium effectiveness on *T. subterraneum* and highly effective on *T. fragiferum* and 7 strains with opposite behaviour.

Second screening

From the preliminary screening, 41 strains were selected for assessing relative effectiveness using TA1 as reference (100%). The results are shown in Table 3.

The second screening (41 strains) indicated that 29 strains used with *T. subterraneum* and 8 used with *T. fragiferum* possessed a symbiotic effectiveness superior to the reference strain TA1, 6 of them being superior to TA1 with both hosts. However, only 7 strains with *T. subterraneum* and none with *T. fragiferum* were significantly different from TA1.

The relative effectiveness of selected native strains (Table 3) using TA1 as reference, showed the good performance of these strains for production of inoculants.

Eleven strains were selected (those marked[a] on Table 3) for future studies. Effectiveness on *T. subterraneum* cv. Clare was the primary criterion for selection, since it is the most widely sown clover. Diversity of host plant origin was considered as a secondary criterion in order to maintain a greater diversity of germplasm. One strain, 129Ts3, was selected in particular, because it showed a great difference in effectiveness on the two host plants, being the lowest with *T. subterraneum*.

Discussion

The strains tested may be considered fairly representative of the nodule population of *Rhizobium leguminosarum* biovar *trifolii*. Vincent (1988), reported that paired nodules from one plant were about as likely to yield different strains, as nodules formed on different plants. Working with a pea crop, Young et al. (1987) found that only 2.5% of the overall variation was due to differences between subsamples.

The wide range of effectiveness obtained from these screening tests (Tables 1 and 2) and the indication that a potential exists for developing legume inoculants from local *Rhizobium* strains

Table 3. Plant dry weight yield (mg) and relative effectiveness of 41 strains of *Rhizobium leguminosarum* biovar *trifolii* on *T. subterraneum* cv. Clare and *T. fragiferum* cv. Palestine, using the strain TA1 as reference

Strain	Clare		Palestine	
	Dry wt./ plant	Relative effectiveness	Dry wt./ plant	Relative effectiveness
21 Ts2	33.8	82	51.1	91
25 Ts1	41.9	105	51.9	94
28 Ts1	42.7	107	49.0	89
34 Ts1	43.1	107	41.4	75
36 Ts3	40.6	102	45.8	84
38 Tc1	44.2	110	48.8	89
46 Ta2	36.7	92	46.9	85
51 Tch1[a]	44.7	112	41.4	76
53 Ts2	44.1	110	57.5	105
76 TR2[a]	48.2	120	53.4	96
89 Ts2	40.1	100	53.7	98
89 Ts3	42.8	107	56.1	101
100 Ts1	42.9	105	46.4	84
102 Ts1[a]	47.8	120	51.4	93
103 Ts1	44.9	112	46.8	85
104 Ts2	35.3	87	46.5	85
108 Ts3	39.1	97	53.3	96
110 Ts3	41.0	102	54.6	100
111 Ta1	44.4	110	49.0	89
111 Ta2	40.9	102	48.0	87
112 Ts1	40.9	102	46.0	84
114 Ta1	35.8	90	41.3	75
117 Ts1	37.5	95	46.4	84
117 Ts2	42.8	107	17.1	31
120 Ts2	42.6	107	56.5	104
121 Ts2	40.1	100	60.7	111
123 Ts1[a]	48.4	122	53.3	95
123 Ts2[a]	52.4	130	54.7	100
126 Ts3	43.6	110	58.7	107
129 Ts3[a]	26.2	65	54.1	98
145 TR2	32.6	82	59.7	109
146 Ta1[a]	47.7	120	53.4	96
147 Ts3[a]	45.1	112	48.0	87
148 Ts1[a]	46.4	115	33.8	62
149 Ts1[a]	45.4	112	56.0	102
149 Ts2[a]	48.7	120	57.5	105
150 Ts4	42.5	107	52.1	95
152 Tt2	42.3	105	52.0	95
152 Tt3	40.9	102	45.4	82
155 Tp2	27.6	70	5.0	9
155 Tp3	34.2	85	44.6	82
TA1	40.4	100	55.4	100
LSD (0.05)	6.0		7.6	
AVR (41 strains)[b]		104		88

[a] Selected strains.
[b] Average of relative effectiveness.

(Table 3) are in agreement with results of workers from other countries. Friedericks et al., (1990) working with isolates from native Ethiopian clover species, found significant differences in effectiveness among strains, which as a group, showed higher rates of N-fixation than the commercial Nitragin inoculant. Working with Indonesian *Rhizobium* strains for *Vigna un-*

guiculata L. and *Macroptilium atropurpureum* Urb., Nurhayati et al. (1988) found a broad spectrum of moderate to highly successful strains (compared to commercial ones) with regard to nodulation and dry matter production; some local strains proved to be superior to those currently imported from Australia.

