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Symbiotic and competitive properties of motility mutants of *Rhizobium trifolii* TA1

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Abstract. Non-motile mutants of *Rhizobium trifolii* defective in either flagellar synthesis or function were isolated by transposon Tn*5* mutagenesis. they were indistinguishable from motile control strains in growth in both laboratory media and in the rhizosphere of clover roots. When each non-motile mutant was grown together with a motile strain in continuous culture, the numbers of motile and non-motile organisms remained in constant proportion, implying that their growth rates were essentially identical. When inoculated separately onto clover roots, the mutants and wild-type did not differ significantly in the number of nodules produced or in nitrogen fixing activity. However, when mixtures of equal numbers of mutant and wild-type cells were inoculated onto clover roots, the motile strain formed approximately five times more nodules than the flagellate or non-flagellate, non-motile mutants, suggesting that motility is a factor in competition for nodule formation.

Key words: *Rhizobium trifolii* – Motility mutants – Nodulating competitiveness – Nodulation – Rhizosphere

Bacteria of the genera *Rhizobium* and *Bradyrhizobium* form symbioses with leguminous plants, resulting in the production of root nodules in which fixation of atmospheric nitrogen occurs.

The successful establishment of introduced inoculant strains of rhizobia depends on their ability to compete with indigenous rhizobial populations for nodulation of host legumes. To maximise the potential benefits of highly effective inoculant strains, it is important to understand the physiological and ecological factors which affect competition with indigenous strains. For example, temperature (Marshall 1964; Somasegaran et al. 1983; Gaworzewska and Carlile 1982) and pH (Munns 1968) are known to influence the growth and persistence of soil rhizobia.

It is unclear how rhizobia in the soil reach the rhizosphere of legumes. Bacterial motility, either random or chemotactic, may be important in this early stage of the interaction; rhizobia are regularly motile, the flagellation being subpolar in the slow-growing strains (*Bradyrhizobium* spp.) and peritrichous in the fast-growing strains (*Rhizobium* spp; De Ley and Rassel 1965). Various *Rhizobium* species have been found to be chemotactic towards specific sugars, sugar alcohols and amino-acids (Currier 1980; Bowra and Dilworth 1981; Burg et al. 1982), or towards components of root exudates form their plant hosts (Currier and Strobel 1976a, b; Gitte et al. 1978). The importance of motility per se to rhizobia in the soil was first investigated by Ames et al. (1980). It was found initially that flagellated or non-flagellated non-motile mutants of *Rhizobium meliloti* produced as many nodules on alfalfa plants as did the wild type. Further work showed, however, that the motile strain of *R. meliloti* had a marked advantage over both types of non-motile mutants when mixed in various unfavourable ratios (Ames and Bergman 1981). Similarly, Hunter and Fahring (1980) found that a slow-migrating mutant of *Bradyrhizobium japonicum* S-110 was much less successful in nodulation than the wild type, and Soby and Bergman (1983) showed that efficient spreading of *Rhizobium meliloti* in various autoclaved soils was dependent on its motility and chemotactic behaviour.

Thus, evidence exists that motility is an important factor in competition between rhizobal strains. However, analysis of the interactions between motile and non-motile strains requires that these be controlled for other ecologically important parameters such as growth rate. In the studies cited above, it is not clear whether motile and non-motile strains had the same growth rates in pure and mixed culture, and whether growth rates of motile and non-motile strains were the same in laboratory media, and, more importantly, in the rhizosphere.

In this report, the interactions of a motile strain of *R. trifolii* with non-motile mutants, both flagellated and non-flagellated, are analysed. The growth of these strains is described in batch and continuous culture in the laboratory, and in the clover rhizosphere, and against this background, the effects of loss of motility on the ability to nodulate in competition with the wild type strain are assessed. Rhizosphere growth and competition experiments were carried out in yellow sand, as this is an important soil type in agricultural areas of Western Australia.

