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Neighbourhood effect of genotypes of *Rhizobium leguminosarum* biovar *trifolii*, *Trifolium repens* and *Lolium perenne*

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Abstract Ryegrass, white clover and *Rhizobium* isolated from the corresponding clover nodules, were harvested from a natural pasture in the Massif Central mountains (France). The specificity between *Lolium*, *Trifolium* and *Rhizobium*, and the genetic diversity of *Rhizobium* were examined. This study showed that:

1) Natural neighbouring combinations of white clover and ryegrass, re-planted together in pots, accumulated a higher biomass than non-neighbouring ones. This increase of mass is higher in the presence of the native strain of *Rhizobium*.
2) When white clover was inoculated with a mixture of *Rhizobium* strains, nodules were more often formed by its native strain.
3) The genetical diversity of the *Rhizobium leguminosarum* biovar *trifolii* was very high, as revealed by electrophoresis of esterases on seven substrates.

These results support the hypothesis that there is a co-adaptation between white clover, ryegrass and *Rhizobium*.

Key words *Rhizobium leguminosarum* biovar *trifolii* · *Trifolium repens* L. · *Lolium. perenne* L. · Co-adaptation · Esterase

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Introduction

Darwin (1859) suggested that "the most important part of an individual's environment may be its immediate neighbours". Starting from this hypothesis, the influence of the neighbourhood of plants on plant growth has been studied by numerous authors (Rhodes and Ngah 1983; Solangaarchchi and Harper 1987), and the role of competition, co-existence and co-evolution in the formation of plant communities has been reviewed (Lüscher and Jacquard 1991).

However, few studies have been devoted to interspecific interactions, and it was only with the work of Turkington and Harper (1979) that interest in this aspect was initiated. They studied the effect of various graminaceous species on some populations of white clover in a natural meadow and concluded that the white clover population was differentiated into subpopulations, each of them being specific to a particular neighbouring grass. Following this study, Aarssen and Turkington (1985) have demonstrated that the total white clover biomass was always higher when it grew with its co-adapted ryegrass genotypes. Evans et al. (1985) found similar results studying Swiss and French populations of white clover and ryegrass. However, this increase of biomass could be due to the presence of nitrogen-fixing *Rhizobium* in symbiosis with white clover. Because of the complexity of the associations between clover and ryegrass and between clover and *Rhizobium*, Turkington (1985) put forward the hypothesis that if clover and ryegrass genotypes adapted themselves to each other, then, in the same way, genotypes of *Rhizobium* could adapt themselves to the clover. In 1989, Chanway et al. demonstrated that the specificity between *Trifolium repens* and *Lolium perenne* was lost if the natural *Rhizobium* strain was absent; in contrast to this work Lüscher et al. (1992) showed that the neighbour specificity between *T. repens* and *L. perenne* existed also when native *Rhizobium* strains were not present.

The objective of the present work was to study the influence of the neighbourhood effect of the three species *L. perenne* L., *T. repens* L. and *R. leguminosarum* biovar *trifolii*, to determine whether there is a specificity between the white clover genotypes and their original *Rhizobium*, and to study the genetic diversity of the population of *Rhizobium*.

Materials and methods

Sampling of material

The biological material was gathered on a transect of 200 m on an increasing fertility gradient in a natural meadow at St. Gervais-en-Auvergne (altitude 750 m) on an acidic brown soil with low potassium content. This type of fertility gradient is frequently found in natural meadows in the Massif Central mountains: where there are preferred cattle resting places, there is a correspondingly high soil nitrogen level due to animal excrement. The biological material used in this study was: *L. perenne* L., *T. repens* L. and *R. leguminosarum* biovar *trifolii*.

The harvest was carried out along a slope, each time taking a triplet, i.e. a core including ryegrass and white clover with its nodules. From each tuft, some tillers from one individual ryegrass and from one natural neighbour white clover stolon were multiplied in pots containing a mixture of sand and mould.

