Long-term effects of crop management on *Rhizobium leguminosarum* biovar *viciae* populations

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Abstract

Little is known about factors that affect the indigenous populations of rhizobia in soils. We compared the abundance, diversity and genetic structure of *Rhizobium leguminosarum* biovar *viciae* populations in soils under different crop managements, i.e., wheat and maize monocultures, crop rotation, and permanent grassland. Rhizobial populations were sampled from nodules of pea- or vetch plants grown in soils collected at three geographically distant sites in France, each site comprising a plot under long-term maize monoculture. Molecular characterization of isolates was performed by PCR-restriction fragment length polymorphism of 16S–23S rDNA intergenic spacer as a neutral marker of the genomic background, and PCR-restriction fragment length polymorphism of a nodulation gene region, *nodD*, as a marker of the symbiotic function. The diversity, estimated by richness in types and Simpson’s index, was consistently and remarkably lower in soils under maize monoculture than under the other soil managements at the three sites, except for the permanent grassland. The highest level of diversity was found under wheat monoculture. Nucleotide sequences of the main rDNA intergenic spacer types were determined and sequence analysis showed that the prevalent genotypes in the three maize fields were closely related. These results suggest that long-term maize monoculturing decreased the diversity of *R. leguminosarum* biovar *viciae* populations and favored a specific subgroup of genotypes, but the size of these populations was generally preserved. We also observed a shift in the distribution of the symbiotic genotypes within the populations under maize monoculture, but the diversity of the symbiotic genotypes was less affected than that of IGS types. The possible effect of such changes on biological nitrogen fixation remains unknown and this requires further investigation.

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1. Introduction

There is increasing interest in gaining knowledge on environmental factors influencing diversity and structure of soil microbial communities since biodiversity has been assumed to guarantee ecosystem stability, productivity and resilience towards disturbance [1]. The nitrogen-fixing symbiosis between soil bacteria, the rhizobia, and leguminous crops is of considerable importance for nitrogen nutrition of plants. Rhizobia are widely distributed in various soil environments, both in the bulk soil and in the rhizosphere of host plants and non-host plants including non-leguminous plants such as wheat, barley and maize [2–6]. Whereas diversity

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and composition of natural populations of rhizobia in symbiotic interactions have been extensively studied [7,8], less information is available about factors that affect them in soils. Several studies have reported the effect of land management and soil physico-chemical characteristics on the genetic diversity of rhizobial populations [9–12]. In particular, Palmer and Young [11] found that the diversity of Rhizobium leguminosarum biovar viciae populations was higher in arable lands than in permanent grasslands.

The general objective of this research was to assess the lasting impact of crop managements on the diversity and the genotypic composition of rhizobial populations. The study reported here focuses on the influence of long-term monocropping. Within soil bacterial populations, certain genotypes are more competent to colonize the rhizosphere of a given crop [13–17]. Resulting differences between bulk soil and rhizosphere populations may be expected to affect bulk soil populations over time by long-term monocropping. Agricultural practices such as plant residue burying and crop-specific pesticide inputs are also factors that may modify soil environment and microbial activity and diversity [13,18–20].

We analyzed indigenous populations of R. leguminosarum biovar (bv.) viciae (Rlv) at three geographically distant experimental sites in France, established on different soil types. The selected sites allowed the comparison of long-term and short-term continuous maize cropping with other crop managements including continuous wheat cropping, crop rotation, and permanent grass. The R. leguminosarum chromosomal types were characterized by using the 16S-23S rDNA intergenic spacer region as a neutral marker previously shown to be representative of variation in the whole genome and allowing differentiation of strains at the intraspecies level [21]. We also aimed to examine whether the different crop management practices influence the diversity of rhizobial populations at the functional level. In many rhizobial species including R. leguminosarum, genes essential for nodulation and nitrogen fixation are carried on a symbiotic (Sym) plasmid, and it was reported that the Sym plasmid genotypes can be randomly distributed in chromosomal backgrounds in natural populations of R. leguminosarum bv. viciae [15,22]. Therefore, variation in Sym genes was analyzed by using the regulation gene nodD, which is essential for nodulation of the host legume as a molecular marker of the Sym function.

