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Effectiveness, competitiveness and persistence of inoculant *Rhizobium* for perennial African clovers in a highland Vertisol

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SUMMARY

The effectiveness of 20 strains of *Rhizobium leguminosarum* bv. *trifolii* was evaluated with *Trifolium semipilosum* and *T. burchellianum* grown in a Vertisol soil in the glasshouse at the International Livestock Research Institute (ILRI) in Addis Ababa. Several effective strains were identified for both species. In *T. semipilosum*, inoculation significantly increased nodule DM and root N yield over the uninoculated control; Ethiopian *Rhizobium* isolates outperformed isolates from other sources for shoot DM and N yield. In *T. burchellianum*, contrast analysis revealed that there was no significant response to inoculation, although one effective strain was identified. Inoculant strains failed to overcome the competitive dominance of indigenous strains as reflected in mean nodule occupancies by inoculant strains of 15 and 7% in *T. semipilosum* and *T. burchellianum*, respectively. The 20 strains showed variable persistence following a 5-week drought period; only two of eight (*T. semipilosum*) and six of eight (*T. burchellianum*) strains were recovered from nodules on seedlings planted in the soil following the drought period. Overcoming the constraints of low nodule occupancy and variable persistence will require further understanding of the competitive interaction and the factors affecting access to nodule infection sites if superior *Rhizobium*–clover combinations are to be identified and developed.

INTRODUCTION

Major constraints to livestock production in sub-Saharan Africa include inadequate quantities and poor quality of forage. Feed supply is primarily constrained by the seasonal rainfall pattern and low soil fertility, especially nitrogen (N) and phosphorus (P). A sustainable approach to meeting the N demands of the cropping system may be to incorporate nitrogen-fixing legumes into the production sequence; legumes can contribute to higher quality feed and improved soil fertility. For many ecosystems where legumes form part of the indigenous flora, persistent *Rhizobium* populations may be highly competitive with introduced inoculant bacteria. It is important, therefore, to identify effective N-fixing combinations of legume genotypes and competitive *Rhizobium* strains. In addition to effectiveness and competitive ability, another important factor in selecting rhizobia for perennial clovers is the ability of the inoculant strains to persist in soil in the absence of their hosts (Howieson 1995).

In the Ethiopian highlands, both annual and perennial *Trifolium* species are the most predominant pasture legumes. Because of their successful adaptation to the region, these species have been given high priority in programmes to improve livestock feed resources in the east African highlands. Agronomic research on annual clovers has been conducted in Ethiopia (Kahurananga & Tsehay 1984; Friedericks *et al*. 1991; Nnadi *et al*. 1993; Tedla *et al*. 1994), but little is known about their symbiotic nitrogen-fixation behaviour. Friedericks *et al*. (1990) described effective *Rhizobium* strains for annual clovers, but these studies were conducted on artificial media and strain competitiveness was not evaluated. In sub-tropical Australia, where the perennial *T. semipilosum* is recommended as a pasture legume for animal production, *Rhizobium* strain-screening studies identified a specific isolate (strain CB782) for inoculant production (Jones &

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Date 1975; Roughley & Date 1986). Such work has not been conducted for perennial clovers in the Ethiopian highlands, which has the most significant population of ruminant livestock in sub-Saharan Africa (Saka *et al*. 1994).

In this work, we describe the evaluation of 20 *Rhizobium* isolates derived from different sources, for effectiveness, competitiveness and persistence with the perennial clover species *T. semipilosum* and *T. burchellianum*.

MATERIALS AND METHODS

Rhizobium strains

The strains and sources of *Rhizobium leguminosarum* biovar *trifolii* that were used are listed in Table 1. All strains were originally isolated from either *T. semipilosum* or *T. burchellianum*. Spontaneous mutants resistant to 400 μg ml^{-1} streptomycin were isolated as described by Kuykendall (1987). All inoculants were prepared in sterile peat following the procedure described in CIAT (1988). The inoculants, which contained at least $5{\cdot}0 \times 10^8$ cells per gram of peat after incubation, were diluted to 1×10^5 cells ml^{-1} with sterile water immediately before inoculation.

Plant materials

Seeds of *Trifolium semipilosum* (accession ILCA7609) and *T. burchellianum* (accession ILCA9764) were obtained from the Gene Bank of the International Livestock Research Institute (ILRI), formerly called International Livestock Centre for Africa (ILCA). The seeds were surface-sterilized with 7% (v/v) calcium hypochlorite and pre-germinated on moist filter paper contained in Petri dishes in the dark (Lupwayi & Haque 1994).

