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Residual Impact of the Biocontrol Inoculant *Pseudomonas fluorescens* F113 on the Resident Population of Rhizobia Nodulating a Red Clover Rotation Crop

U.F. Walsh,[1] Y. Moënne-Loccoz,[2] H.-V. Tichy,[3] A. Gardner,[1] D.M. Corkery,[1] S. Lorkhe,[1] F. O'Gara[1]

[1] BIOMERIT Research Centre, Microbiology Department, National University of Ireland, Cork, Ireland
[2] UMR CNRS Ecologie Microbienne, Université Claude Bernard (Lyon 1), F-69622 Villeurbanne Cedex, France
[3] TÜV Süddeutschland Bau und Betrieb GmbH, ISB, D-79108 Freiburg, Germany

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ABSTRACT

A field trial was previously conducted in which sugarbeet seeds were either untreated, inoculated with the biocontrol strain *Pseudomonas fluorescens* F113Rif, or treated with chemical fungicides. Following harvest of sugarbeet, the field site was sown with uninoculated red clover. The aim of this study was to assess the residual impact of the microbial inoculant (and the fungicide treatment) on the diversity of resident rhizobia nodulating the red clover rotation crop. The percentage of nodules yielding rhizobial isolates after surface disinfection was 67% in the control and 70% in the *P. fluorescens* F113Rif treatment, but only 23% in the chemical treatment. Isolates were characterized by RAPD analysis. The main RAPD cluster (arbitrarily defined at 70% similarity) was prevalent in all three treatments. In addition, the distribution of RAPD clusters followed a log series model, regardless of the treatment applied, indicating that neither the microbial inoculant nor the fungicide treatment had caused a strong perturbation of the rhizobial population. When the *P. fluorescens* F113Rif and control treatments were compared using diversity indices, however, it appeared that the genetic diversity of rhizobia was significantly less in the inoculated treatment. The percentage of rhizobia sensitive to 2,4-diacetylphloroglucinol (Phl; the antimicrobial metabolite produced by *P. fluorescens* F113Rif) fluctuated according to field site heterogeneity, and treatments had no effect on this percentage. Yet, the proportion of Phl-sensitive isolates in the main RAPD cluster was lower in the *P. fluorescens* F113Rif treatment compared with the control, raising the possibility that the residual impact of the inoculant could have been partly mediated by production of Phl. This impact on the rhizobial population took place without affecting the functioning of the *Rhizobium*–clover symbiosis.

Correspondence to: F. O'Gara; E-mail: f.ogara@ucc.ie

Introduction

Many fluorescent pseudomonads produce secondary metabolites (e.g., 2,4-diacetylphloroglucinol; Phl) that are inhibitory to certain fungal and bacterial soil-borne phytopathogens [15, 31, 46, 53, 57]. Phl-producing pseudomonads are thought to contribute to the disease-suppressive ability of certain soils [43, 44, 55], and biological control of several soil-borne diseases may be achieved via inoculation with such bacteria [5, 15, 30, 46, 48]. In certain strains, mutational inactivation of *phl* genes may diminish or abolish biocontrol ability [15, 25], and conversely enhancement of Phl-synthesis ability via genetic modification can lead to increased biocontrol efficacy [11, 30, 50]. In addition, Phl has been detected in the rhizosphere of plants colonized by inoculated or resident Phl-producing pseudomonads [2, 25, 30].

The efficient use of Phl-producing biocontrol *Pseudomonas* inoculants necessitates the release of high numbers of cells into the soil environment, and several studies have focused on the ecological impact of inoculation [10, 56]. Indeed, Phl can inhibit the growth of a range of different terrestrial organisms including bacteria [8, 25, 28, 47], fungi [14, 16, 30, 31], helmintic worms [3], nematodes [9], and even plants [24, 25, 47]. Despite the broad-spectrum activity of Phl, only a few studies have focused on the possible side effects of Phl-producing inoculants on indigenous, nontarget microbial populations present in soil or the rhizosphere [17, 37, 38, 40].

Phl-producing biocontrol pseudomonads whose ecological impact has been investigated include *P. fluorescens* F113, a strain isolated from sugarbeet [51] and capable of reducing the extent of *Pythium*-mediated damping-off disease of sugarbeet [15]. In 1994, a spontaneous rifampicin-resistant mutant of *P. fluorescens* F113 (i.e., strain F113Rif) was released in the field as a sugarbeet seed inoculant. Root disease pressure was absent that year and thus positive effects of the inoculation were not observed. However, as argued by Girlanda et al. [17], this represents the ideal experimental conditions to assess the ecological impact of a biocontrol strain, as possible detrimental effects are not masked by the positive, biocontrol effect. The *P. fluorescens* F113Rif treatment had a significant impact on the community of culturable resident fluorescent pseudomonads colonising sugarbeet seedlings [37], but had no effect on enzymatic activities in the rhizosphere [38] or key agronomic parameters [34].