2. Materials and methods

2.1. Site description and soil sampling

The study was conducted on experimental fields located in three different French geographic areas. The dates of sampling and the cultural histories as well as the main soil characteristics are summarized in Table 1. Different soil samplings were done at the INRA Experimental Station of Grignon (Yvelines) in TTF4 and M62 plots. The TTF4 field was a randomized block design including two 23-year-old treatments in three replicates, a wheat monoculture (Grignon W) and a wheat-maize rotation (Grignon WMR) waiting for maize sowing at the time of sampling. Soil samples were collected in each of the six plots (three samples per plot of 30 m × 12 m) to a depth of 20 cm after removal of the 2-cm top layer. The soil was sieved at 5 mm and the three sub-samples from each plot were mixed together before use. Plot M62 was about 150-m distant from TTF4. This field had been continuously cultivated with maize for 36 years (Grignon M). The sampling procedure was the same as for TTF4. One of the TTF4 plots was subdivided in three subplots in 1997 after 24 years under wheat monoculture. One half of the plot was maintained under wheat. One quarter was cultivated under maize, which included atrazine application (Grignon WMA), like in the other maize-cultivated plots used in this study. The last part of the plot received atrazine on the soil maintained bare fallow (Grignon WBA). The rate of atrazine application was 1 kg ha⁻¹ of active ingredient corresponding to 0.5 mg kg⁻¹ soil. Soils samples (three per plot) were collected from Grignon WMA and WBA plots after two consecutive years of treatment.

The second site was located at the experimental farm of an agricultural technical school (La Côte Saint André, Rhône-Alpes). Soil samples were collected in two 1 km-distant fields. Field 5B had been continuously cultivated with maize for 11 years (Côte St André M). Field 11 was under common crop rotation including the cultivation of rye-grass, pea, barley, wheat, oil seed rape, alfalfa, and finally maize during the two years before sampling (Côte St André R). The fields were randomised in three plots (60 m × 40 m each) and three soil sub-samples per plot were collected and pooled as for Grignon sampling.

A third site of sampling was at Hagetmau (Landes). Soil samples were collected from three plots of an experimental field of the Agronomy Laboratory of INRA (Bordeaux). These plots had been continuously cultivated with maize for 31 years (Hagetmau M). Thirty soil samples were collected in each of the three plots (50 m × 4 m) to a depth of 20 cm and mixed together before sieving at 5 mm. A similar sampling was done in a permanent at least 8-year-old grassland (Hagetmau G) adjacent to the maize field experiment.

All arable lands were under conventional agricultural managements including tillage, fertilization, pesticide treatments (fungicides and/or herbicides). In particular, all maize cultures were treated with atrazine.

Soil samples were stored at 4 °C and analyzed within two weeks after collection.
2.2. Counting of rhizobial populations

Most probable numbers (MPN) of *R. leguminosarum* bv. *viciae* were estimated by plant tests [23] as previously described [24].

2.3. *Rhizobium leguminosarum* bv. *viciae* isolation

*Rhizobium leguminosarum* bv. *viciae* strains were isolated from root nodules of pea (*Pisum sativum* cultivar Solara) plants grown in pots (four plants per pot) filled with Grignon soils (three pots per plot) and Côte St André soils (two pots per plot) in greenhouse conditions as described previously [15]. Ten to 30 nodules per pot were randomly collected on the root systems with an equal number for each plant (30 per plot for Grignon W and WMR, and Côte St André R and M, i.e., 90 per treatment; 30 per soil sub-sample with a total of 90 for plot Grignon M; 20 per soil sub-sample with a total of 60 for plots Grignon WMA and WBA). The *R. leguminosarum* bv. *viciae* populations from Hagetmau plots under maize monoculture were initially sampled from vetch nodules as part of another study. Therefore, we also used vetches to sample the population from the Hagetmau grass plot. *R. leguminosarum* bv. *viciae* were isolated from nodules of vetch plants (*Vicia sativa* cv. Cristal) grown in pots (two plants per pot) filled with soil and Terragreen (1:1 v/v) in a growth chamber with 16 h daylight at 22 °C, 400 μE m⁻² h⁻¹ and 19 °C night cycle. Five replicates were made for each plot and 3–6 nodules per pot were collected (30 per plot for Hagetmau M, i.e., a total of 90 for this treatment; 20 per soil subsample, i.e., a total of 100 for Hagetmau G). The procedure of isolation of *Rlv* from nodules was as described by Vincent [23]. The isolates were maintained on MGY agar medium [24] at 4 °C. The final number of isolates per treatment that were maintained in collection and/or further characterized is given in Table 2.

