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Comparative analysis of the genetic structure of a *Rhizobium meliloti* field population before and after environmental release of the highly competitive *R. meliloti* strain GR4

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Abstract

Rhizobium meliloti strain GR4, which exhibits a highly competitive ability for alfalfa root nodule occupancy, was used in a field release experiment in Granada, Spain. In order to analyze the ecological impact of the GR4 release, we characterized the *R. meliloti* indigenous population of the field site by ERIC-(enterobacterial repetitive intergenic consensus) PCR and IS (insertion sequence) fingerprinting. Both fingerprinting methods resulted in the same grouping of the isolates. Data obtained were compared with a previous analysis by plasmid based sequence-specific PCR. Isolates belonging to the major infective group, as defined by dominant plasmid types, were shown to have identical or nearly identical ERIC and IS fingerprint patterns. Hence, we conclude that all three typing methods are suited to characterize the genetic structure of the field population. The possible impact of the introduction of strain GR4 was examined two years after its release in its original environment. No effect on the genetic structure of the indigenous *R. meliloti* field population was observed.

Keywords: *Rhizobium meliloti*; Population genetics; Polymerase chain reaction; IS fingerprinting; ERIC fingerprinting; Deliberate release; Persistence

1. Introduction

For almost a century rhizobial inoculants have been used to increase the yield of leguminous crops, based on the ability of these bacterial species to infect the legumes' root system eliciting nitrogen-fixing nodules. However, the control of the infection process under field conditions is still an unsolved problem, which hinders the exploitation of superior nitrogen-fixing rhizobia as biological fertilizers [1–3].

Different studies have noted the difficulty of replacing indigenous strains with strains provided as inoculants. Distinct approaches have been used to control infection in the field, such as high inoculant

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doses, bactericidal agents in conjunction with resistant strains, inoculant formulation or placement, as well as selection of host and bacterial genotypes [1–4]. In relation to the latter, it is well known that strains selected as highly competitive and efficient under controlled conditions (laboratory or greenhouse) do not always behave in the same manner after their release into the field environment. Nevertheless, phenotypic and genotypic diversity among rhizobial strains is an important basis of strain selection for symbiotic and ecological characteristics [5,6].

Rhizobium meliloti strain GR4 exhibits a highly competitive phenotype in the laboratory which is linked to a plasmid (pRmeGR4b)-borne genetic region, the so-called *nfe* locus (nodulation formation efficiency) [7–9]. Strain GR4 was isolated in 1975 from the Estación Experimental del Zaidín (EEZ) field-site in Granada. Recent analysis by sequence-specific PCR based on primers derived from the *nfe* region and from the *repC* gene of plasmids pRmeGR4b and pRmeGR4a, respectively, has indicated that strain GR4 belongs to the major infective group of the indigenous *R. meliloti* population in the EEZ [10]. In this work we further characterize the *R. meliloti* population of the Zaidín field site by sequence-specific PCR with plasmid borne primers [10], by ERIC-PCR [11], and by IS fingerprint analysis [12–15], in order to understand the dominance of nodulation by particular genotypes of the indigenous population. In addition, we address the question of how the introduction of an indigenous strain (GR4) in its same field environment influences the infectiveness of the native population.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Recently, 100 *R. meliloti* field isolates obtained from *Medicago sativa* root nodules have been analyzed by plasmid-based sequence-specific PCR [10]. Twenty-two isolates, representing all plasmid groups, were chosen for further characterization (Table 1). Among these 22 isolates the ratio of a- and b-type plasmids and IS*Rm3* harbouring bacteria reflects that of the initially characterized 100 field isolates.

R. meliloti strains were cultured at 28°C in TY medium [16] or minimal medium [17]. A spontaneous GR4 [6] derived strain resistant to streptomycin (Sm, 250 μg ml^{-1}) and spectinomycin (Sp, 100 μg ml^{-1}), showing wild-type growth rate and symbiotic properties, was used in the field release experiment. As additional identification marker, we took advantage of the observation that strain GR4 and derivatives exhibit a non-mucoid phenotype on plates whereas the field isolates used form large mucoid colonies.