The inoculant was found at low levels in the rhizosphere of uninoculated red clover sown at the experimental site in 1995 [34]. However, bacterial inoculants may interact with a diverse range of microorganisms in the soil ecosystem, and under these conditions it is possible that some of the ecological effects triggered by the inoculation with *P. fluorescens* F113Rif were indirect and may only be evident after an extended period, perhaps even after inoculant decline [13, 19, 22]. Essentially, *P. fluorescens* F113Rif had no effect on the *Rhizobium*–clover symbiosis, as indicated by nodulation score and clover yield results [34], as well as broader aspects of crop performance such as the levels of readily available nutrients in the rhizosphere (determined by electro-ultrafiltration [EUF] analysis) and nutrient contents of the foliage of red clover [35]. In another investigation, inoculation with *P. fluorescens* F113 actually had a positive effect on nodulation of soybean by *Bradyrhizobium japonicum* [6]. Whether or not the *P. fluorescens* F113Rif treatment had any impact on the diversity of resident populations of clover-nodulating rhizobia was not dealt with in our previous work [34, 35] and is, therefore, the focus of the current investigation.

Thus, the objective of this study was to assess, by comparison with an untreated control, the residual effect of inoculation of sugarbeet seeds with *P. fluorescens* F113Rif on the diversity of nodule rhizobia of a subsequent red clover crop. *Rhizobium leguminosarum* has proved to be a useful bioindicator of ecological perturbations such as contamination by heavy metals [20], corresponds to a key bacterial taxon with regard to soil fertility, and is widespread in Irish farm soils [32]. A third treatment consisting of a commercial sugarbeet seed treatment with proprietary chemical fungicides was also included since the latter are routinely used in sugarbeet farming for seedling protection.

Materials and Methods

Pseudomonas Inoculant

P. fluorescens F113 was isolated from the rhizosphere of field-grown sugarbeet [51]. *P. fluorescens* F113Rif is a spontaneous rifampicin-resistant mutant of strain F113 that grows and produces Phl like the wild type *in vitro* [4] *P. fluorescens* F113Rif inhibits phytopathogenic *P. ultimum* under *in vitro* conditions and controls the extent of damping-off of formulated sugarbeet seeds in soil microcosms prepared with soil naturally infested by *Pythium* spp. [36].

Experimental Field Site

The field site was located near Bandon (County Cork, Ireland). The soil corresponds to a Brown Podzolic soil (topsoil: loam, 9.5% organic matter, pH 7.2; [34]) and the slope of the field is about 2% south–north. The field was cropped with barley for 10 years prior to the experiment. The Bandon field site has been described in detail elsewhere [34].

Sugarbeet Field Trial

Sugarbeet 'Accord' was sown on April 15, 1994, at the rate of 104,300 seeds ha^{-1}. The seeds were sown 17 cm apart on the row and the distance between rows was 56 cm. The treatments studied included (i) an untreated control treatment, (ii) seed inoculation with the biocontrol agent *P. fluorescens* F113Rif (6.0 log CFU $seed^{-1}$, using a proprietary seed formulation [34]), and (iii) a commercial seed treatment (22 mL ha^{-1}) consisting of the fungicides thiram (6.7 g active ingredient ha^{-1}) and Previcur N (i.e., propamocarb; 15.6 mL active ingredient ha^{-1}) incorporated into the seed pellet. Except for the seed treatment, all farming practices in the experiment were similar to those currently used by farmers [34].

Clover Field Trial

The field was plowed in March 1995 and uninoculated red clover (*Trifolium pratense* 'Merviot') was sown on May 4, 1995 (33.7 kg seeds ha^{-1}). No fertilizer was used. All clover shoots were harvested on August 8 (i.e., at 90 days) and October 12 (i.e., at 158 days). Root systems present in the first 34 cm of soil (i.e., the first two horizons) were sampled at the first harvest and *P. fluorescens* F113Rif was recovered at 2.0 log CFU $(\text{root system})^{-1}$, using sucrose asparagine plates containing rifampicin (detection limit was 1.4 log CFU $(\text{root system})^{-1}$) followed by Random Amplified Polymorphic DNA (RAPD) analysis to confirm inoculant identity [34].

Isolation and Characterization of Rhizobia from Clover Nodules

Root segments harboring nodules were sampled on July 25 (i.e., at 86 days) from the first 34 cm of soil (i.e., the first two soil horizons) and stored at −20°C in 20% glycerol solution. Plots from blocks I–V (described by Moënne-Loccoz et al. [34]) were studied and 20 nodules were taken per plot (one nodule from each of 20 plants). For isolation of clover rhizobia (which was done 6 months later), the nodules were thawed, surface-disinfected [42], and aseptically crushed. The resulting suspension was used to streak yeast extract–mannitol plates and one isolate was randomly chosen from each nodule. The isolates were kept at −20°C in 20% glycerol solution.