2.4. Characterization of *R. leguminosarum* bv. *viciae* by plasmid profiling and PCR fingerprinting

For rhizobial isolates from Grignon W and WMR, plasmid content of all *R. leguminosarum* bv. *viciae* isolates (a total of 189) was analyzed by agarose gel electrophoresis for a preliminary classification as previously described [22]. Only representatives of each plasmid profile (62 isolates representing 44 distinct plasmid profiles) were further characterized by PCR-restriction fragment length polymorphism (RFLP) analysis of 16S–23S rDNA IGS with restriction enzyme *Hae*III according to Laguerre et al. [15,21]. All other isolates (a total of 475) were directly characterized by PCR-RFLP analysis of 16S–23S rDNA IGS, since such approach is easier and less time-consuming than plasmid
profiling. The symbiotic genotype of isolates was also characterized by PCR-RFLP of the nodulation gene region \(\text{nodD-}F\) with restriction enzyme \(\text{HaeIII}\) as described previously [15].

### 2.5. Data analysis

The distributions of genotypes among populations were statistically compared among and within treatments by analysis of molecular variance (AMOVA version 1.55, 1995; [25]). To estimate the number of dominant types, we calculated the Simpson inverse diversity index, \(1/D = 1/\sum[n_i(n_i - 1)/N(N - 1)]\), where \(n_i\) is the number of the \(i\)th type and \(N\) is the number of individuals in the population [26]. Rarefaction analysis was done and confirmed a posteriori that the size of our sampling (the numbers of isolates) was high enough to allow direct comparisons of genotype richness (number of types) between populations using the EstimateS program (http://viceroy.eec.uconn.edu/estimates, version 6.0b1 for Windows, 2000).

### 2.6. Sequencing of rDNA 16S–23S IGS regions

The sequences of the 16S–23S rDNA IGS regions were obtained after cloning the PCR products by using a pGEM-T easy Vector kit (Promega). The result of cloning was checked by PCR amplification by using the vector plasmid primers T7 and SP6 according to the procedure described by Novagen and RFLP analysis using \(\text{HaeIII}\). The PCR products presenting the expected restriction patterns were sequenced with primers T7 and SP6 by Genome Express S.A. (Meylan, France). Sequencing produced 560–950 nucleotides for each DNA strand and a 1120–1352 bp sequence was reconstructed for each IGS region. The sequences were checked by mapping restriction sites using the software provided by the Centre de Ressources INFOBIOGEN (http://www.infobiogen.fr).

### 2.7. Phylogenetic analysis

Multiple sequence alignment was performed with Clustal W (version 1.8, June 1999 [27]) and manually corrected using the GeneDoc software (version 2.6.002, http://www.psc.edu/biomed/genedoc). The nucleotide sequences of 3' and 5' ends of the 16S and 23S rDNAs were eliminated based on comparison with the \(E.\) \(coli\) rRNA operon sequence [28] and with the 23S rDNA sequence of the \(R.\) \(leguminosarum\) biovar (bv.) \(viciae\) type strain [29]. The similarities between the aligned IGS sequences were calculated by the Jukes-Cantor method (see http://www.infobiogen.fr). A phylogenetic tree was inferred from the multialignment by the maximum likelihood method using the fastDNAml software (http://www.infobiogen.fr). The sequences have been