Gibson et al. (1975) examined *Rhizobium trifolii* populations from 8 regions of Australia over 5 years and found that the effectiveness ratings for any region were between 62 and 93% of their standard strain (TA1). His results (in contrast to the wide range of effectiveness we found) may indicate that the strains he isolated were derived from a limited number of *Rhizobium* genotypes introduced into those 8 regions. Lie and Göktan, (1984) and Lie et al., (1987), working with pea plants (*Pisum sativum* L. cv. Rondo), also verified that the symbiotic variation among the European *Rhizobium* populations was relatively small. All the strains ranged from 65 to 110% in effectiveness, using the Dutch *Rhizobium* strain PF2 as control (100%). However, strains from Middle East populations, one of the gene centres for peas, included some that were completely ineffective, partly effective or effective.

Contrasting with the results of Gibson et al., (1975), Bergersen et al., (1971) found a wide range of symbiotic effectiveness in soils of south-eastern Australia, and suggested that the limited introduction of *Rhizobium trifolii* into their country with the introduction of clovers had been overcome by naturalized population evolution or by introduction of alien strains.

Working with *Trifolium repens* in the United Kingdom, Holding and King (1963) showed that the effectiveness of a large proportion (73%) of natural *Rhizobium trifolii* strains was unsatisfactory. They found that the mean effectiveness of *Rhizobium* populations was significantly correlated with per cent base saturation, pH, exchangeable calcium and exchangeable magnesium. Similar results were obtained by Hagedorn, (1978) in Southwest Oregon soils where the effectiveness of *Rhizobium trifolii* populations was predominantly poor and correlated with soil base status and phosphorus levels. However, these results do not agree with ours, where strains isolated from soils with a poor base status (podzols) seemed on the average to possess an effectiveness not inferior to those isolated from other soils (Table 1).

The results in Tables 1 and 2 show that soil type and plant origin did not influence general effectiveness of natural populations of *Rhizobium leguminosarum* biovar *trifolii*. Brockwell and Katznelson, (1976) in soils from Israel also found that the soil type did not influence the general effectiveness of natural populations.

All the strains we isolated from *T. pratense* (Table 2) were ineffective or medium effective on *T. subterraneum*, but not on *T. fragiferum*.. This suggests that the former clover species can be highly specific symbiotically. This hypothesis is supported by the results of Vincent (1954). He reported that rhizobia isolated from *T. pratense* were ineffective on *T. subterraneum*, while Robinson (1969) also found that isolates from *T. pratense* were less effective on *T. subterraneum* than on *T. pratense*.

It should be mentioned that strains with a good performance on both *T. subterraneum* and *T. fragiferum* were isolated from native spontaneous clovers. The effectiveness of the strains isolated from subclovers (grouped in Table 2) being greater with *T. fragiferum* cv. Palestine than with *T. subterraneum* cv. Clare support the results of Brockwell and Katznelson (1976), where the least responsive species to inoculation, could capture the most effective strains when used as trap hosts.

The occurrence of a broad spectrum of strains may be attributed to evolution and adaptation during centuries and to the diversity of clovers. The majority (68%) of the strains in this study showed similar indices of effectiveness for both hosts. Thus, in spite of the interactive nature of effectiveness (Brockwell and Katznelson, 1976; Mytton, 1976; 1984; Mytton and Livesey, 1983; Mytton et al., 1984), the strain itself, seems to play a very important role.

This study points to the heterogeneity of native strains, which could probably be larger, if the nodule collections were not limited to the more prominent nodules. This methodology, however, can partially contribute to the selection of medium and highly effective strains. These may form a good basis for the production of high quality inoculants, capable of replacing the Australian strains used up to the present.

The results obtained from these limited collections of *Rhizobium* strains, certainly call for further collections and evaluation work.

Acknowledgements

We thank Prof. Marques-Pinto, Polytechnic of Coimbra, for comments on the study, Prof. A V Garcia, Technical University of Lisbon and Dr. D Eskew, University of Tennessee, for assistance in preparing this manuscript.

References

Bergersen F J, Brockwell J, Gibson A H and Schwinghamer E A 1971 Studies of natural populations and mutants of *Rhizobium* in the improvement of legume inoculants. Plant and Soil Spec. Vol. 3–16.

Brockwell J 1981 A strategy for legume nodulation research in developing regions of the old world. Plant and Soil 58, 367–382.

Brockwell J and Katznelson J 1976 Symbiotic characteristics of *Rhizobium trifolii* from Israel in association with 10 species of *Trifolium*. Aust. J. Agric. Res. 27, 799–810.

Chatel D L and Parker C A 1973 The colonization of host-root and soil by rhizobia. I. Species and strain differences in the field. Soil Biol. Biochem. 5, 425–432.

Chatel D L and Greenwood R M 1973 The colonization of host root and soil by rhizobia. II. Strain differences in the species *Rhizobium trifolii*. Soil Biol. Biochem. 5, 433–440.

Chatel D L, Shipton W A and Parker C A 1973 Establishment and persistence of *Rhizobium trifolii* in Western Australian soils. Soil Biol. Biochem. 5, 815–824.

Date R A 1982 Collection, isolation, characterization and conservation of *Rhizobium*. *In* Nitrogen Fixation in Legumes Ed. J M Vincent. pp 95–109. Academic Press, Sydney.