Table 1
Isolates from the Granada field site representing the plasmid-based sequence-specific-PCR groups [10]

Characteristics	Strains
pRmeGR4b type	A14, A111, A315 and A318
pRmeGR4b + a type	A13, A19, A213, A216 and A313
pRmeGR4a type	A12, A214 and A218
IS*Rm3* [a]	A18, A219, A312, A314, A316, A319
None [b]	A17, A212 and A215

[a] Isolates that do not carry any of the indicated plasmid types, but posses IS*Rm3*.
[b] Isolates that did not yield amplification products with any of the plasmid and IS-specific PCR primer used.

2.2. Isolation of R. meliloti field strains

R. meliloti strains were isolated from surface-sterilized root nodules of alfalfa [18] grown in the field or in pots prepared with soil from the field site located at the Estación Experimental del Zaidín in Granada (South East of Spain). Pots were placed in a plant growth chamber for 30 days.

2.3. Preparation of cell lysate DNA

Plasmid based sequence-specific PCR reactions were carried out as described previously [10]. For ERIC-PCR reactions bacterial cells grown on TY agar plates were suspended in 100 μl of water containing 50 mM NaOH and 0.25% SDS. The suspension was boiled for 5 min, centrifuged for 10 min at 15 000 $\times$ g at room temperature and the cell lysate was stored at 4°C for 24 h.

2.4. PCR conditions and oligonucleotide primers

PCR-mediated amplifications were carried out as described by Villadas et al. [10], and ERIC-PCR

reactions were performed according to de Bruijn [11]. Oligonucleotide primers for plasmid based sequence-specific PCR (pRmeGR4a-type *repC* primers C1 and C2, and pRmeGR4b-type *nfe* primers P6 and F2) were as described [10]. For ERIC-PCR the ERIC2 primer [11] was used as a single primer. It has been reported previously that primer ERIC2 alone yielded amplification patterns which were nearly identical to those obtained with the primer set ERIC1R and ERIC2 [19].

2.5. IS fingerprinting

Total DNA was isolated according to Meade et al. [20]. After *Eco*RI digestion aliquots of DNA were electrophoretically separated in an 0.8% Tris-acetate agarose gel and vacuum blotted onto nylon filters (Hybond-N, Amersham), according to the manufacturer's instructions. The DNA probes of IS elements IS*Rm3* [21], IS*Rm2011-1*, IS*Rm2011-2*, IS*Rm220-12-3*, IS*RmUSDA1024-1*, IS*Rm102F34-1*, IS*Rm-MVII-10* [15] were labelled with digoxigenin-11-dUTP (DIG, Boehringer). Hybridization was carried out at high stringency conditions (68°C, 5 × SSC) according to the supplier's instructions. Washing was carried out twice, 5 min each, in 0.1 × SSC, 0.1% SDS at room temperature and twice, 15 min each, in 0.1 × SSC, 0.1% SDS at 68°C. Detection of the hybridization signals was performed as specified by Boehringer.

2.6. Analysis of ERIC and IS fingerprint data

ERIC-PCR pattern and IS fingerprint patterns of strains were captured using a CCD camera (Cybertech, Berlin). ERIC-PCR patterns were imported into a database and comparison of these patterns were carried out with the WinCam 2.0 software (Cybertech) using the Pearson correlation for all bands from 200 to 1200 bp. For derivation of the dendrogram the UPGMA (unweighted pair group with mathematical averaging) method was used.

2.7. Cell culture and seed coating

The GR4 Sm^r, Sp^r derivative strain was grown in 100 ml of TY medium supplemented with 250 μg of streptomycin per ml and 100 μg of spectinomycin per ml. The culture was incubated in shake flasks at 28°C to early stationary phase and then washed twice with sterile phosphate buffered saline. After the second wash, pelleted cells were suspended in 1 ml of sterile water, providing a final density of 2.5×10^{12} cells ml^{-1}. The cell suspension was inoculated directly into 1.4 g of non-sterile peat carrier and immediately 2 ml of a sucrose solution 30% (w/v) was added. The inoculated peat was used to coat the seeds (140 g of alfalfa cv. Aragón) to an estimated rate of 10^6 cells per seed. This process was carried out in the same afternoon of planting, in the first week of March 1993.