Methods

Organism

Rhizobium trifolii TA1 (J. M. Vincent, University of NSW, Sydney), is a wild type strain which nodulates subterranean clover (*Trifolium subterraneum*). Derivatives of this strain used in this study are shown in Table 1.

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Table 1. Strains of *Rhizobium trifolii*

Strain	Derivation	Antibiotic resistance ($\mu g\ ml^{-1}$)	Motile	Flagella
TA1	Wildtype	–	+	+
MNF1000	Spontaneous $Str^{R\,a}$ mutant of TA1	Str (100)	+	+
MNF1003	Spontaneus $Rif^{R\,a}$ mutant of MNF1000	Str (100) Rif (50)	+	+
$Tn5^+$ *derivatives of MNF 1000*				
MNF1002	Spontaneous Rif^R mutant of motile MNF1000::Tn*5*	Str (100) Kan (50) Rif (50)	+	+
MNF1005	Non-motile mutant	Str (100) Kan (50)	–	–
MNF1012	Non-motile mutant	Str (100) Kan (50)	–	+

Phenotypic designation: Kan = kanamycin; Rif = rifampicin; Str = Streptomycin

[a] Str^R and Rif^R mutants of TA1 arose spontaneously at frequencies of ca 10^{-7} and 10^{-8} respectively

Media

Bacteria were grown at 28° C on YMA medium (Vincent 1970), containing 1.5% agar (Difco), and supplemented with antibiotics as required. Antibiotics (Sigma Chemical Co.) were used at the following concentrations ($\mu g \cdot ml^{-1}$): streptomycin 100, kanamycin 50, rifampicin 50. Cycloheximide ($50\ \mu g \cdot ml^{-1}$) was included in all plates used for the rhizosphere experiment. Batch cultures were grown at 28° C in the liquid minimal salts medium of Brown and Dilworth (1975), containing glucose (10 mM), NH_4Cl (10 mM) and phosphate at 0.3 mM; the pH was maintained at 7 with 20 mM HEPES buffer or at 6 with 20 mM 2-(N-morpholino)ethane-sulphonic acid (MES). Higher concentrations of HEPES buffer abolished motility. Continuous cultures were grown at 28° C in the liquid minimal salts medium (MSM) of Brown and Dilworth (1975), containing arabinose (1 mM), NH_4Cl (10 mM) and phosphate at 0.3 mM; the pH was maintained at 6 with 20 mM 2-(N-morpholino)ethane-sulphonic acid (MES). Batch cultures for use as inocula for continuous cultures were grown in the same medium.

In transduction experiments, kanamycin-resistant transductants were selected on TY medium (Beringer 1974) containing kanamycin at $50\ \mu g \cdot ml^{-1}$.

Mutagenesis

Mutants defective in motility were obtained by Tn*5* mutagenesis using the suicide plasmid vector pJB4JI as described by Beringer et al. (1978). Kanamycin-resistant (i.e. $Tn5^+$) prototrophic *R. trifolii* were selected on Y medium (Beringer 1974) containing glycerol (10 mM), succinate (10 mM) and kanamycin ($100\ \mu g \cdot ml^{-1}$). Single colonies were stabbed into yeast extract swarm plates (pH 6.0) containing $50\ \mu g \cdot ml^{-1}$ kanamycin and $100\ \mu g \cdot ml^{-1}$ streptomycin, as described by Ames et al. (1980) to check for movement from the centrally inoculated point. $Tn5^+$ clones were screened at pH 6 because optimum motility was seen on swarm plates at this pH.

Transduction

Bacteriophage RL38 was grown on donor strains of *R. trifolii* as described by Buchanan-Wollaston (1979) for *R. leguminosarum*. Transducing lysates were added to recipient bacteria at a multiplicity of infection of approximately 0.1, and the mixtures incubated at 28° C for 3 h. Bacteria were then washed twice in saline (0.9%, w/v), and plated on solid TY-kanamycin medium.