Inoculation and culture of plants

The plants were grown in pots filled with 1 kg of soil from the ILRI Headquarters research farm in Addis Ababa. The soil was a P-deficient (1 μg P g^{-1} soil) pH 6 Vertisol, the predominant soil type in the Ethiopian highlands. Phosphorus was applied as triple superphosphate at 25 μg P g^{-1} soil in all pots. Ten seedlings, which were later thinned to six, were planted in each

Table 1. *Sources of the* Rhizobium *strains used in the experiments*

Strain	Host spp.*	Source†	Other designations	Geographical origin	Comments
CB782	Ts	CSIRO	Unknown	Unknown	Recommended strain in Australia
ILCA108	Ts	GLRS	Unknown	Zimbabwe	
ILCA372	Ts	NifTAL	TAL909, MAR705	Zimbabwe	
ILCA393	Ts	NifTAL	TAL978, CIAT22	Zimbabwe	
ILCA108str1	Ts	CSIRO/ILCA	None	Zimbabwe	Strep. resist. mutant of ILCA108
ILCA108str2	Ts	GLRS/ILCA	None	Zimbabwe	Another strep. resist. mutant of ILCA108
ILCA372str	Ts	NifTAL/ILCA	None	Zimbabwe	Strep. resist. mutant of ILCA372
ILCA393str	Ts	NifTAL/ILCA	None	Zimbabwe	Strep. resist. mutant of ILCA393
ILCA402	Ts	UBC	2167A	Ethiopia	
ILCA405	Ts	UBC	2260C	Ethiopia	
ILCA406	Ts	UBC	2264F	Ethiopia	
ILCA407	Ts	UBC	2264H	Ethiopia	
ILCA408	Ts	UBC	2215C	Ethiopia	
ILCA406str	Ts	UBC/ILCA	None	Ethiopia	Strep. resist.‡ mutant of ILCA406
ILCA120	Tb	ILCA	None	Ethiopia	
ILCA303	Tb	ILCA	None	Ethiopia	
ILCA313	Tb	ILCA	None	Ethiopia	
ILCA120str	Tb	ILCA	None	Ethiopia	Strep. resist. mutant of ILCA120
ILCA303str1	Tb	ILCA	None	Ethiopia	Strep. resist. mutant of ILCA303
ILCA303str2	Tb	ILCA	None	Ethiopia	Another strep. resist. mutant of ILCA303

* Ts, *T. semipilosum*, Tb, *T. burchellianum*.

‡ CSIRO, Commonwealth Scientific and Industrial Research Organization, Australia; GLRS, Grasslands Research Station, Zimbabwe; NifTAL, Nitrogen Fixation in Tropical Agricultural Legumes Project, Hawaii; ILCA, International Livestock Centre for Africa, Ethiopia. Now called International Livestock Research Institute (ILRI); UBC, University of British Columbia, Canada. UBC/ILCA or similar combinations mean that the parent strain was obtained from UBC, but the mutant strain was isolated at ILCA.

‡ Strep. resist., Streptomycin resistant.

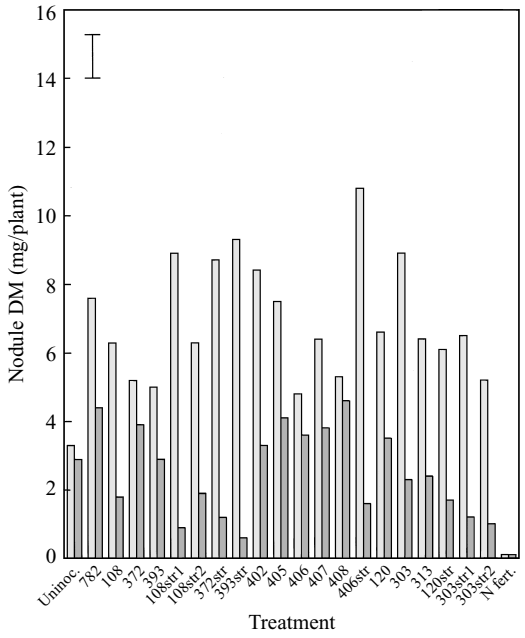

Fig. 1. Effect of inoculation on nodule dry matter (DM) of *T. semipilosum* (□) and *T. burchellianum* (▨). The strains designated 'str' were streptomycin-resistant mutants. The vertical bar represents S.E. (86 D.F.).

pot. Each seedling was inoculated at planting by dripping 1 ml of peat inoculant suspension containing 1×10^5 cells ml^{-1} onto the radicle. The plants were grown in a glasshouse with day and night temperatures of 21–25 and 13–17 °C, respectively.