Date R A and Norris D O 1979 *Rhizobium* screening of *Stylosanthes* species for effectiveness in nitrogen fixation. Aust. J. Agric. Res. 30, 85–104.

Dudman W F and Brockwell J 1968 Ecological studies of root-nodule bacteria introduced into field environments. 1. A survey of field performance of clover inoculants by gel diffusion serology. Aust. J. Agric. Res. 19, 739–747.

Friedericks J B, Hagedorn C and Vanscoyoc S W 1990 Isolation of *Rhizobium leguminosarum* (biovar *trifolii*) strains from Ethiopian soils and symbiotic effectiveness on African annual clover species. Appl. Environ. Microbiol. 56, 1087–1092.

Gibson A H 1972 The influence of the environment and managerial practices on the legume-*Rhizobium* symbiosis. *In* A Treatise on Dinitrogen Fixation. Section IV: Agronomy and Ecology. Eds. R W F Hardy and A H Gibson. pp 393–450. Wiley, New York.

Gibson A H 1980 Methods for legumes in glasshouses and controlled environment cabinets. *In* Methods for Evaluation Biological Nitrogen Fixation. Ed. F J Bergersen. pp 139–184. Wiley, Brisbane.

Gibson A H 1987 Evaluation of nitrogen fixation by legumes in the greenhouse and growth chamber. *In* Symbiotic Nitrogen Fixation Technology. Ed. G H Elkan. pp 321–369. Marcel Dekker, New York.

Gibson A H, Curnow R W, Bergersen F J, Brockwell J and Robinson A C 1975 Studies of field populations of *Rhizobium*: Effectiveness of strains of *Rhizobium trifolii* associated with *Trifolium subterraneum* L. pastures in South-Eastern Australia. Soil Biol. Biochem. 7, 95–102.

Hagedorn C 1978 Effectiveness of *Rhizobium trifolii* populations associated with *Trifolium subterraneum* L. in Southwest Oregon soils. Soil Sci. Soc. Am. J. 42, 447–451.

Holding A J and King J 1963 The effectiveness of indigenous populations of *Rhizobium trifolii* in relation to soil factors. Plant and Soil 18, 192–198.

Jensen H L 1942 Nitrogen fixation in leguminous plants. 1. General characters of root-nodule bacteria isolated from species of *Medicago* and *Trifolium* in Australia. Proc. Linn. Soc. NSW 67, 98–108.

Lie T A 1981 Environmental physiology of the legume-*Rhizobium* symbiosis. *In* Nitrogen Fixation. Vol. 1: Ecology. Ed. W J Broughton. pp 104–134. Clarendon Press, Oxford.

Lie T A and Göktan D 1984 Gene centres, a source for genetic variants in symbiotic nitrogen fixation: The symbiotic response of the cultivated pea to *Rhizobium leguminosarum* strains from Europe and the Middle East. Plant and Soil 82, 359–367.

Lie T A, Göktan D, Engin M, Pijnenborg J and Anlarsal E 1987 Co-evolution of the legume-*Rhizobium* association. Plant and Soil 100, 171–181.

Mytton L R 1976 The relative performance of white clover genotypes with rhizobial and mineral nitrogen in agar culture and in soil. Ann. Appl. Biol. 82, 577–587.

Mytton L R 1984 Developing a breeding strategy to exploit quantitative variation in symbiotic nitrogen fixation. Plant and Soil 82, 319–335.

Mytton L R and Livesey C J 1983 Specific and general effectiveness of *Rhizobium trifolii* populations from different agricultural locations. Plant and Soil 73, 299–305.

Mytton L R, Brockwell J and Gibson A H 1984 The potential for breeding an improved lucerne-*Rhizobium* symbiosis. 1. Assessment of genetic variation. Euphytica 33, 401–410.

Nurhayati D P, Diatloff A and Hoult E H 1988 The effectiveness of some Indonesian strains of *Rhizobium* on four tropical legumes. Plant and Soil 108, 171–177.

Robinson A 1969 host selection for effective *Rhizobium trifolii* by red clover and subterranean clover. Aust. J. Agric. Res. 20, 1053–1060.

Vincent J M 1954 The root-nodule bacteria as factors in clover establishment in the red basaltic soils of the Lismore district, New South Wales. I.A. survey of native strains. Aust. J. Agric. Res. 5, 55–60.

Vincent J M 1970 A Manual for the Practical Study of the Root-Nodule Bacteria. IBP Handbook No. 15. Blackwell Scientific Publications, Oxford.

Vincent J M 1988 Ecological aspect of the root-nodule bacteria: Competition and survival. *In* Nitrogen Fixation

by Legumes in Mediterranean Agriculture. Eds. D P Beck and L A Materon. pp 165–172. Martinus Nijhoff Publishers, Dordrecht, The Netherlands.
Young J P W, Demetriou L and Apte R G 1987 *Rhizobium* population genetics: Enzyme polymorphism in *Rhizobium leguminosarum* from plants and soil in a pea crop. Appl. Environ. Microbiol. 53, 397–402.