RAPD analysis was carried out using primer DAF-4 [37, 58], as described [7], and banding profiles were obtained using an Automated Laser Fluorescent sequencer (Amersham Pharmacia Biotech, Freiburg, Germany). The profiles were compared with the help of the WinCam2.2 software (Cybertech, Berlin, Germany) and RAPD clusters were defined at an arbitrary 70% similarity level (Dice coefficient) following UPGMA clustering. Sensitivity of the isolates to synthetic Phl was assessed based on their ability to grow on yeast extract–mannitol plates amended with Phl at 5 and 50 μg mL^{-1}.

Assessment of Rhizobial Diversity

Abundance distribution of RAPD clusters was investigated using rank abundance plots and assessing the relevance of major ecological models (e.g., geometric series, log series, broken stick [29]), as done by Girlanda et al. [17]. Expected [29] and observed values for the abundance of RAPD clusters were compared using ψ^2 tests at the $P < 0.05$ level.

The ratio between numbers of RAPD clusters and isolates was computed. Diversity indices may be useful to document the impact of Phl-producing biocontrol pseudomonads on resident populations [17, 39], and here the genotypic diversity of nodule rhizobia was evaluated with regard to the number of RAPD clusters identified (i.e., richness), using Shannon's H' [52], and the distribution of isolates among those RAPD clusters (i.e., evenness), using Shannon's E [52]. The number of isolates available per plot in the *P. fluorescens* F113Rif treatment and untreated control was either 13 or 14. Consequently, H' and E were obtained using 13 randomly chosen isolates from each plot studied to eliminate possible biases linked to the differences in the number of isolates from one plot to the next. Strain evenness was computed from H' and the total number of strains (S), as follows: $E = H'/\ln S$. In addition, the percentage of isolates belonging to the main RAPD cluster (i.e., Berger-Parker's dominance index d) was also computed. Diversity estimates are considered to be normally distributed [29].

Relationship between Rhizobial Diversity and Field Site Characteristics

The relationship between rhizobial diversity and field site characteristics was assessed by Pearson correlation analysis ($P < 0.05$; SPSS 10.0, SPSS Science, Chicago, IL), using ecosystem functioning data obtained in previous investigations [34, 35, 38]. Rhizobial data ($n = 13$) were taken from blocks I–V for the control and the *P. fluorescens* F113Rif treatments and from blocks I, II, and IV for the chemical treatment (as no isolates were obtained for that treatment in blocks III and V). Field site characteristics included the 1994 sugarbeet data related to crop performance at 14 and 48 days after sowing (plant emergence; 3 treatments, blocks I–V, $n = 13$) and at harvest (root yield, root contents in sugar, α-amino nitrogen, potassium, and sodium, sugar extractability, juice purity and alkalinity, and recoverable sugar; 3 treatments, blocks I–V, $n = 13$), and rhizosphere enzymatic activities at harvest (acid and alkaline β-galactosidases, urease, *N*-acetylglucosaminidase, acid and alkaline phosphatases,

phosphodiesterase, and arylsulfatase; control and *P. fluorescens* F113Rif treatments, blocks I–III, $n = 6$), as well as the 1995 clover data corresponding to chemical composition of rhizosphere soil at 96 days after sowing (pH and EUF levels of readily available nutrients including organic nitrogen [sum of both EUF fractions], nitrate [sum of both fractions], phosphorus [EUF fractions 1 and 2], calcium [EUF fractions 1 and 2], magnesium [sum of both fractions], potassium [EUF fractions 1 and 2], sodium [EUF fraction 1], boron [sum of both fractions], copper [sum of both fractions], zinc [sum of both fractions], manganese [sum of both fractions], molybdenum [sum of both fractions], iron [sum of both fractions], aluminum [sum of both fractions]; 3 treatments, blocks I–III, $n = 8$), clover nodulation at 82 days (nodulation score; 3 treatments, blocks I–V, $n = 13$), and composition of clover shoots at 90 and 158 days (foliage dry matter, crude fiber percentage, fiber yield, and contents in total nitrogen, nitrate, calcium, potassium, magnesium, sodium, chlorine, phosphorus, sulfur, boron, copper, zinc, manganese, molybdenum, cobalt, selenium, iodine, iron, and aluminum; 3 treatments, blocks I–V, $n = 13$).

Statistical Design and Analyses

The design of the field experiment was a 7×7 Latin square, of which three of the treatments were studied in the current work. Data were studied by analysis of variance followed when appropriate by Fisher's LSD tests (SPSS 10.0; $P < 0.05$) to compare treatments ($n = 5$). Arcsin values of the square root of data were used when dealing with percentages or ratios. In addition, the effect of the *P. fluorescens* F113Rif treatment on (i) the total number of Phl50-sensitive isolates and (ii) the number of Phl50-sensitive isolates in the main RAPD cluster was assessed using ψ^2 tests ($P < 0.05$), after pooling isolates from all blocks and expressing data as numbers of isolates.