Harvesting, assessment of nodulation and determination of dry matter and nitrogen

Plants were harvested 14 weeks after planting by cutting at soil level; shoots were dried at 70 °C for 48 h and weighed. Roots and nodules were recovered by washing the potted soil through a sieve. The nodules were then detached from the roots and counted. Roots and nodules were dried separately at 70 °C for 48 h and weighed. Shoot and root samples were ground and analysed for total N content after Kjeldahl digestion (Tadesse *et al.* 1991).

Symbiotic effectiveness

Twenty strains (Table 1) were evaluated to compare the response of (*a*) clover species inoculated with strains isolated from the same host compared with strains isolated from the other species, (*b*) clover species inoculated with imported *Rhizobium* strains compared with local isolates, and (*c*) clover species to inoculation with antibiotic resistant *Rhizobium* mutants. Using two controls (uninoculated and N-fertilized clover), the experiment was conducted as a factorial experiment comparing species (2) (*T. semipilosum* and *T. burchellianum*) and inoculation treatment (22) (including controls). The experiment was designed as a randomized complete block with three replicates. Nitrogen was applied (to the N-fertilized clover) as urea at an equivalent rate of 150 kg N ha^{-1}, split into 30 kg N ha^{-1} weekly applications during the first 5 weeks of the experiment. The plants were grown in the glasshouse as described, and were harvested 14 weeks after planting for determination of nodule number, nodule, shoot and root dry matter (DM), and shoot and root N yields.

Strain competitiveness

In the eight treatments involving streptomycin-resistant mutants, nodule occupancy was assessed in addition to nodulation, plant DM and N content determinations. For nodule occupancy analysis using the antibiotic resistance marker (Kuykendall 1987), at least 40 nodules were collected at random from each treatment. The nodules were surface-sterilized with 7% (v/v) calcium hypochlorite and squashed with a sterile glass rod. Nodule extracts were streaked onto yeast extract mannitol agar (YEMA) with and without 400 μg ml^{-1} streptomycin and cultured at 28 °C. The number of nodules containing the inoculant strain was expressed as a percentage of the total number of typed nodules.

Strain persistence

At planting, the eight treatments using streptomycin resistant isolates were duplicated in an additional three replicates. To evaluate strain persistence, watering was stopped at 14 weeks (coincident with the harvest of the other experiments). After 5 weeks, watering was resumed. None of the plants re-grew, and fresh seedlings of both clover species were re-planted without inoculation. This second crop was harvested 14 weeks after planting for assessment of nodulation, nodule occupancy and plant DM. A strain reinfection index was calculated as follows:

$$\text{SRI} = 100 \times (n_2 \times o_2)/(n_1 \times o_1)$$

where: SRI = Strain reinfection index, n_2 = Total number of nodules per plant in the second crop, o_2 = Proportion of nodules occupied by the original inoculant strain in the second crop, n_1 = Total number of nodules per plant in the first crop, and o_1 = Proportion of nodules occupied by the inoculant strain in the first crop. SRI values evaluate the relationship between first and second crops with respect to both total nodulation and nodule occupancy.

Statistical analysis

Data for strain effectiveness, competitiveness and persistence were analysed by standard analysis of variance (ANOVA) techniques. The 22 treatment means

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for each genotype in the strain effectiveness experiment were separated by orthogonal contrasts. The eight treatment means in the strain competitiveness and persistence evaluations were separated by range analyses. ANOVA were also performed by legume species. All statistical analyses were performed using MSTAT-C computer software (Michigan State University 1988).

RESULTS

Symbiotic effectiveness

The data revealed that *T. semipilosum* produced significantly higher DM and N yields than *T. burchellianum*. Within clover species, differences in nodule DM between different treatments were observed only for *T. burchellianum*, where strain ILCA408 produced a high nodule DM (Fig. 1). In both clover species, there were significant differences between treatments in root DM (Fig. 2), shoot N and root N yields (Fig. 3), but not in shoot DM. In *T. semipilosum*, strain ILCA372str produced high root DM and N yields and strain ILCA313 produced a high shoot N yield. The combination of *T. burchellianum* with strain ILCA393 resulted in high root DM, shoot N and root N yields.