2.8. Site preparation and planting protocol

The EEZ field site used in the field release experiment was ploughed until two weeks before planting. This is the same field from where strain GR4 was isolated in 1975 and it has been without alfalfa for the 8 years between its isolation and its re-release. A plot of 2 × 12 m was seeded with the inoculated coated seeds by hand. The control uninoculated plot was also prepared 1 m apart from the inoculated field.

2.9. Evaluation of the R. meliloti field population

The infective *R. meliloti* population in the soil was determined by most probable number of rhizobia (MPN) analysis, 25 tubes with alfalfa plants grown on nitrogen-free medium [18] were inoculated with five-fold serial dilutions of soil suspensions as described [22]. In the release site, nodules elicited in each of the 25 tubes, after surface sterilization were crushed in sterile water and tested for the presence of the introduced GR4 derivative strain by plating on TY agar supplemented with streptomycin (250 μg ml^{-1}) and spectinomycin (100 μg ml^{-1}). The isolates showing the antibiotic resistances and the non-mucoid phenotype of the introduced GR4 (Sm, Sp) strain were further confirmed by PCR using GR4 specific primers [10]. Moreover, the presence of the released strain was also measured by plating serial dilution of the corresponding soil suspension on selective (Sm, Sp) TY agar including 150 μg ml^{-1} of cycloheximide. The putative GR4 (Sm, Sp) colonies were further confirmed by sequence-specific

PCR [10]. Both estimation methods for the released strain, MPN and direct plating on agar, gave similar results.

2.10. Nodule occupancy

Thirty days and three months after planting, the roots of 10 plants each were randomly collected from the control and inoculated plots. In each case 50 nodules were surface sterilized, crushed in sterile water and the containing bacteria were plated on TY agar with and without streptomycin plus spectinomycin. The released strain GR4 could also be distinguished from the indigenous *R. meliloti* population by its non-mucoid morphology and it was further confirmed by PCR using GR4 specific primers [10]. Seven months after the release, the alfalfa root system was longer than 30 cm and very much spread into the soil. At this time it was very difficult to identify new nodules. To solve this problem nodule occupancy analyses performed 7, 14, 17 and 24 months after planting were carried out in the greenhouse. We have observed that there is no effect over the population isolated directly from the field one month after planting alfalfa (when it is possible to pick up easily the nodules) or in the greenhouse with the corresponding soil samples over the same period (one month). Soil samples of 10 cm depth were taken by using a cylinder of 10 cm diameter and used to prepare the pots. At least 5 different soil samples each were taken from the control and inoculated fields. 4 pots were prepared per soil sample. Nodules were collected (one month after planting alfalfa) from the different pots and 60 to 100 nodules randomly chosen were further analyzed in each case for the presence of the released strain GR4 as described above.

3. Results and discussion

3.1. Characterization of selected isolates of a natural R. meliloti population using ERIC and IS fingerprint techniques

Recently, an *R. meliloti* field population consisting of 100 isolates sampled at the Estación Experimental del Zaidín (EEZ) field site had been characterized by sequence-specific PCR with primers derived from the IS elements IS*Rm3* and IS*Rm4*, the plasmid origin of replication (pRmeGR4a *repC* locus) and plasmid pRmeGR4b specific DNA sequences [10]. In this work we analyzed the potential impact of the environmental release of *R. meliloti* strain GR4 on this indigenous population. Therefore, we asked whether grouping of isolates based on their indigenous plasmid content, correlated with their chromosomal background, i.e. whether the plasmid based isolate typing method correctly described the genetic structure of the field population. For this purpose 22 isolates representative for all plasmid groups were chosen for further characterization. In order to analyze the association of certain plasmid types with the genotype of these isolates, the ERIC (enterobacterial repetitive intergenic consensus) and IS typing methods were applied.