Results and Discussion

Isolation of Nodule Rhizobia and Yield of Recovery

Root nodule isolates were obtained from approximately two-thirds of the nodules in the control and *P. fluorescens* F113Rif treatments, but only for about a quarter of the nodules in the chemical treatment (Table 1). Nodulation scores were not statistically different for the three treatments [34] and the residual impact of the chemical treatment was thought to be of small magnitude, if any [34, 35]. However, nodules were noticeably smaller in the chemical treatment (which did not show in the nodulation score), which may account for the low isolation yield from surface-disinfected nodules in that treatment. Previous work has shown that chemical fungicides (including thiram) can affect rhizobia as well as the legume–*Rhizobium* symbiosis [21, 23]. In summary, the use of chemical fungicides on sugarbeet seeds resulted in a lower recovery yield of nodule rhizobia the following year, when compared with the control, and this negative effect (presumably linked to the smaller size of nodules) did not take place in the inoculation treatment.

Genetic Fingerprints of Nodule Rhizobia and Distribution of RAPD Clusters

Rhizobial diversity was high overall, as clustering of RAPD data at an arbitrarily chosen similarity level of 70% yielded a total of 47 different RAPD clusters (illustrated in Fig. 1). However, as many as 30 of the 160 isolates studied be-

Table 1. Effects[a] of inoculation of sugarbeet seeds with *P. fluorescens* F113Rif and of a chemical fungicide treatment (in 1994) on nodule rhizobia in 1995

	Untreated control	Inoculation with F113Rif	Chemical fungicides
Isolation yield			
Percentage of nodules yielding rhizobial isolates	67.0 (2.7)[b] a	70.0 (0) a	23.0 (21.1) b
Genetic diversity			
Ratio between numbers of RAPD clusters and isolates	0.792 (0.091) a	0.614 (0.064) b	0.821 (0.093) a
Shannon's richness index (H')[c]	0.974 (0.056) a	0.781 (0.041) b	NA
Shannon's evenness index (E)[c]	0.416 (0.009) a	0.376 (0.012) b	NA
Percentage of isolates in cluster RAPD-1[d]	22.3 (4.7) a	41.4 (3.2) b	31.0 (10.3) a
Sensitivity to Phl			
Percentage of Phl50-sensitive isolates	19.5 (10.1) a	20.5 (12.8) a	13.1 (12.5) a
Percentage and number of Phl50-sensitive isolates in cluster RAPD-1[de]	33.3% [5 of 15]	3.4% [1 of 29]	28.6% [2 of 7]

[a] Letters are used to indicate the statistical relationships between treatments when data were subjected to ANOVA ($P < 0.05$).
[b] Standard deviation of data.
[c] Shannon's diversity indices were computed using 13 randomly chosen isolates for each treatment in each of the five blocks. The chemical treatment was not included since too few isolates were available (NA, not applicable).
[d] Only three of five replicates were used for the chemical control, as isolates could not be obtained at two of the plots.
[e] Data were obtained considering all isolates together rather than taking means over the four replicates.