Preliminary analysis of nodule DM indicated that the original host from which the isolates were derived

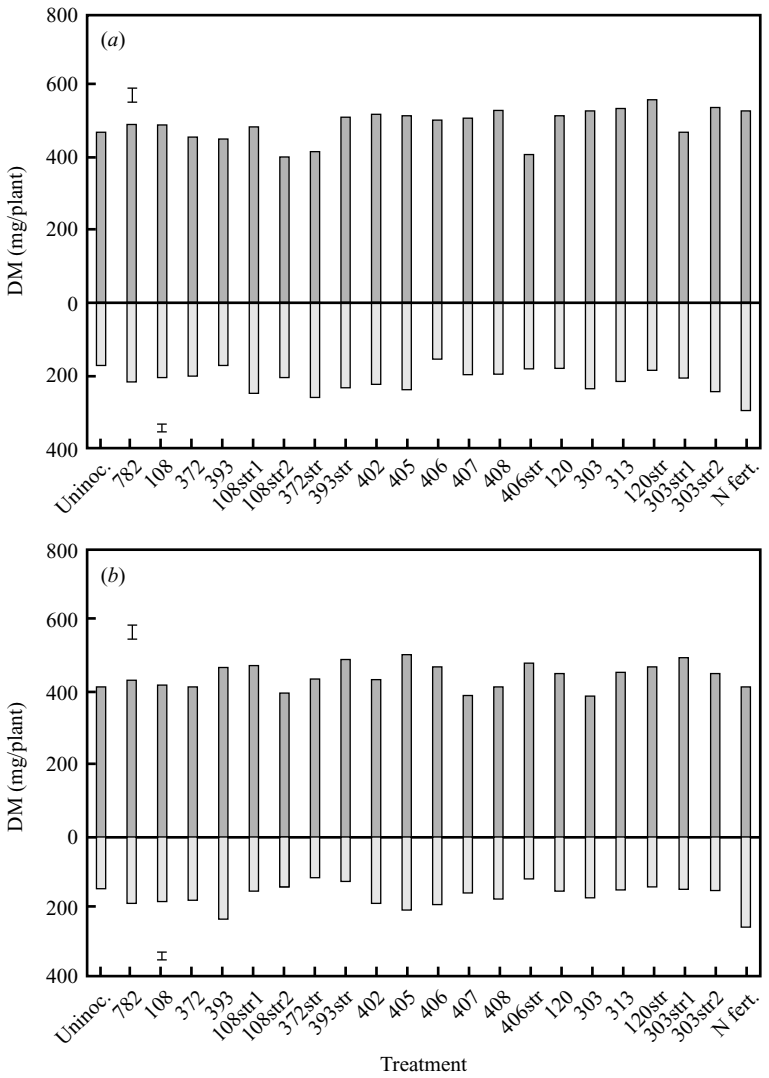

Fig. 2. Effect of inoculation on shoot (▨) and root (□) dry matter (DM) of (*a*) *T. semipilosum* and (*b*) *T. burchellianum*. The strains designated 'str' were streptomycin-resistant mutants. Vertical bars represent S.E. (42 D.F.).