Light microscopy

Mounts were examined for motility at a magnification of $\times 400$ in an Olympus phase contrast microscope.

Nodulation tests

R. trifolii and its mutants were tested in a modified version of the Fahreus agar flask nodulation system described by Beringer (1974) where boiling tubes (20 cm $\times$ 2.5 cm diam.) replaced conical flasks. The tubes were kept in a growth cabinet with light and dark cycles of 12 h at $21° \pm 3°$ C for up to 4 weeks. Acetylene reduction assays were performed on intact plants at 25° C following the method of Trinick et al. (1976).

Seedlings

Seed of *T. subterraneum* cv. Mount Barker was obtained from the Western Australian Department of Agriculture. Seed was surface sterilized for 30 s in 70% ethanol followed by 3 min in 0.2% (w/v) $HgCl_2$ and six successive washes in sterile deionised water. The surface sterilized seed was then allowed to imbibe water for 24 h prior to pregermination on water agar for a further 24 h before sowing.

Soil

The soil was yellow sand from Jandakot, Western Australia, and was treated by two passages through a steam sterilizer at 90° C for 90 min.

Inoculation

Bacterial cultures were grown to an OD_{600} of 0.1 – 0.2 in glucose-NH_4^+ minimal salts, and the resultant suspensions were then diluted in sterile saline to between 1×10^3 and 1×10^5 cells $\cdot\ ml^{-1}$. Viable counts of inoculant cells were determined on plates of YMA and/or YMA containing appropriate antibiotics using the Miles and Misra drop-plate method (Vincent 1970).

Growth rates

Mean generation times were determined by growth in glucose minimal salts liquid medium, samples being removed at hourly intervals for absorbance measurements at 600 nm in a Varian 635 spectrophotometer.

Growth in mixed continuous culture

Strains were grown initially in 50-ml batch cultures (in 250 ml conical flasks) with shaking at 28° C for 36 h before being used to inoculate the continuous culture vessel. A 5 ml aliquot of motile strain MNF1003 (Rif^R Str^R) was added to 45 ml of fresh medium contained in a 50 ml round-bottomed, Quick-fit culture vessel. The vessel was continuously sparged with sterile air and the temperature maintained at 28° C. When the initial inoculum had grown sufficiently, a dilution rate of 0.1 h^{-1} was established. After steady state had been reached (approximately 2–3 days), 5 ml of a culture of the non-motile strain (either MNF1005 [Kan^R Str^R] or MNF1012 [Kan^R Str^R]) having approximately the same OD_{600} as the motile strain, was introduced into the culture vessel. Samples of ca. 5 ml of the mixed culture were withdrawn daily, using sterile syringes and needles, and dilutions plated onto YMA plates containing the appropriate antibiotics for identification of each strain. Growth of the mixed rhizobial culture was followed in this way over a period of at least 5 days after the introduction of the non-motile strain into the culture vessel.

Growth in the rhizosphere

Ninety pots each containing 3 kg steam treated yellow sand were prepared. Ten pregerminated clover seedlings with radicles of length 3 mm were inoculated with 0.5 ml of either non-motile (MNF1005), motile (MNF1003), or a mixed suspension of both motile and non-motile rhizobia containing 2×10^4 cells · ml^{-1}. Each treatment comprised 30 pots. The seedlings were covered lightly with sand, a layer (1 cm) of sterile vermiculite added, and a plastic bag secured over the top of the pot to prevent loss of water. The pots were kept in root cooling tanks at 21° ± 1° C for up to 11 days. Ten pots of each treatment were harvested after 4, 7 and 11 days.