### Table 2

| Soil Distribution of isolates (%) in IGS types | Number of isolates | Number of genotypes | Diversity index |
|-----------------------------------------------|--------------------|---------------------|----------------|
| Grignon W                                     | 14                 | 2                   | 11.6           |
| Grignon WMA                                   | 21                 | 2                   | 9.9            |
| Grignon WBA                                   | 23                 | 2                   | 8.2            |
| Grignon WMR                                   | 62                 | 3                   | 2.5            |
| Grignon M                                     | 100                | 1                   | 1.1            |
| Coët Si André R                               | 20                 | 1                   | 1.0            |
| Coët Si André M                               | 13                 | 1                   | 1.1            |
| Hagetmau G                                   | 100                | 1                   | 1.1            |
| Hagetmau M                                   | 86                 | 1                   | 1.1            |

- The results for 3–5 subpopulations per treatment (3–5 replicate plots or soil subsamples, see Section 2) were pooled.
- a The results were pooled for IGS types present in only one treatment.
- c Simpson inverse index.
deposited in the GenBank database under Accession Nos. from AY491944 to AY491966.

3. Results

3.1. Impact of crop management on the size of Rlv populations

The results of MPN estimates are summarized in Table 1. Differences in Rlv densities were observed among the soils and the treatments. The rhizobial counts in Côte St André soils were higher than in the other soils, and the lowest number was obtained in the Grignon M soil. However, no statistically significant difference in counts was found among crop managements by comparing pairs of soils at the same site and sampling date, i.e., Grignon W and WMR, and Côte St André R and M. In particular, maize monoculture did not appear to affect Rlv densities as compared to crop rotation at Côte St André. At Grignon, the rhizobial number in the soil under long-term maize monocropping was about 100 times lower than in soils under wheat and crop rotation, but since the sampling years were different, variation in climatic conditions may have influenced the results.

3.2. Impact of crop management on the diversity of chromosomal types within Rlv populations

The results of diversity measurement and analysis of the genetic structure of R. leguminosarum bv. viciae populations based on PCR-RFLP of 16S–23S rDNA IGS are summarized in Table 2. A total of 27 IGS types was recorded among the 654 R. leguminosarum bv. viciae isolates. Diversity of IGS types was estimated by considering genotype richness and Simpson diversity indices, which integrate both richness and evenness of genotypes. Diversity of the R. leguminosarum bv. viciae populations was influenced by crop management history at the three geographical sites. The effect of treatment on the differentiation of the R. leguminosarum bv. viciae populations was also significant and statistically established (p < 0.05) by AMOVA (Table 3).

The highest level of diversity was found in pea-nodulating populations sampled from the Grignon plots under wheat monoculture during 23 years (Grignon W) with a total of 17 IGS types. No prevalence of particular types was observed. The lowest levels of diversity were found in the three soils under long-term maize monoculture (Grignon M, Côte St André M, and Hagetmau M). A single genotype largely predominated in the populations sampled from these soils grouping 95–100% of isolates. Generally, all the genotypes detected in Hagetmau soils were also found in Côte St André soils and the populations sampled under maize monoculture at both sites were similar although Hagetmau populations were sampled from vetch nodules and Côte St André populations from pea nodules. By contrast the most abundant genotype in these soils (IGS type 16) was not detected in the Grignon populations (all sampled from pea nodules). Conversely, the single genotype (IGS type 1) detected in Grignon M soil was not found at the two other sites. Only one genotype (IGS type 6) was common to all the fields sampled except Grignon M.

The populations from the Grignon soil under long-term wheat/maize rotation (Grignon WMR) showed an intermediate level of diversity compared to those sampled from Grignon W and WMR. The frequency of IGS type 1 was higher in Grignon WMR than in Grignon W (62% and 14%, respectively). The analysis of the populations sampled at Côte St André (R and M) confirmed the lower diversity of IGS types under maize monoculture than under crop rotation. Two other genotypes were prevalent with IGS type 16 within the population sampled from the crop rotation.