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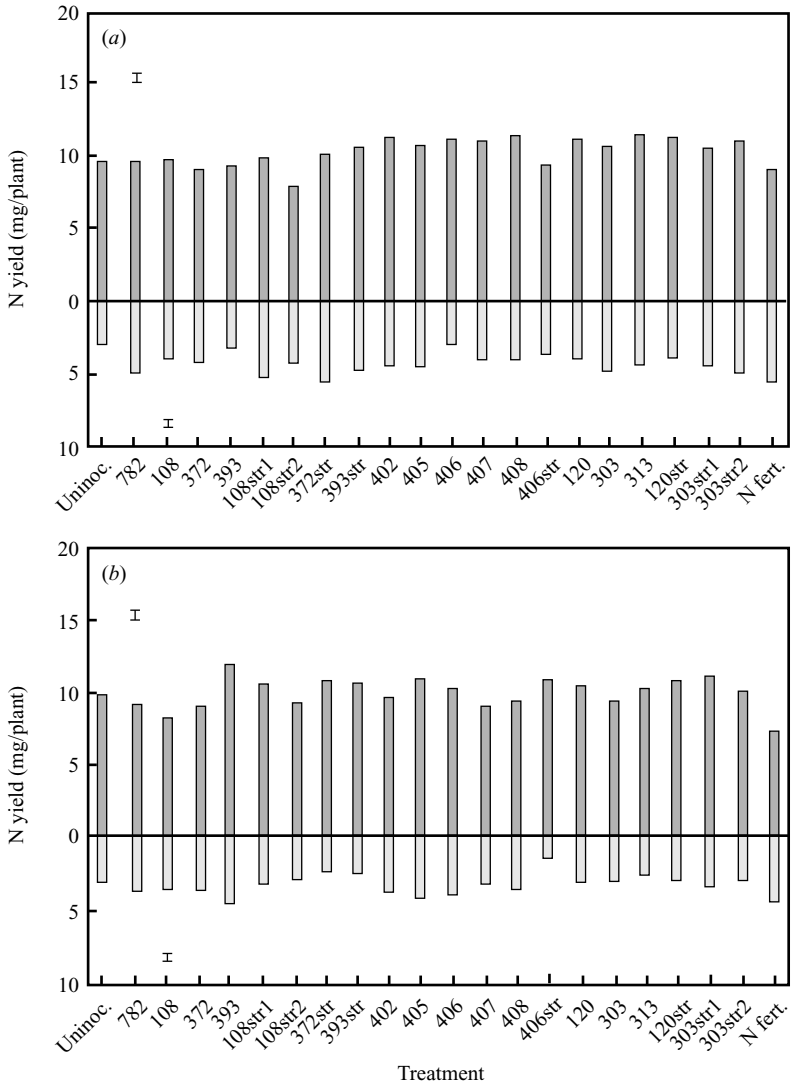

Fig. 3. Effect of inoculation on shoot (▨) and root (□) N yields of (*a*) *T. semipilosum* and (*b*) *T. burchellianum*. The strains designated 'str' were streptomycin-resistant mutants. Vertical bars represent S.E. (42 D.F.).

had no significant effect on nodulation of either clover species. Consequently, all 22 treatments were used in contrast analyses for each clover species. In contrast analysis, groups of treatments are compared, and the results do not necessarily reflect responses to individual treatments as described above. These analyses (results not presented) showed that in *T. semipilosum*, inoculation produced higher nodule DM and root N yields than uninoculated plants, but these increases were not reflected in significantly different shoot or root DM yields. Nodulation was significantly suppressed by supplemental nitrogen, but *T. semipilosum* responded to N fertility with significantly higher root DM and N yields, although no significant difference was observed for shoot DM. Local *Rhizobium* isolates were superior to imported strains in both shoot DM and N yield. Whereas parent strains were associated with higher shoot DM than mutant strains, the latter induced a higher response in plant root DM and N. In *T. burchellianum*, inoculation showed no significant advantage in any of the parameters measured compared to uninoculated plants. N-fertilized treatments were superior in root DM and N yield, but showed no difference in shoot DM yield and were inferior to inoculated treatments in shoot N yield. No difference was observed between the performance of local and

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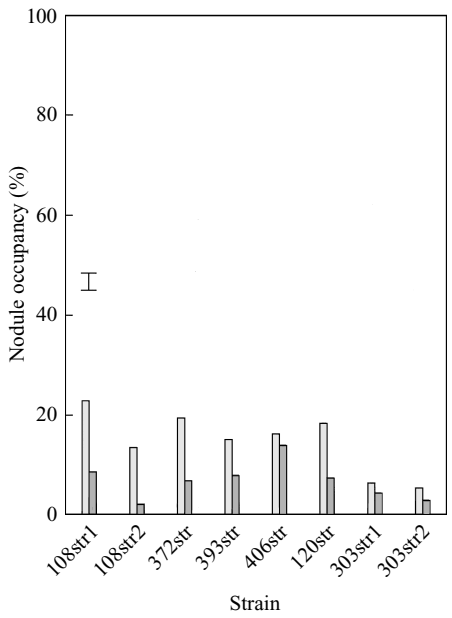

Fig. 4. Nodule occupancies (%) of inoculant strains on *T. semipilosum* (□) and *T. burchellianum* (▨). All the strains were streptomycin-resistant mutants. The vertical bar represents S.E. (30 D.F.).

imported strains, and parent strains were generally superior to their mutant counterparts.