To assay the rhizosphere population, 10 plants from each pot were removed with minimal damage to the root system and shaken lightly to remove excess soil. The roots were shaken at maximum speed for 30 min in 50 ml sterile saline [0.85% (w/v) NaCl] in a 100 ml flat bottle on a Griffin wrist arm action flask shaker. Motile (MNF1003) and non-motile (MNF1005) organisms were counted using the Miles ad Misra drop-plate method (Vincent 1970) on YMA plates containing appropriate antibiotics. The tap roots were weighed and their length measured.

Competition between motile (MNF1002) and non-motile (MNF1005 or MNF1012) rhizobia for nodulation of clover

Twenty-nine pots, each containing 3 kg steam treated yellow sand, were prepared. Five pregerminated clover seedlings per pot were each inoculated with ca. 1.5×10^3 cells of either non-motile (MNF1012), motile (MNF1002), a mixed suspension of both motile and non-motile (MNF1002 + MNF1012, or MNF1002 + MNF1005) or no rhizobia. The motile strain MNF1002 carries Tn*5*; insertion of this transposon has no effect upon motility or growth rate of this strain, and it is indistinguishable in these properties from the $Tn5^-$ strain MNF1003. The purpose of using MNF1002 in competition with MNF1005 and MNF1012 is to control for any effects that Tn*5* itself might have upon nodulation competitiveness. The mixed inoculum treatments each comprised six pots, the single inoculum treatments five pots each and the control treatment two pots. The seedlings were covered lightly with sand and then with a layer (1 cm) of sterile vermiculite. Watering was by means of a centrally positioned polyvinylchloride tube (diam. 2 cm, length 20 cm) which reached the base of the pot. Pots were watered daily to field capacity, suing the nutrient solution of Broughton and Dilworth (1971).

All pots were kept in a root cooling tank at 21° ± 1° C for 7–8 weeks before harvesting. After this time the plants were carefully removed and approximately 100 nodules of various sizes were picked from the separately inoculated treatments and approximately 400 nodules of various sizes were picked from the mixed-inoculum treatments. The strain of *R. trifolii* in the nodule was identified as described below.

Nodule typing

Nodules were surface sterilized by treatment for 1 min with 95% ethanol followed by immersion in aqueous $HgCl_2$ [0.1% (w/v)] for 3–4 min. They were then washed thoroughly in at least six changes of sterile tap water (Vincent 1970).

To check for double occupancy, each of a sample of 10 nodules from the plants receiving the mixed inoculum was crushed aseptically using a sterile orange stick, and streaked to single colonies on a YMA plate. Ten colonies from each nodule isolation were patched onto YMA master plates and replica plated after 4–5 days incubation on agar containing streptomycin plus kanamycin, or streptomycin plus kanamycin plus rifampicin. Each of the nodules tested yielded only one kind of single colony (i.e. all Rif^R or all Rif^s); thus, none appeared to contain a mixed population. The remaining nodules were crushed as above, but patched directly onto master plates and strains identified from their antibiotic resistances as above. It is highly unlikely that spontaneous mutation of non-motile mutants to rifampicin resistance leads to erroneous identification of nodule occupants, as the frequency of such mutation in free-living cultures of *R. trifolii* TA1 is ca. 10^{-8}.

Results and discussion

Properties of motility mutants

The parental strain was *Rhizobium trifolii* MNF1000, a Str^R derivative of strain TA1 (Table 1). Seven mutants, which were either completely non-motile or otherwise defective in motility were identified in the screening of approximately 1,500 clones of *R. trifolii* MNF1000 which had been mutagenised with Tn*5*. Of these MNF1005 and MNF1012, with non-leaky phenotypes, were chosen for further analysis.

Control experiments showed that all strains retained their antibiotic resistance markers through plant passage and were as effective as TA1 on *Trifolium subterraneum* c.v. Mount Barker. All strains formed red nodules in the same time and in approximately equal numbers in the modified flask system described by Beringer (1974). Representative values for acetylene reduction at 50 ± 7 days by each strain were: MNF1002, 450 nmol min^{-1} g^{-1}, MNF1005, 403 ± 14 nmol min^{-1} g^{-1}; MNF1012, 322 ± 74 nmol min^{-1} g^{-1}.