The ERIC2 primer [11,19] was used for the PCR amplification and generation of strain specific fingerprint patterns (Fig. 1). Based on these PCR fingerprints a similarity dendrogram of all isolates was derived using the WinCam software. When using a similarity threshold of 80% the isolates were classified into nine groups. The threshold of 80% was chosen since analysis of independent ERIC-PCR reactions of the same strain run in different gels yielded similar values in the range from 80–100% (unpublished data). Fig. 1 shows the ERIC-PCR derived patterns of the 22 isolates together with the dendrogram derived and the resulting groups. The most prominent group contained ten isolates (45% of the subpopulation tested; group I) including strain GR4. Three further groups contained two or three isolates, and five isolates exhibited individual patterns.

As a second approach to characterize the isolates we investigated the presence and copy number of IS elements. A set of seven IS elements isolated from different *R. meliloti* strains was used for hybridization with total DNA. The presence of the IS elements in the isolates and their copy number are shown in Fig. 2. Isolates displaying identical or nearly identical IS fingerprint pattern for all elements tested were grouped in the same IS fingerprint group. The fingerprints of the 10 isolates of group A, although differing slightly in the copy number of one or the other IS element, exhibited a very similar basic IS pattern and represented the main IS fingerprint group. Corre-

spondingly, the isolates of the two B and J subgroups each showed nearly identical patterns.

This classification was most obvious for the IS*Rm2011-2* fingerprints since IS*Rm2011-2* was the only element present in virtually every isolate tested, and had by far the highest copy number (9 to 19). The only exception was A17 (one copy). It was reported previously that IS*Rm2011-2* generally shows an average high copy number and a wide distribution in *R. meliloti* strains [12,14]. The other six elements are present in some of the isolates with a copy number of 1 to 9. Two isolates, A215 and