The third geographical site, Hagetmau, offered the opportunity to compare a field under maize monoculture to permanent grassland (Hagetmau G). The level

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Table 3

| Comparison of treatments | Variance components | Among treatments | Among subpopulations within treatments | Within subpopulations |
|--------------------------|---------------------|-----------------|---------------------------------------|----------------------|
| Grignon W/WMR/M          | 24.8 (p = 0.04)     | 11.9 (p < 0.001)| 63.3 (p < 0.001)                      |
| Grignon W/WMR            | 9.3 (p = 0.1)       | 14.3 (p < 0.001)| 76.4 (p < 0.001)                      |
| Grignon WMR/M            | 15.0 (p < 0.001)    | 22.0 (p < 0.001)| 63.0 (p < 0.001)                      |
| Grignon W/M              | 45.0 (p < 0.001)    | 4.8 (p < 0.001) | 50.2 (p < 0.001)                      |
| Grignon WBA/WMA          | 0 (p = 0.5)         | 1.1 (p = 0.3)   | 98.9 (p = 0.7)                        |
| Grignon WBA/WMA          | 0 (p = 0.7)         | 5.3 (p < 0.001)| 94.7 (p < 0.001)                      |
| Côte St André R/M        | 28.6 (p < 0.001)    | 2.3 (p = 0.05)  | 69.1 (p < 0.001)                      |
| Hagetmau G/M             | 3.1 (p < 0.001)     | 0.5 (p = 0.7)   | 96.4 (p = 0.1)                        |
| Côte St André M/Hagetmau M| 0.1 (p = 0.4)      | 0.3 (p = 0.3)   | 99.6 (p = 0.5)                        |
| Côte St André R/Hagetmau G| 17.3 (p < 0.001) | 0.9 (p = 0.04) | 81.8 (p < 0.001)                      |

* The results are given in percentage of the total variance which is the sum of the three components: the variance among treatments, among subpopulations within treatments (3–5 subpopulations per treatment), and within subpopulations. The values within parentheses are the probability of having a more extreme variance component and F statistics than the observed values by chance alone.
of diversity of the population from Hagetmau G was nearly as low as that from Hagetmau M, IGS type 16 being also prevalent in the grassland (85% of isolates).

The short-term effect (over two years) of maize cropping separate from the effect of atrazine application, the herbicide associated with maize cropping was investigated by sampling plots Grignon WMA and WBA (Table 1). These plots were previously under wheat monocropping (subplots of Grignon W). Grignon WMA was cropped with maize and treated with atrazine. Grignon WBA was left bare but was treated with atrazine. The levels of diversity were high and comparable to that observed initially after 23 years of wheat monocropping (Grignon W). No significant variation of genetic structure was found between the three treatments Grignon W, WMA and WBA. This result showed that short-term treatments have not influenced the diversity and the genetic structure of the pea-nodulating Rlv populations. Therefore, this result also suggests the stability of these populations over at least two years.

Fig. 1. Maximum-likelihood tree from 1323 trees examined and derived from the alignment of 16S–23S rDNA IGS sequences of R. leguminosarum bv. viciae (Rlv) isolates from the current study (in bold-faced letters), Rlv and R. leguminosarum bv. phaseoli reference strains from our collection, and the type strains (T) of R. leguminosarum and the related species R. etli and R. tropici; 1661 base positions were considered. Branch length at each node was significantly positive (p < 0.01). Only the IGS types (HaeIII restriction patterns) identified or identical to those identified in the current study are indicated.
3.3. Genetic relatedness among IGS types

In addition to differences in nucleotide sequences, length polymorphism in restriction fragments could be generated by variations in length of IGS regions between strains and, for some strains, between the three copies of the rRNA operon as previously discussed [21]. In order to estimate relatedness between the IGS types defined by PCR-RFLP, complete rDNA IGS sequences of seventeen representative strains of *R. leguminosarum* bv. *viciae* were determined, five from the current study and twelve from a previous one [21]. This sample represented thirteen distinct IGS-RFLP types including ten detected in the current study. Sequencing of rDNA IGS of one *R. leguminosarum* bv. *phaseoli* strain, and of the type strains of two closely related rhizobial species, *R. etli* and *R. tropici* was also achieved.