Competitive ability

There were significant differences between strains and clover species in nodule occupancies by inoculant strains (Fig. 4). Only 15 and 7% of the nodules, on average, were occupied by inoculant strains in *T. semipilosum* and *T. burchellianum*, respectively. Part of the difference in nodule occupancy between the clover species may be attributable to the generally lower nodulation by streptomycin-resistant strains compared to parent strains in *T. burchellianum*. However, in *T. semipilosum*, where there were no significant differences between parent and mutant strains, even the maximum nodule occupancy of 23% by strain ILCA108str1 (Fig. 4) is low. The most competitive strain in *T. burchellianum* was ILCA406str with 14% nodule occupancy.

Persistence

The second crop produced less nodule and shoot DM than the first crop in both clover species (Table 2). Surprisingly, the second *T. burchellianum* crop had greater root DM than the first crop (Table 2). Nodule

Table 2. *Response of two consecutive crops of* T. semipilosum *and* T. burchellianum *to inoculant applied to the first crop*

Strain	Nodule DM (mg plant^{-1})		Shoot DM (mg plant^{-1})		Root DM (mg plant^{-1})	
	Crop 1	Crop 2	Crop 1	Crop 2	Crop 1	Crop 2
T. semipilosum						
ILCA108str1	8·9	0·8	483·3	333.3	246·4	167·6
ILCA108str2	6·3	0·4	400·0	366·7	203·6	163·7
ILCA372str	8·7	0·2	516·7	288·9	257·8	130·8
ILCA393str	9·3	0·4	511·1	338·9	230·6	160·6
ILCA406str	10·8	0·9	411·1	361·1	177·4	210·2
ILCA120str	6·1	1·0	566·7	422·2	178·8	177·6
ILCA303str1	6·5	2·3	477·8	422·2	199·8	194·1
ILCA303str2	5·2	0·5	544·4	372·2	235·3	170·0
S.E. (14 D.F.)	2·5	0·4	34·0	41·7	23·7	24·6
T. burchellianum						
ILCA108str1	0·9	0·2	477·8	288·9	156·0	216·4
ILCA108str2	1·9	0·9	400·0	416·7	141·2	202·3
ILCA372str	1·2	0·0	438·9	355·6	116·6	140·7
ILCA393str	0·6	0·1	494·4	338·9	129·7	154·5
ILCA406str	1·6	1·7	488·9	388·9	118·0	243·3
ILCA120str	1·7	0·3	477·8	300·0	138·4	191·5
ILCA303str1	1·2	1·3	505·6	388·9	144·8	208·0
ILCA303str2	1·0	0·0	461·1	366·7	146·7	183·1
S.E. (14 D.F.)	0·4	0·5	47·0	38·0	20·9	25·7

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Table 3. *Strain reinfection indices for eight antibiotic-resistant strains*

Strain	Strain reinfection index*	
	T. semipilosum	*T. burchellianum*
ILCA108str1	0	74
ILCA108str2	0	44
ILCA372str	0	1
ILCA393str	0	0
ILCA406str	16	25
ILCA120str	15	18
ILCA303str1	0	7
ILCA303str2	0	0
S.E. (14 D.F.)	3	29

* Where the index is zero, the strain was not detected in the second crop (i.e. $o_2 = 0$). When a strain was detected only in the second crop (i.e. if $o_1 = 0$ but $o_2 > 0$), it was given an index of 100.

occupancy by inoculant strains was generally less in the second crop of *T. semipilosum* than in the first, but most strains occupied a greater percentage of nodules in the second crop of *T. burchellianum* than in the first. Strain reinfection indices (Table 3) show that strains ILCA406str and ILCA120str reinfected *T. semipilosum* the most, but there were no significant differences in reinfection indices between strains in *T. burchellianum* despite a wide range in values. This was due to high variability of the data as the standard error shows (Table 3).

DISCUSSION

Inoculation with *Rhizobium* was not inherently superior to either the uninoculated or the N-fertilized treatments for either of the perennial clover species tested. However, for both clovers, inoculation gave shoot DM yields which were equivalent to the N-treatment and produced higher shoot N yields. 12–13 weeks after planting, N-fertilized plants appeared slightly chlorotic; this observation may reflect exhaustion of the N supply or the leaf discoloration which has been reported for *T. semipilosum* in Australia (Roughley & Date 1986). Since the uninoculated plants did not show a similar chlorosis, it appears unlikely that the observation was a consequence of simple nitrogen limitation; the impact of chlorosis may have been to reduce the overall performance of the N-fertilized plants.

For both clover species, root DM and N yields were more responsive to inoculation and N fertilizer than shoot DM and N yields. Root growth is traditionally ignored in such trials since it is not a component of economic yield and is assumed to be less responsive to nitrogen. Our results indicated the importance of assessing root parameters in nitrogen fixation and nutrient response analysis. Since these studies were conducted in non-sterile native soils, the presence of other microflora may also contribute to the observed root responses (Srinivasan *et al*. 1996).