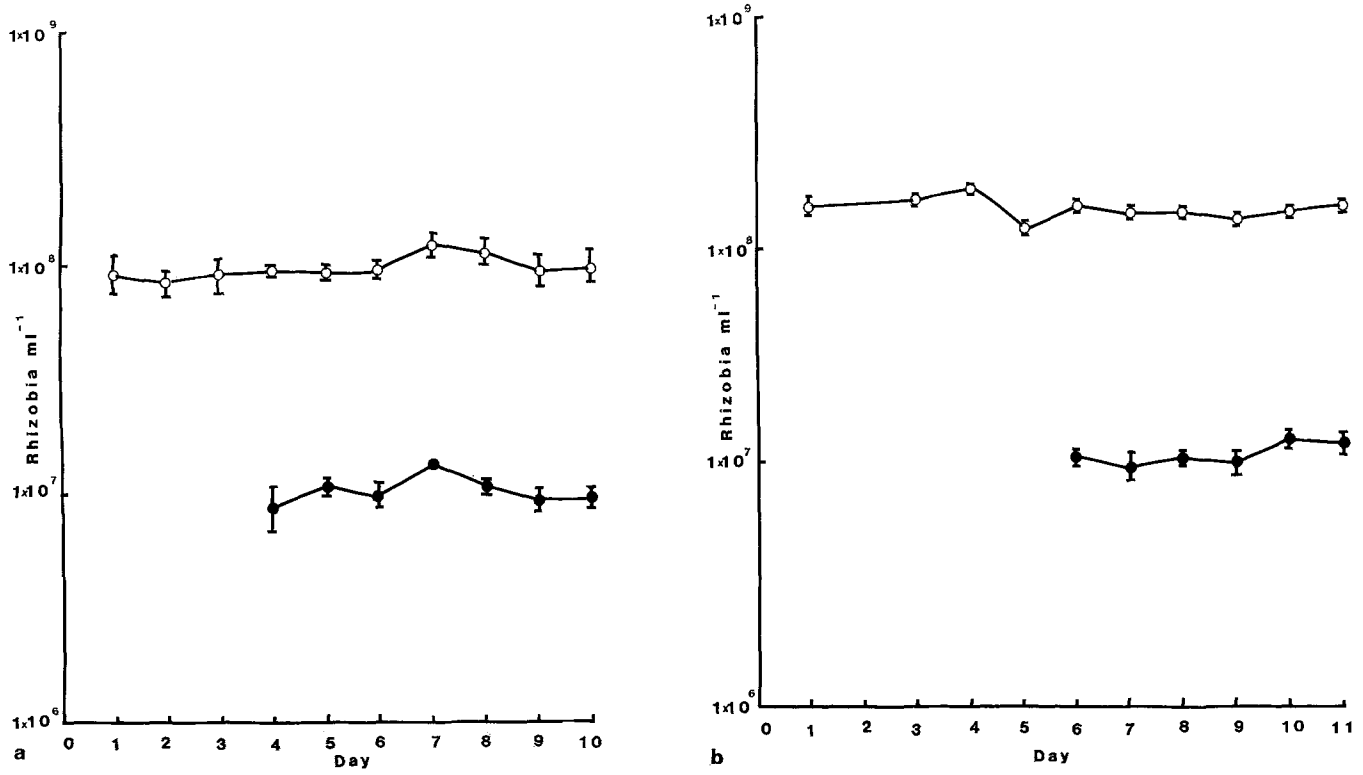

Fig. 1. **a** Population density of motile strain MNF1003 (○) and non-motile strain MNF1012 (●) in mixed continuous culture, over 10 days. *Vertical bars* show the standard deviation of the bacterial counts. **b** Population density of motile strain MNF1003 (○) and non-motile strain MNF1005 (●) in mixed continuous culture over 11 days. *Vertical bars* show the standard deviation of the bacterial counts

Microscopy

Phase contrast microscopy showed that under conditions where the wild type was highly motile, MNF1005 displayed only Brownian motion and cells of strain MNF1012 pivoted about their own poles.