The sizes of the sequenced IGS varied from 966 to 1198 bp. The similarity values among the *R. leguminosarum* IGS sequences ranged from 69.6% to 99.8% and the values between the *R. leguminosarum* sequences and those of *R. etli* and *R. tropici* type strains from 66.6% to 83.2%. Sequencing of multiple strains with identical IGS RFLP types (types 4 and 16 on Fig. 1) showed that the sequences were identical or very similar. Some RFLP types were closely related, such as IGS types 1 and 16 (>90% similarity). The differences between IGS RFLP types 1 and 16 were explained by the insertion of 29 nucleotides in the IGS type 1 sequence at position from 823 through 852, which did not exist in the IGS type 16 sequences. For strain L143, PCR amplification of the IGS yielded two bands with different sizes. Three clones were sequenced, two with the same size of PCR products (1088 bp) and one with a 72 bp larger IGS. The three clones showed closely related nucleotide sequences (89.0–97.5% similarity). By contrast, strain L165 had two distantly related IGS (70.6% similarity). The presence of tRNAIle and tRNAAla was detected in all the sequences determined in this study at positions from 373 through 450, and from 589 through 664 of the aligned sequences, respectively. The tRNA gene sequences were identical among the three species *R. leguminosarum*, *R. etli* and *R. tropici*. Two exceptions were the tRNAIle gene sequence of *R. leguminosarum* bv. *viciae* PC-22 (IGS type 12) and the tRNAAla sequence of *R. leguminosarum* bv. *viciae* AI-4 (IGS type 16) which showed 10 differences and one difference in nucleotides, respectively.

3.4. Diversity of symbiotic genotypes

The regulatory gene *nodD* essential for nodulation of the host legume was used as a functional marker of the symbiotic function of *R. leguminosarum* bv. *viciae*. Isolates from Grignon and Côte St André soils were analyzed by PCR-RFLP of the *nodD* gene region. Eleven

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Table 4

| Soil         | Diversity of *nod* types | Diversity of IGS types | Diversity of *nod*/*IGS* types associations | Diversity of *nod*/*IGS* type associations a |
|--------------|--------------------------|------------------------|-------------------------------------------|-------------------------------------------|
| Grignon W    | 4 (7)                    | 6 (2.3)                | 6 (2.3)                                   | 6 (2.3)                                   |
| Grignon WMR  | 3 (2.1)                  | 3 (2.1)                | 3 (2.1)                                   | 3 (2.1)                                   |
| Grignon M    | 1 (2.1)                  | 1 (2.1)                | 1 (2.1)                                   | 1 (2.1)                                   |
| Côte St André R | 11 (8)                  | 11 (8)                 | 11 (8)                                    | 11 (8)                                    |
| Côte St André M | 3 (6)                   | 3 (6)                  | 3 (6)                                     | 3 (6)                                     |

a The *nod* types are identified by letters, the IGS types by numbers. The results of three subpopulations per treatment were pooled.

b The results were pooled for 10 *nod* types and 16 IGS types represented by fewer than 5% of isolates within each population.

c Diversity estimated by the number of genotypes (first value) and Simpson inverse index (value in parentheses).
distinct nod types (or Sym genotypes) were detected. The nod gene was less polymorphic than the rDNA IGS, but certain IGS types were associated with different symbiotic genotypes, and a total of 37 associations were detected (Table 4). For example, IGS type 1 was associated with four different Sym genotypes and three of these associations were found in Grignon soil under maize monoculture. Like for IGS types, richness of Sym genotypes was lower under maize monoculture than under wheat monoculture and crop rotations. However, the differences between the crop managements were less remarkable than when using the rDNA IGS as a molecular marker. Simpson diversity index was low for the three treatments at Grignon, which reflected that two nod types grouped more than 80% of isolates within each population. We also observed a shift of the distribution of isolates in symbiotic genotypes. The nod type g was the most abundant in Grignon W and WMR (69% and 64% of isolates, respectively), while nod type d was prevalent in Grignon M (63% of isolates), these two nod types being genetically distant as previously shown [15]. At Côte St André, two nod types not detected at Grignon predominated with nod type g (Table 4). Treatment effect on the distribution of isolates in nod types was significant (p < 0.001) at both Grignon and Côte St André by AMOVA (25% and 8.3% of the variance, respectively). Only the Grignon populations were statistically differentiated (p < 0.01) by analysis of the frequencies of the association of nod and IGS types (variability among treatments = 13.4% of the variance).