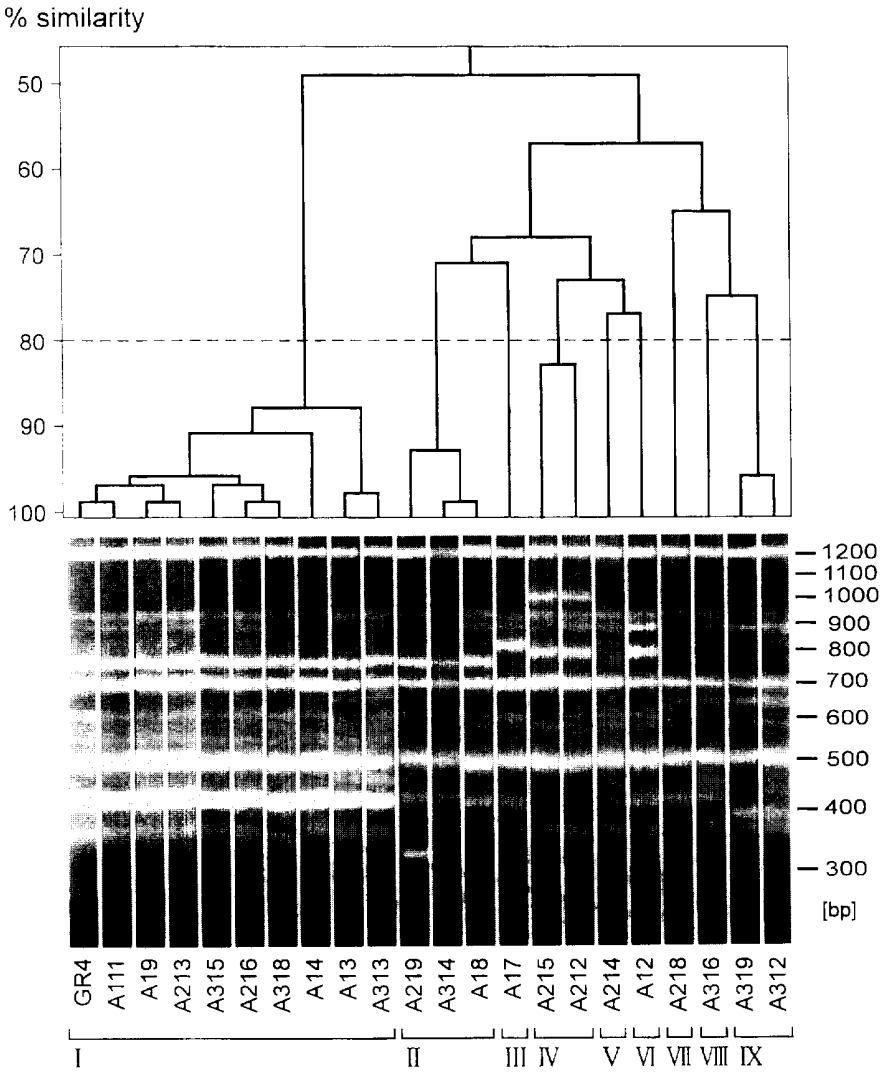

Fig. 1. ERIC-PCR grouping of the 22 isolates from the EEZ field site. The PCR patterns of the isolates obtained with the ERIC2 primer, their dendrogram and their similarity groups are shown. The PCR pattern from 200 to 1200 bp were imported into a database identifying a difference of 1.2% as equal size. Comparison of PCR fingerprint patterns were done using the Pearson correlation. The UPGMA method was used for calculating the corresponding dendrogram. With a similarity level of 80% the 22 isolates could be classified into 9 groups.

A212, only carry IS*Rm2011-2*. It should be noted that the A13 group and isolate A111 harbour all seven IS elements tested.

3.2. Comparison of ERIC and IS fingerprint groups to the distribution of a- and b-type plasmids

Fig. 3 summarizes the classification of isolates based on their ERIC-PCR and IS fingerprint patterns. When comparing these groupings it was obvious that each ERIC group harboured isolates with a very similar or even identical IS fingerprint pattern. Since these IS fingerprints confirmed the ERIC based grouping, we conclude that both methods reflected the genetic relatedness among the isolates. We further suggest that each ERIC/IS group represents an individual strain or at least a group of derivatives of one strain. Isolates within one IS group displayed the same basic fingerprint pattern. Minor variations in copy number might be due to transposition events in the case of additional copies. Furthermore, slightly different patterns might also be a result of recombinations (inversions or deletions) between two copies of the same IS element. We conclude that the ERIC pattern reflects the genetic structure of the rhizobial population, whereas the most detailed differentiation among isolates is possible by comparing IS fingerprints.

Although the different ERIC groups were very homogenous with regard to their IS distribution patterns, some subgroups lacked one or the other element, e.g. IS*RmMVII-10* in GR4 and A213 (Fig. 2). Assuming that each ERIC and IS fingerprint group represented related individuals, either GR4 and A213 lost this element or the remaining isolates of IS group A might have acquired this elements by horizontal gene transfer. The differences in copy number of IS elements are most likely to be due to transpositional events.

We further compared the distribution of pRmeGR4a- and b-type plasmids within this population [10] to the ERIC based grouping of isolates. A