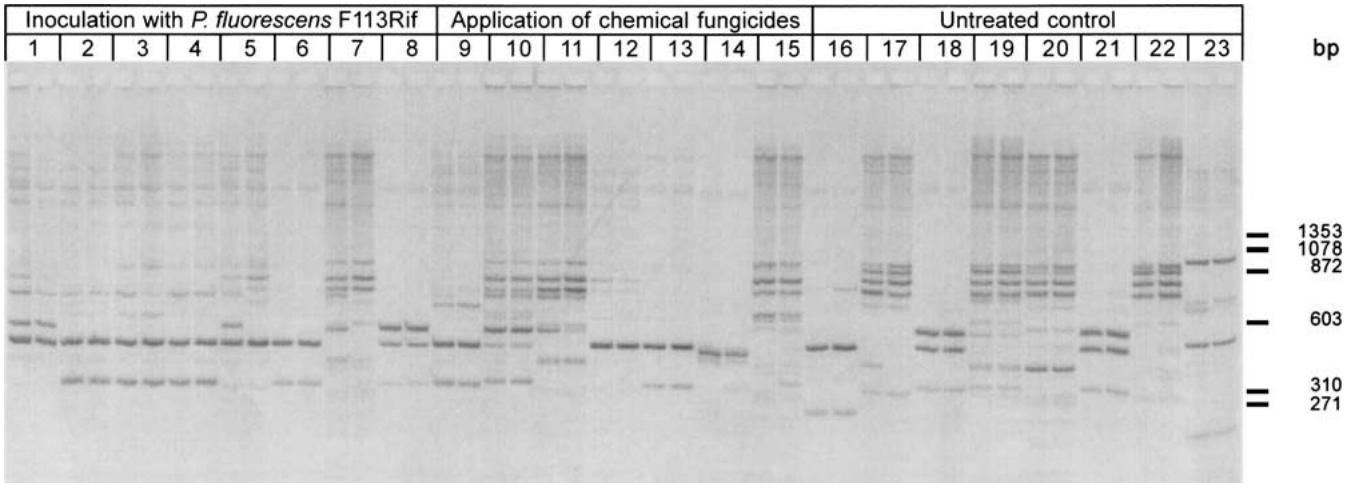

Fig. 1. Illustration of the diversity of RAPD profiles found in the study. The gel was silver-stained (as described by Bassam et al. [1]) prior to being scanned. Each numbered lane corresponds to one rhizobial isolate and shows the results of two independent RAPD analyses done per isolate. Overall, reproducibility was good. For certain strains, differences in banding pattern were found when the RAPD procedure was repeated (noticeably for rhizobia in lanes 5, 7, 17, and 23), and similarity analysis was carried out using reproducible bands only. Band sizes can be estimated from the size marker ΦX174-RF-DNA (*Hae*III digest; Amersham Pharmacia Biotech), and bands corresponding to (from top to bottom) 1353, 1078, 872, 603, 310, and 271 bp are indicated. Four of the 23 isolates shown here (i.e., in lanes 2, 3, 4 and 13) belong to the main RAPD cluster (i.e., RAPD-1), whereas the isolates in lanes 8, 18, and 21 belong to a second RAPD cluster. RAPD profiles for isolates grouped into one RAPD cluster differed from one another by the presence or intensity of minor bands that are often difficult to see in the picture.

longed to a single RAPD cluster (designated cluster RAPD-1). Cluster RAPD-1 was prevalent in all three treatments.

When rank abundance plots of RAPD clusters were studied for the three treatments (Fig. 2), it appeared that data fitted the log series model in each case, which is an indication that the *P. fluorescens* F113Rif and chemical treatments did not have a major impact on the diversity of nodule rhizobia. This is in accordance with the observation that major disturbance to microbial and other populations may be associated with a switch to the geometric model [29, 59]. Likewise, the Phl-positive biocontrol pseudomonad *P. fluorescens* CHA0 had no major impact on the distribution models of fungal taxa associated with cucumber roots [17].

Rhizobial Diversity

Although the *P. fluorescens* F113Rif treatment did not have an impact in terms of isolation yield, as observed with the chemical treatment, inoculation of sugarbeet seeds with the biocontrol pseudomonad did result in a statistically significant reduction in the genetic diversity of nodule rhizobia. This is indicated by (i) a reduction of approximately 20% in the ratio between the number of RAPD clusters and the number of isolates, (ii) a 20% reduction of Shannon's richness index (*H'*; i.e., the abundance of RAPD clusters), (iii) a 10% reduction of Shannon's evenness index (*E*; i.e., how equally abundant the RAPD clusters are), and (iv) a 86% increase in the percentage of isolates in cluster RAPD-1 (i.e., Berger-Parker's dominance index of the main RAPD cluster) (Table 1).

A reduction in the genetic diversity of clover rhizobia can take place following ecological perturbation, such as heavy metal contamination [20]. However, a previous investigation with Phl-producing *Pseudomonas* inoculants has shown only a limited effect on the population structure of a *Sinorhizobium meliloti* consortium in microcosms under gnotobiotic conditions [41]. The observed negative impact of *P. fluorescens* F113Rif inoculation on rhizobial diversity correlates with an enrichment of strains belonging to cluster RAPD-1, probably because of a negative effect on rhizobia belonging to other RAPD clusters. Whether this impact took place during the sugarbeet growing season or when rhizobia colonized clover roots the following year is not known. The former possibility is rather unlikely, as the inoculant was not found outside of the rhizosphere of inoculated sugarbeet (data not shown) and the rhizosphere represented only a small part of the total soil. In summary, inoculation of sugarbeet seeds with the biocontrol strain *P. fluorescens* F113Rif had a residual,