Transductional analysis of mutant strains

Lysates of phage RL38 were grown on MNF1005 and MNF1012 and used to transduce kanamycin resistance into wild-type TA1. At least 10 kanamycin resistant transductants from each strain were tested for their motility. All of the transductants were non-motile, indicating that the lack of motility in MNF1005 and MNF1012 is due to insertion of Tn*5* and that there is only one copy of Tn*5* per genome.

Growth rates

The mutant strains MNF1000, MNF1002, MNF1003, MNF1005 and MNF1012 were found to grow as well as TA1 in glucose (10 mM) minimal salts medium, pH 6, with a mean generation time of 5.3 h $\pm$ 0.3 h. Thus the transposon insertions which abolish motility in MNF1005 and MNF1012 do not exert any pleiotropic effect on the growth of these strains.

Growth in mixed continuous culture

Once it had been established that all strains had the same growth rate in pure batch culture, it was necessary to determine if these rates remained equal in mixed cultures. This was done by monitoring the composition of mixed (motile and non-motile) populations in carbon-limited chemostats (Fig 1a, b). It is clear that the size of each subpopulation remained unchanged over at least 5 days. Thus, under these conditions, there appear to be no differential growth rates of motile and non-motile strains in mixed culture.

Rhizosphere populations

Although non-motile strains of rhizobia have previously been found to grow as well as motile strains in the laboratory (Ames and Bergman 1981), the behaviour of such strains in the rhizosphere appears not to have been examined. It is important to measure this parameter, before comparing nodulation properties of motile and non-motile strains, to ensure that any difference between them is due to their motility difference and not merely to a difference in growth rates in the rhizosphere.

The rhizosphere populations of motile and non-motile strains of *R. trifolii* were followed from 4 to 11 days after inoculation, both with single inocula and with a mixed inoculum. Since root length and root weight were directly proportional ($r = 0.86$, $P < 0.01$), populations of rhizobia were expressed as numbers · (mm root length)$^{-1}$.

Numbers of rhizobia (mm root length)$^{-1}$ for both strains inoculated separately had risen from approximately 45 rhizobia · recovered (mm root length)$^{-1}$, at day 4, to 560 rhizobia · (mm root length)$^{-1}$ at day 11 (Fig. 2a). When both MNF1003 and MNF1005 were inoculated together, cell numbers per mm root length rose from approximately