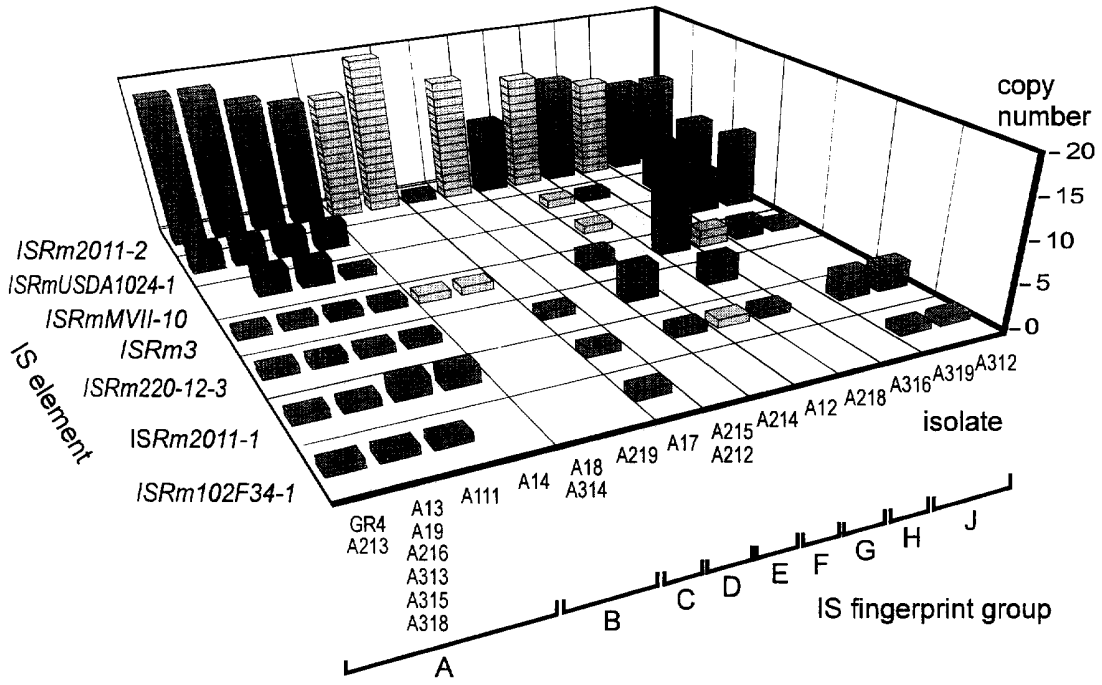

Fig. 2. Distribution and copy number of seven IS elements in the 22 isolates. For each isolate the presence or absence of the seven IS elements and their copy number is shown. Isolates showing an identical fingerprint pattern for all IS elements used are arranged underneath one another. Classes of isolates with similar fingerprint patterns are as indicated.

isolate	ERIC-PCR groups	IS fingerprint groups	plasmid types (a/b)
GR4	I	A_1	a + b
A213			
A13		A_2	
A19			
A216			
A313			
A315			b
A318			
A111			
A14		A_3	
A18	II	B	-
A314			
A219			
A17	III	C	-
A215	IV	D	-
A212			
A214	V	E	a
A12	VI	F	a
A218	VII	G	a
A316	VIII	H	-
A319	IX	J	-
A312			

Fig. 3. Comparison of isolate groupings according to their ERIC pattern (Fig. 1), IS fingerprint (Fig. 2), and presence of a- and b-type plasmids according to [10]. Dashed lines indicate a subgrouping based on slight differences in IS or plasmid patterns.

pRmeGR4b-type plasmid was present in all isolates of ERIC group I, but absent in the members of the remaining ERIC groups. This result may reflect a preferential association of pRmeGR4b with a certain genetic background. Analogously, a correlation between indigenous sym plasmids and chromosomal genotypes in field populations of *Rhizobium leguminosarum* had been reported previously [23,24]. In contrast to pRmeGR4b, a pRmeGR4a-type plasmid was present in only some isolates of ERIC group I which therefore harbour both plasmid types, and in three further isolates (A12, A214, and A218) representing individual ERIC groups (Fig. 3). The presence of pRmeGR4a-type plasmid in ERIC groups other than group I may reflect horizontal gene transfer events. It should be noted, that plasmid pRmeGR4a of strain GR4 is self-transmissible while plasmid pRmeGR4b is only mobilizable by pRmeGR4a [25].

From our comparison of typing methods we conclude that all three methods were suited to describe the genetic structure of the field population.

3.3. Initial field site characteristics and release of strain GR4 (Sm, Sp)

The Estación Experimental del Zaidín field site from where the population investigated was isolated had experienced no alfalfa cultivation for the last 8 years. Thus, the field population isolated [10] and further characterized in this work reflects an *R. meliloti* indigenous population that had remained in the soil for a long period of time in the absence of the host plant. Most-probable-number analysis (MPN) of the soil determined before planting alfalfa (March 1993) showed that the number of indigenous *R. meliloti* cells (per gram of dried soil) were in the range of $2.7–18.6 \times 10^4$ (confidence limits 95%). The same estimation was obtained in the control field one month later, after planting alfalfa, whereas in the inoculated field it was in the range of $5.4–42.6 \times 10^4$. At this time, the release strain, GR4 Sm, Sp was estimated in the range of $2.7–18.6 \times 10^4$. Thus, we assumed that the release strain at this time was roughly at the same level in the soil than the indigenous population.