4. Discussion

The results reported in the present study clearly concern only the subcomponents of the Rlv populations able to form nodules on peas or vetches, the host plants used to trap rhizobial genotypes. In a previous study, comparison between free-living soil populations of R. leguminosarum bv. viciae and pea-nodulating populations has indicated that pea plants are able to discriminate among the diversity of rhizobial genotypes that are present in soil and that relatively small differences in frequency of R. leguminosarum bv. viciae genotypes between soils appear to strongly influence the distribution of Rlv genotypes in pea nodules [15]. Therefore, we assumed that nodule populations are more sensitive to variation in free-living populations and, therefore, may be also a more valuable biological indicator of environmental disturbances than free-living populations. However, the pea populations from Grignon and Côte St André, and the vetch populations from Hagetmau cannot be strictly compared. We have previously reported that more diversity was revealed by trapping R. leguminosarum bv. viciae populations with vetches than with peas [15], although the same IGS genotypes predominated in nodules formed with both plant species (G. Laguerre, unpublished data). Therefore, higher diversity may be expected by using vetches than by using peas as trap plants.

A correlation between long-term maize monocropping and low levels of diversity of R. leguminosarum bv. viciae was observed in the current study. The other finding was the consistent predominance of IGS RFLP types that were closely related at the nucleotide sequence level in the three soils under long-term maize monocropping, independently of environmental variables including soil characteristics, year of sampling, and geographical area.

The comparison between soils sampled in 1996 and 1999 from the same field initially continuously cropped with wheat over 23 years suggests the stability of the genotypic composition of Rlv populations over time. A previous study also indicated the stability of R. leguminosarum bv. viciae populations during several seasons [15]. However, we also found differences in the composition of Rlv populations based on RFLP genotyping which may be related to differences in soil characteristics without relation to crop management. Côte St André and Hagetmau soils are roughly similar and different from Grignon soils based on soil textures, cation exchange capacity and pH value ranges. Although the populations from Côte St André and Hagetmau were sampled from two different host legumes, we observed that these populations were very similar and clearly different from the Grignon populations.

The effect of maize cropping on the diversity of Rlv was already detectable by introduction of maize in rotation with wheat as compared to continuous wheat cropping. This may be species-specific since no difference in diversity of total microbial communities and of Bacillus-related communities was observed by comparison of soils under long-term common agricultural rotation and long-term maize monoculture [30,31]. Similarly, no difference in genotype richness was found for another bacterial species, Pseudomonas corrugata investigated in the same samples of Grignon soils W and WMR [32], although the genetic structures of these populations were different among the two crop managements.

In contrast to maize monocropping, the highest level of diversity was recorded in Grignon soils under wheat monoculture as compared to soils under common agricultural rotation at Côte St André or at other geographical sites in silty clay soils with physico-chemical characteristics similar to those of Grignon soils [15]. Therefore, we conclude that the impact of long-term monocropping on the diversity of R. leguminosarum bv. viciae is strongly dependent on the crop species and/or the agricultural practices involved. This result may be also species-specific since it was previously reported that microbial diversity was higher under wheat in rotation than under continuous wheat [33]. Addition-
ally, a low level of diversity was recorded for the *R. leguminosarum* bv. *viciae* population sampled from the grassland at Hagetmau, a result especially significant since this population was sampled from vetches. This result corroborates those of Palmer and Young [11], who have previously reported that diversity of pea-nodulating *Rlv* populations was generally lower in permanent grasslands than in arable lands.