The response of these clover species also indicated that DM yields were less responsive than N yields. It may be inferred that an absence of response to inoculation or N fertilization in DM yield does not necessarily reflect a lack of underlying improvement in the N nutrition of the plant.

Strains isolated from Ethiopian soils were, as a group, superior to those derived from other sources in increasing shoot DM and N yield of *T. semipilosum*. This observation is consistent with the comparison of 35 Ethiopian isolates compared with 18 strains from elsewhere in Africa for effectiveness on five annual *Trifolium* species (Friedericks *et al*. 1990). In contrast, there was no significant difference between indigenous and imported strains in the performance of *T. burchellianum*. As none of the imported strains had been isolated from a *burchellianum* host, their equivalent performance to the indigenous strains would have seemed contradictory in the absence of the nodule occupancy results. With maximum nodule occupancies $< 15\%$, it is clear that the test soils contained indigenous populations of *Rhizobium* which were highly competitive for nodule sites. Given the high level of specificity which we have observed for the infection and inoculation of these African perennial clovers (F. B. Holl, unpublished), the observed nodulation and occupancy data are consistent with the behaviour of these species in response to inoculation.

Although strain differences in plant response were observed, suggesting that some strains may produce a superior plant response, the critical observation in these studies is the low level of nodule occupancy which was observed. Estimates of indigenous populations of clover *Rhizobium* in highland Ethiopian soils range from 0 to > 25000 cells per gram (data not shown). Significant populations of indigenous *Rhizobium* are known to be successful competitors for nodule infection sites (Streeter 1994); even populations as low as 50 cells per gram of soil have been reported to reduce or eliminate the inoculation response (Thies *et al*. 1991). Plant host, *Rhizobium* strain and environmental factors are among the many factors which can influence the competitive interaction of *Rhizobium* in the soil (Dowling & Broughton 1986; Triplett 1990; Streeter 1994). Poor competitive ability as reflected in the low nodule occupancy data is an important logistical constraint to the development of appropriate commercial inoculants. Streeter (1994) has reviewed ways in which nodule occupancy by inoculant strains can be increased. The biotechnological means are reviewed in greater detail by Maier & Triplett (1996), but Giller & Cadisch (1995) argue

that, particularly for farmers in developing countries, immediate enhancement and exploitation of biological nitrogen fixation are possible by simply implementing existing technical knowledge.

Persistence of the inoculant *Rhizobium* strains was inferred through a reinfection index. Although it is self evident that reinfection cannot occur unless a strain persists, it is also possible that a strain may persist in the soil but fail to reinfect plants. However, the concept of a reinfection index integrates survival traits with those of recolonization and renewed nodulation. These are key characteristics of a good inoculant strain and therefore, in practical terms, this concept is more useful than persistence.

There was wide variation between strains in reinfection. Not surprisingly, Ethiopian strains, especially those from *T. semipilosum*, were more persistent than imported strains, some of which were completely lost in the short 5-week drying period. As extensive drying is a regular feature of Ethiopian Vertisols during the dry season, such sensitivity to drought could have significant practical consequences. Because reinfection was a function of survival, competitive ability and nodulation in our calculations, the low nodulation response in the second crop resulted in a small sample size for nodule typing. The methodology may thus have underestimated the persistence of the inoculant strains. Nevertheless, it was clear that population numbers and competitive ability of the inoculant strains were insufficient to displace the dominance of the indigenous bacteria for nodule formation.

While these data provide encouraging support for the development of locally adapted *Rhizobium* inoculants for perennial Ethiopian clovers, they emphasise the critical limitation to inoculant development in the failure of inoculant strains to compete with adapted populations of indigenous strains. Overcoming these constraints will require further understanding of the competitive interaction and the factors affecting access to nodule infection sites if superior *Rhizobium*–clover combinations are to be identified and developed.

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REFERENCES

CIAT (Centro Internacional de Agricultura Tropical) (1988). *Legume–Rhizobium Symbiosis*: *Methods Manual for Evaluation*, *Selection and Agronomic Management*. Cali: CIAT.

Dowling, D. N. & Broughton, W. J. (1986). Competition for nodulation of legumes. *Annual Review of Microbiology* **40**, 131–157.