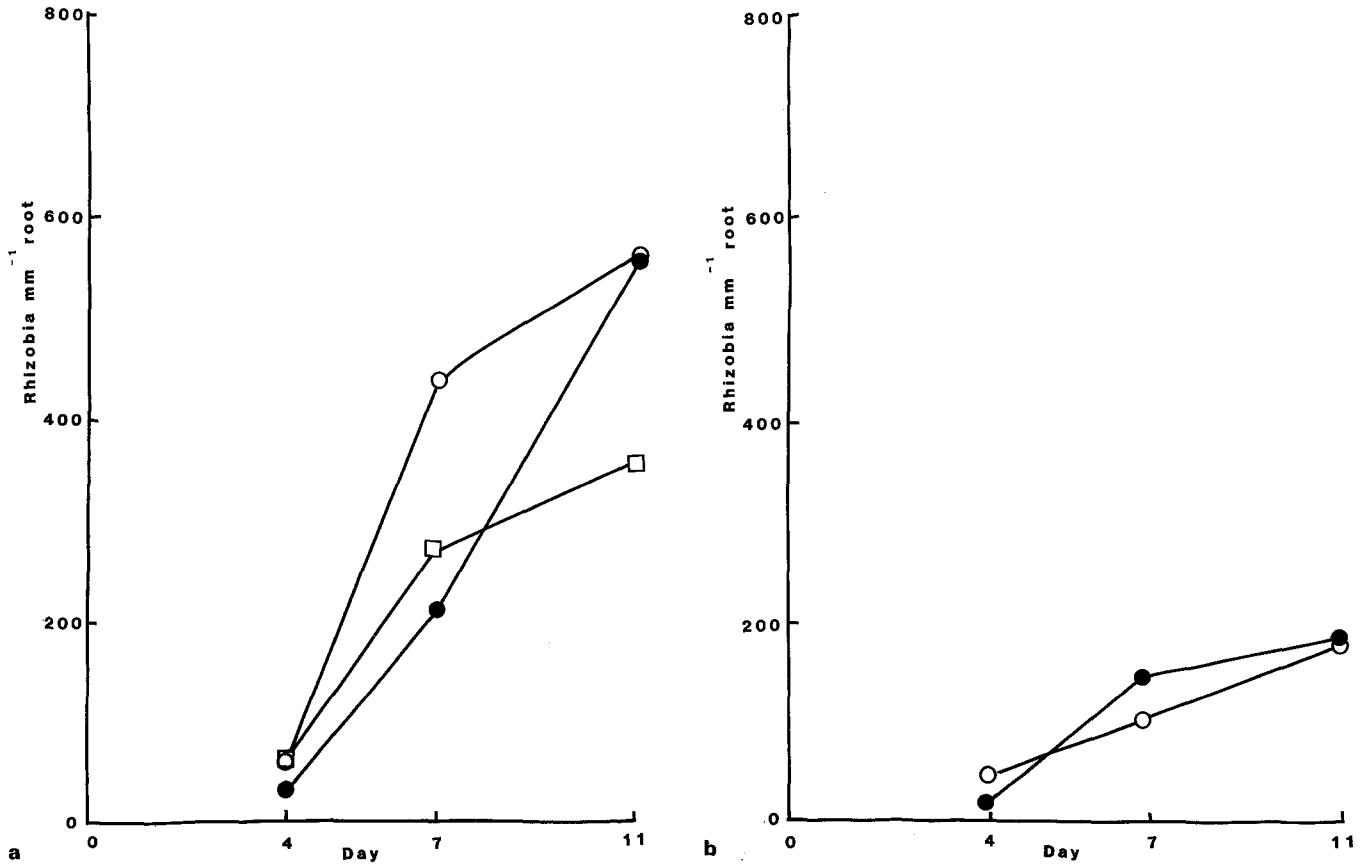

Fig. 2. **a** Rhizosphere population densities of motile and non-motile *Rhizobium trifolii* inoculated from separate culture. ○ MNF1003; ● MNF1005 (non-motile). Sum of Rhizosphere population densities of motile and non-motile *R. trifolii* inoculated from mixed culture (■). **b** Rhizosphere population densities of motile and non-motile *R. trifolii* inoculated from mixed culture. ○ MNF1003 (motile); ● MNF1005 (non-motile)

Table 2. Nodule numbers produced per plant (± SEM) after inoculation with separate (motile or non-motile) or mixed (motile + non-motile) strains

Strain	None	MNF1000 + MNF1012	MNF1002 + MNF1005	MNF1012	MNF1002	MNF1005
Number	10	25	25	18	22	19
Nodule no	0	106 ± 8	119 ± 8	147 ± 18	152 ± 17	153 ± 15

The one-way ANOVA statistical analysis shows no significant differences between pairs of means for nodule number whichever inoculum strain or mixture was applied

30 at 4 days to approximately 180 at 11 days for both strains (Fig. 2b).