So far we have not elucidated how continuous maize cropping affects the diversity of *Rlv* and favors specific rhizobial genotypes; this would require further studies. Various factors may be involved including the selective effect of maize rhizosphere or buried plant residues, and of pesticide inputs. Rhizobia are common inhabitants of maize rhizosphere [2,5,6]. Some *R. leguminosarum* strains were reported to be efficient colonizers of maize roots [34,35], and one strain was found to be more efficient in colonization of maize than in colonization of wheat [35]. Alternatively, application of atrazine, an herbicide widely used to control broad-leaved weeds in maize fields may also contribute to explain the present results. It was reported that the long-term use of the herbicides atrazine and metolachlor have significantly lowered the diversity of the methanotroph community of the bulk soil of a maize monoculture [36]. Long-term atrazine applications also induced significant changes in abundance of specific functional groups of microorganisms [20]. On the other hand, repeated field applications of atrazine stimulate atrazine degradation [37], which may be related to the increased abundance in atrazine-degrading communities [38]. Since some rhizobia and other phylogenetically related soil isolates have been shown to be able to mineralize atrazine [39–41], we have analyzed a sample of *R. leguminosarum* bv. *viciae* isolates from maize monoculture for atrazine mineralization (data not shown). None were able to degrade atrazine by incubation experiments in a liquid medium supplemented with 14C-labeled atrazine as the sole source of nitrogen and monitoring the appearance of intermediate metabolites by HPLC according to Topp et al. [41]. However, the possibility that these rhizobia cometabolize atrazine within mixed mineralizing consortia cannot be excluded as well as the possible loss of the mineralizing ability since the strains were not conserved in presence of atrazine as a selective pressure.

Differences in *R. leguminosarum* bv. *viciae* densities were observed between the soils and the treatments, but comparisons between crop managements can be done only for pairs of soils sampled at the same site and sampling date. In these cases, no effect of crop managements was observed. In particular, 11 years of maize monoculture did not significantly affect *Rlv* densities as compared to crop rotation at Côte St André. However, we cannot discard the possibility that longer maize monocropping may dramatically reduce the size of *R. leguminosarum* bv. *viciae* populations because of the low number observed in the Grignon soil sampled after 36 years of continuous maize cropping. The higher numbers in the Côte St André soils as compared to the other soils may be due to soil type, climatic conditions and cultivation history (conventional rotation with a diversity of crop in Côte St André soil under rotation and before maize monocropping in the other plot) while the other arable soils analyzed were submitted to either long-term monocropping (Grignon W and M; Hagetmau M) or rotation between only two crops including maize (Grignon WMR). Further analysis by resampling at the same date the Grignon plots examined in this study and also sampling additional Grignon fields submitted to various other crop managements could be done to confirm these hypotheses.

Nevertheless, the range of *Rlv* densities in the studied soils in which no host legume was cropped (except for Côte St André R) was generally similar to those reported in arable soils including host legumes in rotation [15]. This result suggests that the potential of soil for symbiotic nitrogen fixation had not been affected by the crop managements, although no specific agronomical survey was carried out to assess this function. At least we observed that the roots of the trap plants used to sample the *R. leguminosarum* bv. *viciae* populations were well nodulated and that the plants did not show evidence of nitrogen-limited growth suggesting that the numbers of symbiotically efficient rhizobia were not a limiting factor. In addition, the impact of maize monocropping on rhizobial diversity was less pronounced for Sym genotypes than for rDNA IGS types. The explanation is that the diversity of Sym genotypes was somewhat conserved within the predominant genomic backgrounds, and therefore the risk of loss of the Sym function is attenuated.

In conclusion, this study shows that long-term maize monocropping in contrast to other soil management practices including wheat monocropping significantly decreased the diversity of *R. leguminosarum* bv. *viciae* populations, but their abundance was not affected at least after 11 years of continuous maize cropping. In spite of the overall intra-species diversity revealed by analyzing several hundreds of isolates, we found that maize monocropping has selected a specific subgroup of *R. leguminosarum* bv. *viciae* independently of soil environment. We also observed a shift in the distribution of the Sym genotypes within the populations under maize monoculture. It would be interesting to assess the resilience of these bacterial populations by reintroducing crop rotation in the maize fields. Although biological nitrogen fixation was probably not affected, the risk of loss of this important agronomical and ecological function in soils due to a decrease of diversity should be further investigated.
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