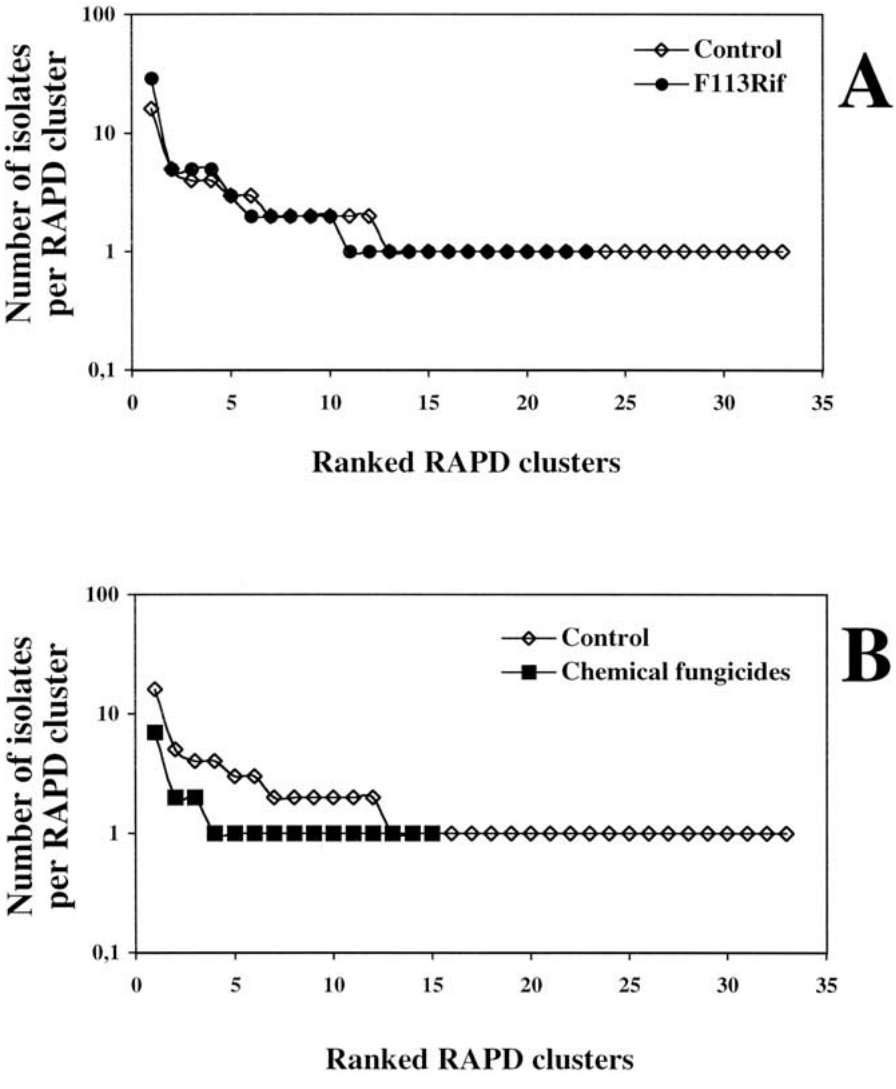

Fig. 2. Comparison of rank abundance plots of the rhizobial RAPD clusters for the *P. fluorescens* F113Rif treatment (A) and the chemical fungicide treatment (B) with that for the untreated control. The fit to the log series model was significant in each treatment ($P < 0.05$).

negative impact on the genetic diversity of rhizobia isolated from clover in the subsequent year.

Sensitivity of Nodule Rhizobia to Phl

Approximately 2% and 18% of the rhizobial isolates were sensitive to Phl at 5 and 50 μg mL^{-1}, respectively. Therefore, clover rhizobia appear to be more sensitive to Phl than fluorescent pseudomonads [37, 39], but less sensitive than (i) many pathogenic bacteria [8, 25] and (ii) rhizosphere bacterial isolates [40]. No significant differences were observed between treatments in terms of the percentage of isolates sensitive to Phl50 (Table 1). Similar findings were obtained when the total numbers of Phl50-sensitive rhizobia were compared using ψ^2 tests (data not shown).

However, when only isolates in the main cluster (i.e., RAPD-1) were considered, it appeared that those isolates differed from one another in terms of sensitivity to Phl50. Diversity among RAPD-1 isolates could be expected since RAPD clusters were defined at a similarity level of only 70%. As many as 5 of 15 RAPD-1 isolates were Phl50-sensitive in the control, but only 1 of 29 in the *P. fluorescens* F113Rif treatment (Table 1; ψ^2 test significant at $P = 0.006$). This difference may result from exposure of clover rhizobia to Phl produced by the inoculant, which in turn could have selected for Phl-tolerant strains. Likewise, inoculation with a trifolitoxin-producing *R. etli* resulted in the inhibition of taxonomically related, trifolitoxin-sensitive bacteria in the rhizosphere of bean [49]. Here, *P. fluorescens* F113Rif was found at only 2.0 log CFU (root system)$^{-1}$ at the first clover harvest, but it could be that the pseudomonad was present in the rhizosphere at higher levels (compatible with efficient Phl production [45]) at earlier stages of clover development based on the results of Carroll et al. [4]. The possibility that *P. fluorescens* F113Rif could have been present as viable but nonculturable