Friedericks, J. B., Hagehorn, C. & Vanscoyok, S. W. (1990). Isolation of *Rhizobium leguminosarum* (biovar *trifolii*) strains from Ethiopian soils and symbiotic effectiveness on African annual clover species. *Applied and Environmental Microbiology* **56**, 1087–1082.

Friedericks, J. B., Hagehorn, C. & Reneau, R. B. (1991). Evaluation of African annual clovers to moisture stress in two Ethiopian highland soils. *Plant and Soil* **133**, 271–279.

Giller, K. E. & Cadisch, G. (1995). Future benefits from biological nitrogen fixation: an ecological approach to agriculture. *Plant and Soil* **174**, 255–277.

Howieson, J. G. (1995). Rhizobial persistence and its role in the development of sustainable agricultural systems in Mediterranean environments. *Soil Biology and Biochemistry* **27**, 595–610.

Jones, R. M. & Date, R. A. (1975). Studies on the nodulation of Kenya white clover (*Trifolium semipilosum*) under field conditions in south-east Queensland. *Australian Journal of Agriculture and Animal Husbandry* **15**, 519–526.

Kahurananga, J. & Tsehay, A. (1984). Preliminary assessment of some annual Ethiopian *Trifolium* species for hay production. *Tropical Grasslands* **18**, 215–217.

Kuykendall, L. D. (1987). Isolation and identification of genetically marked strains of nitrogen fixing microsymbionts of soybeans. In *Symbiotic Nitrogen Fixation Technology* (Ed. G. H. Elkan), pp. 205–220. New York: Marcel Dekker.

Lupwayi, N. Z. & Haque, I. (1994). *Legume–Rhizobium Technology Manual*. Environmental Sciences Working Document No. 29. Addis Ababa: International livestock Centre for Africa.

Maier, R. J. & Triplett, E. W. (1996). Toward more productive, efficient, and competitive nitrogen-fixing symbiotic bacteria. *Critical Reviews in Plant Sciences* **15**, 191–234.

Michigan State University (1988). *MSTAT-C*, *A Microcomputer Program for the Design*, *Management and Analysis of Agronomic Research Experiments*. East Lansing: Michigan State University.

Nnadi, L. A., Haque, I. & Mugwira, L. M. (1993). Phosphorus response and mineral composition of Ethiopian highland *Trifolium* (clover) species. *Communications in Soil Science and Plant Nutrition* **24**, 641–656.

Roughley, R. J. & Date, R. A. (1986). The effect of strain of *Rhizobium* and of temperature on nodulation and early growth of *Trifolium semipilosum*. *Experimental Agriculture* **22**, 123–131.

Saka, A. R., Haque, I., Saidi, A. N., Lupwayi, N. Z. & El-Wakeel, A. (1994). *Forage Legumes in Crop–Livestock Systems of Sub-Saharan Africa*. Environmental Sciences Division Working Document No. 24. Addis Ababa: International Livestock Centre for Africa (ILCA).

Srinivasan, M., Petersen, D. J. & Holl, F. B. (1996). Influence of indoleacetic acid-producing *Bacillus* isolates

on the nodulation of *Phaseolus vulgaris* by *Rhizobium etli* under gnotobiotic conditions. *Canadian Journal of Microbiology* **42**, 1006–1014.

Streeter, J. G. (1994). Failure of inoculant rhizobia to overcome the dominance of indigenous strains for nodule formation. *Canadian Journal of Microbiology* **40**, 513–522.

Tadesse, T., Haque, I. & Aduayi, E. A. (1991). *Soil*, *Plant*, *Water*, *Fertilizer*, *Animal Manure and Compost Analysis*. *Plant Sciences Division Working Document No*. *B13*. Addis Ababa: International Livestock Centre for Africa (ILCA).

Tedla, A., Sherrington, J. & Muhamad-Saleem, M. A. (1994). Integration of forage and food crops grown sequentially on Vertisols under rain-fed conditions in the mid-altitude Ethiopian highlands. *Experimental Agriculture* **30**, 291–298.

Thies, J. E., Singleton, P. W. & Bohlool, B. B. (1991). Influence of the size of indigenous rhizobial populations on establishment and symbiotic performance of introduced rhizobia on field-grown legumes. *Applied and Environmental Microbiology* **57**, 19–28.

Triplett, E. W. (1990). The molecular genetics of nodulation competitiveness in *Rhizobium* and *Bradyrhizobium*. *Molecular Plant–Microbe Interactions* **3**, 199–206.