A two-way analysis of variance showed that the population densities of motile and non-motile rhizobia in the rhizosphere of clover plants did not differ significantly, whether they had been inoculated from mixed or separate cultures (Fig. 2a, b). As expected, numbers of organisms, whether motile or not, and whether inoculated from mixed or separate cultures, were found to increase significantly with harvest time ($P < 0.01$), but there was no strain × time interaction.

If the total numbers of rhizobia from a mixed inoculum in the rhizosphere were compared with those from a single inoculum of either MNF1003 or MNF1005 by means of a two-way analysis of variance, the total mixed population was not significantly less than that for either the motile or non-motile organism ($P < 0.01$; Fig. 1a) alone.

Any lack of success of the non-motile strain when competing with the wild type motile strain cannot therefore be attributed to a failure to adequately reproduce in the rhizosphere.

Nodule formation by motile and non-motile strains

The importance of motility to nodulation was investigated by inoculating clover plants with motile (MNF1002), or non-motile (MNF1005 or MNF1012) or a mixture of motile and non-motile rhizobia. Plants were harvested after 7–8 weeks and plant weight, nodule number and rhizobial phenotype determined (Table 2). The data were analysed by one-way ANOVA using the Student-Newman Keuls procedure with the SPSSX statistical package, omitting the uninoculated control values.

Table 3. Nodule occupancy (%) when mixed inocula of non-motile [MNF1005 (a) or MNF1012 (b)] and motile (MNF1002) rhizobia were applied to clover plants

a) Strain	MNF1005	MNF1002
% Nodule occupancy (362 nodules tested)	16.9	83.1
b) Strain	MNF1012	MNF1002
% Nodule occupancy (415 nodules tested)	16.1	83.9

Whether plants had been inoculated with mixed cultures of motile and non-motile rhizobia (MNF1002 + MNF1012 or MNF1002 + MNF1005) or a single culture of motile (MNF1002) or non-motile (MNF1005 or MNF1012) rhizobia, the numbers of nodules produced were similar. This indicates that the non-motile strains were as efficient as the motile wild type for nodule production. However, plant weights for clover inoculated with MNF1012 alone were significantly higher ($P < 0.05$) than for plants given any other single or mixed inoculum. We do not know why this increased plant weight effect occurred with plants which had been inoculated only with the non-motile strain MNF1012.

With mixed inocula, non-motile strains appeared to be at a disadvantage for nodule formation relative to the wild-type (Table 3). Although the inoculum had a ratio of motile to non-motile cells of between 0.9 and 1.1, a ratio of approximately 5:1 was consistently observed for nodules produced by them. This applied whether the non-motile mutants bore flagella or not.

This disproportionate nodulation by the non-motile strain is similar to that found by Ames and Bergman (1981), who showed that a motile strain of *R. meliloti* had a marked advantage over either flagellated or non-flagellated non-motile derivatives mixed at various unfavourable ratios. Other evidence consistent with this result is provided by Hunter and Fahring (1980) who showed that a "slow migrating" mutant of *Bradyrhizobium japonicum*, S-110, had a much lower nodulation potential than did motile wild type cells.

The experiments described here indicate that the non-motile organisms are as effective in nodule production and nitrogen fixation as are the wild types. Further, the motile and non-motile organisms appeared to be identical in growth rates in batch and continuous culture in the laboratory, and to produce rhizosphere populations of similar sizes on clover roots. The marked difference in nodulation which consistently appeared when motile and non-motile bacteria were presented in a mixed inoculum suggests a form of competition between the two strains on the root hair surface during the infection process. This in turn seems to depend only on the ability of the bacteria to swim normally, since the non-motile strain which possesses flagella behaved in the same way as the strain which does ot.

These strains offer further opportunities to explore how motility affects rhizobial success in other functions, such as movement or colonization away from an inoculation point on a root, and movement accompanying root growth. The experiments reported here were carried out in sand, which is an important soil type in Western Australia. The conclusions drawn from these experiments may not apply to other soil types, in which root environments may be quite different.

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