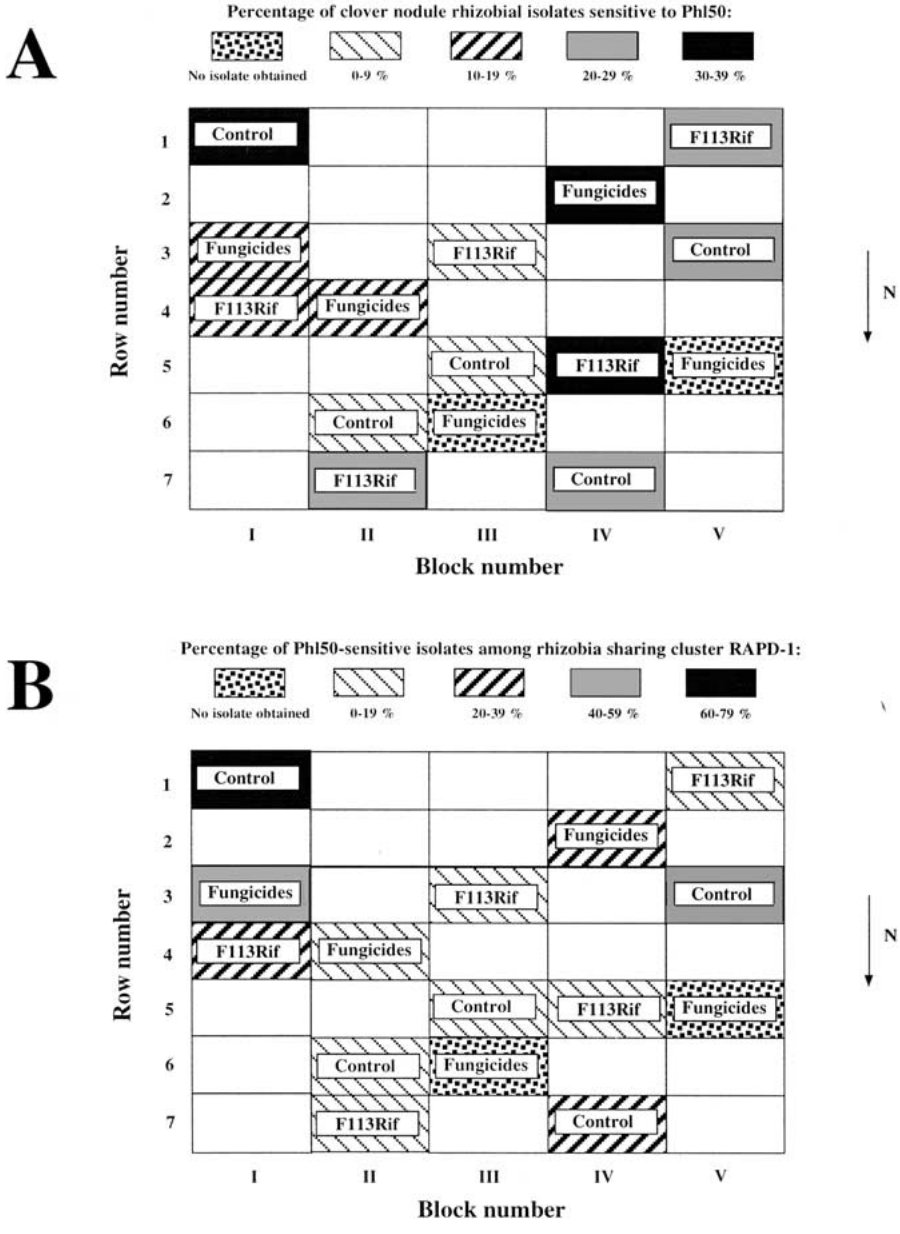

Fig. 3. Relevant section of the 7 × 7 Latin square used for the experiment and spatial distribution of Phl50-sensitive clover nodules rhizobia. Only three of seven treatments (i.e., untreated control, inoculation with *P. fluorescens* F113Rif, and treatment with chemical fungicides) were under investigation in this work, using blocks I–V of the Latin square. Each plot was 3.2 m × 12 m. The percentage of isolates sensitive to Phl50 is shown in A, whereas in B this percentage was computed considering only the subpopulation of isolates belonging to the main RAPD cluster. No data is shown for the chemical fungicide treatment in III-6 and V-5, as isolation of rhizobia was not successful in those plots.

(VBNC) cells, as found with another Phl-producing pseudomonad [18], was not assessed because the ability of VBNC cells to produce Phl has not been established. In summary, the impact of the *P. fluorescens* F113Rif treatment may have involved the production of Phl, but Phl production could explain only part of the impact as no effect on Phl-sensitive rhizobia was found when all rhizobial isolates were taken together.

Relationship with Field Site Heterogeneity and Symbiosis Functioning

Soils are spatially heterogeneous, and at Bandon the heterogeneity of the field site can be illustrated using Phl50 sensitivity data (Fig. 3), as the percentage of sensitive isolates fluctuated considerably from one block to the next within each of the three treatments (e.g., from 8% to 30% in the control and 0% to 33% in the *P. fluorescens* F113Rif treatment; Fig. 3A). When only RAPD-1 isolates were considered, it appeared that sensitive isolates were less numerous seemingly in the center of the experimental field, regardless of the treatment applied (Fig. 3B), which raises the possibility that some of the results could have been linked, at least in part, to the spatial variability of field properties rather than the experimental treatments applied.

To assess this possibility, correlation analyses were carried out between parameters related to the rhizobial

Table 2. Significant correlations (Pearson correlation coefficient; $P < 0.05$) between diversity of nodule rhizobia[a] and parameters indicative of ecosystem functioning in 1994 (treated sugarbeet crop) and 1995 (uninoculated clover crop)

	Shannon's evenness index (E)[b]	Percentage of isolates in cluster RAPD-1[c]	Percentage of Phl50-sensitive isolates[c]
Sugarbeet rhizosphere enzymatic activity			
N-Acetylglucosaminidase			−0.946 (0.004)[d]
Sugarbeet root composition			
Sodium content		0.600 (0.030)	
Clover rhizosphere EUF levels			
Sodium (fraction 1)	−0.852 (0.031)	0.809 (0.015)	
Clover foliage composition			
Manganese at 90 days			0.556 (0.048)
Potassium at 90 days			0.620 (0.024)
Potassium at 158 days			0.573 (0.041)
Fiber % at l58 days			0.561 (0.046)
Fiber yield at 158 days			0.555 (0.049)
Sodium at 158 days			−0.569 (0.042)