3.4. Persistence of the release strain GR4 in the field

The persistence in the soil of the release strain was followed for a period of two years. The estimations of the number of cells of the release strain, as well as the indigenous *R. meliloti* population, after the release are graphically represented in Fig. 4. The inoculant strain exhibited stronger variations than the indigenous population, with a 10^3 fold reduction two years after its release. Similar reduction was observed in autumn of the first year (7 months after the release), but the population of the inoculant strain showed a 10^2 fold increase the next spring (14 months after the release). It is not possible to conclude the reasons for the observed changes, but they could be related with environmental factors such as temperature, water supply, etc. that affected the introduced strain more drastically than the indigenous population.

3.5. Nodule occupancy by the release strain

As observed with the persistence in the soil, the percentage of nodules occupied by the released strain GR4 also suffered strong variations. The percentage of nodule occupancy by the inoculant strain during the two years is also shown in Fig. 4. Although the release strain suffered more than 10^2 fold decrease from August, 1994 (17 months after the release) to March, 1995 (24 months after the release), no significant differences in the number of nodules occupied by the inoculant strain were observed, which might be explained by the competitive ability of the strain.

3.6. Impact over the indigenous R. meliloti population

A total of 17 months after the release, when the level of the inoculant strain in the soil rose and was stabilized during 3 months at the level of 10^4, we tested whether the indigenous population suffered any change in response to the inoculation. Five different soil samples each from the control and the inoculated fields were taken and used to prepare 4 different pots for each soil sample. Between 100–150 nodules were collected from each set of pots. Finally, 500 field isolates each were analyzed from the control and inoculated fields by plasmid pRmeGR4a- and b-type specific PCR [10] and classified within three groups (Table 2): group 1, containing those isolates harbouring plasmid pRmeGR4b and pRmeGR4a + b types [10] which corresponds to ERIC pattern I (Fig. 3); group 2, containing those isolates harbouring only plasmid pRmeGR4a type [10] and corresponding to ERIC patterns V, VI and VII; and group 3, containing those isolates that do not carry any of the former plasmid types and corresponding to the remaining ERIC patterns. Statistical treatment of the results shown in Table 2, indicate that the field inoculation with strain GR4 (Sm, Sp) did not result in a significant change ($P > 0.05$) in any of the groups described above. Although it seems clear that no variation occurred in the major infective group (40% of the infective population) in response to the field release of strain GR4 (included in the same PCR group), we cannot rule out small effects in any of the other PCR groups. These small effects perhaps were not detected because they were grouped for the corresponding analysis. Thus, larger sampling population would be required to detect such possible small variations.

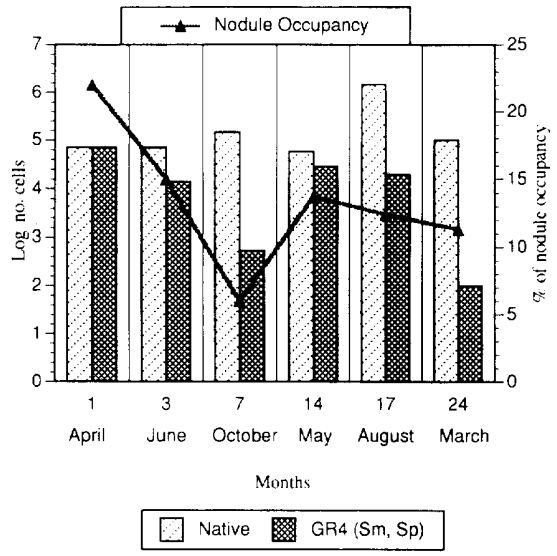

Fig. 4. Evaluation of the *R. meliloti* native population and the persistence and nodule occupancy of the released strain GR4. Measurements were carried out in April 1993 (1), June 1993 (3), October 1993 (7), May 1994 (14), August 1994 (17) and March 1995 (24).

Table 2
Comparison of the *Rhizobium meliloti* indigenous population in the control and release field 17 months after inoculation

Groups	Plasmid type	Percentage of isolates	
		Control field	Inoculated field
1	pRmeGR4b and pRmeGR4a + b type	39.6 ± 6.30	41.8 ± 3.19
2	pRmeGR4a type	14.2 ± 5.71	14.0 ± 3.49
3	–	46.2 ± 10.10	44.2 ± 4.13

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