[a] No correlation was found when considering the ratio between numbers of RAPD clusters and isolates, Shannon's richness index (H'), or the percentage of Phl50-sensitive isolates in cluster RAPD-1.
[b] Shannon's diversity indices were computed using 13 randomly chosen isolates for the control and *P. fluorescens* F113Rif treatments in each of the five blocks. The chemical treatment was not included since too few isolates were available.
[c] Only three of five replicates were used for the chemical control, as isolates could not be obtained at two of the plots.
[d] The P value is indicated in parentheses.

population in red clover nodules and spatialized parameters indicative of ecosystem functioning in both years of the study. A total of 10 statistically significant correlations were found (Table 2), relating to two sugarbeet and seven clover parameters. Only one of these parameters (i.e., sodium EUF level in the clover rhizosphere) was significantly influenced by the sugarbeet treatments [34, 35, 38], which strengthens the hypothesis that spatial field heterogeneity was more influential than the sugarbeet seed treatments in the shaping of the resident rhizobial population present in red clover nodules. Similar findings on the importance of spatial heterogeneity were obtained when assessing the effect of bovine slurry deposition on the genetic structure of nodulating *R. leguminosarum* bv. viciae [27].

Correlation Analysis and Rhizobial Diversity

Correlation analysis may be useful to identify potential mechanisms implicated in the shaping of resident microbial populations. For instance, a negative correlation was found between *N*-acetylglucosaminidase activity and the percentage of rhizobial isolates sensitive to Phl50 (Table 2). Whether or not *N*-acetylglucosaminidase activity in the sugarbeet rhizosphere can actually affect rhizobial ecology and nodulation of a subsequent legume is not known. Perhaps *N*-acetylglucosaminidase(s) can target Nod factor precursors or interfere with Nod factors in the rhizosphere, as results obtained with other chitinolytic enzymes suggest [26, 54]. This, in turn, could arguably affect nodulation by certain rhizobia.

The percentage of isolates in cluster RAPD-1 was positively correlated with the sodium content of sugarbeet roots (Table 2). Interestingly, this percentage was also positively correlated with the sodium content of the clover rhizosphere, and the latter parameter was negatively correlated with the evenness index *E*. Thus, these findings suggest that higher sodium contents in soil favored RAPD-1 rhizobia in their competition with the other indigenous clover-nodulating rhizobia. Certain clover rhizobia are sensitive to sodium toxicity, which can be linked to the presence of particular plasmids [33].

As many as six statistically significant correlations were obtained between the percentage of Phl50-sensitive isolates and clover foliage composition, noticeably (i) sodium content at the second harvest (of interest because three other correlations concerning sodium had already been found), (ii) potassium content (found at both clover harvests) and (iii) the two fiber parameters (i.e., both percentage and yield) at the second harvest (Table 2). Plots with a higher percentage of Phl50-sensitive nodule isolates were the ones with higher foliage contents in potassium and fiber, which is of interest in agronomic terms. Since neither the percentage of isolates sensitive to Phl50 (Table 1) nor any of the five clover parameters listed above were influenced significantly by the sugarbeet treatments [35], it is suggested that fluctuations in the percentage of Phl50-

sensitive isolates were related to field site heterogeneity rather than previous applications of the Phl-producing biocontrol pseudomonad.

Ecological Significance

Effective biocontrol requires an impact on target pathogens, but nontarget, resident microorganisms may be (directly and/or indirectly) affected as well. The ecological impact of *Pseudomonas* inoculants on nontarget, resident populations in the rhizosphere of inoculated plants is thought to be transient [12, 39, 40], and thus potential effects on rotation crops and associated microorganisms have, until now, been neglected. In this work, evidence has been obtained demonstrating that introduced Phl-producing pseudomonads can have statistically significant residual effects on the diversity of resident rhizobia nodulating a red clover rotation crop, and that Phl production is unlikely to be the main mechanism involved. This impact on diversity can be considered of moderate magnitude when compared with that of chemical pesticides or heavy metals [20, 23].

In terms of ecological significance, it is important to understand how an observed impact on the diversity of resident populations can affect ecosystem functioning. In this work, clover rhizobia were chosen as bioindicators of ecological perturbation as (i) they are key beneficial nontarget microorganisms and (ii) the nature and specificity of their contribution to plant growth and development are well known. Despite the observed negative residual impact of *P. fluorescens* F113Rif on the diversity of the clover nodule isolates, this effect did not translate into a significant impact on the functioning of the clover–*Rhizobium* symbiosis [34] or broader aspects of crop performance [35]. Therefore, it can be concluded that the impact of the biocontrol inoculant appears to be of little ecological significance.

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