Four of these nine triplets (numbered 1, 2, 3 and 4) were used to study relationships between neighbouring plants. To determine if a dominant strain of *Rhizobium* was present, 39 strains of *Rhizobium* were isolated from the previously gathered nine clovers, and their ability to nodulate was verified in aseptic tube cultures of *T. repens* (Vincent 1970). The isolates originating from the top of the field were labelled **a**, from the middle **b**, and from the lower part **c**.

Characterizing the genotypes

Nine genotypes of clover and ryegrass were analysed by electrophoresis using two enzymatic markers: phosphogluco-isomerase and esterases (Hayward and McAdam 1977; Michaelson-Yeates 1986; Aung and Evans 1987).

Thirty nine isolates of *Rhizobium* were analysed by electrophoresis. For this purpose, the *Rhizobium* isolates were grown in 50 ml of yeast extract mannitol broth (YEM) (Vincent 1970) on a shaker until the beginning of the stationary phase. Cells were centrifuged (20 min at 10 000 g at 4°C), then washed twice in extract buffer (Tris 0.075 M, glycine 0.062 M, pH = 8.7). The pellet was taken up in 2 ml of the same buffer and sonicated (Sonifier 450 Branson, 3 min, strength 5, 50% of active cycle). The lysate was centrifuged (20 min, 15 000 g at 4°C) and the supernatant kept at −80°C in aliquots of 100 µl; the protein concentration of the extract was determined by the Bradford (1976) method. The bacterial extracts were diluted to half strength in indubiose at 1% and placed, together with a drop of bromophenol blue, in wells of polyacrylamide-agarose (7% and 0.8% respectively) gels. A constant electric current of 7 V cm^{-1} was applied until the migration front had run 14 cm (Uriel 1966; Goullet and Picard 1985).

The enzymatic systems used were chosen following results obtained previously with other species of *Rhizobium* (Young 1985; Eardly et al. 1990). The superoxide dismutase was stained by the method of Pasteur et al. (1987) and esterases (tested on seven substrates: α and β naphtyl-acetate, α and β naphtyl-butyrate, α and β naphtyl-propionate and indoxyl-acetate) were identified by the method described by Uriel (1961). The revealed bands were fixed in acetic acid (7%) and in a mixture of glycerol (1%) and ethanol (70%); the gels were dried in an air flow between two sheets of cellophane (Tebu DO80) and kept at room temperature.

Competition experiment

It will be shown later that the analysis of the enzymatic systems employed did not show the presence of a dominant strain of *Rhizobium* in each clover (see Fig. 8). Therefore, isolates of *Rhizobium* which were from the larger nodules were chosen (usually, a nodule contains only one strain of *Rhizobium*).

After vegetative multiplication, the clones of the four chosen triplets were planted in pots in interspecific associations between natural neighbours and non-natural neighbours following a factorial design for the study of competition (Fig. 1). One white clover stolon, one ryegrass plant with three tillers, and one heavy inoculum of *Rhizobium* (about 10^9 bacteria per plant) were put in each pot. The experiment was carried out in an air-conditioned greenhouse under day light.

The following denominations were employed:

neighbours $R_iL_iT_i$: pots containing the three species coming from the same place (R for *Rhizobium*, L for *Lolium* and T for *Trifolium*),
semi-neighbours $T_iL_iR_j$: pots containing clover and ryegrass from the same place,
semi-neighbours $T_iR_iL_j$: pots containing clover and *Rhizobium* from the same place,
semi-neighbours $R_iL_iT_j$: pots containing *Rhizobium* and ryegrass from the same place,
non-neighbours $R_iL_jT_k$: pots containing species originating from different places.

To avoid casual contamination by other strains of *Rhizobium*, pots were carefully washed with a sodium hypochlorite solution; clover stolons and ryegrass tillers were soaked for 1 min in calcium hypochlorite (7%) and rinsed five times in sterile de-ionized water; the soil mixture (black turf 70% and sand 30%) was sterilized three times during 1 h at 120°C. Sterile water was used for watering and

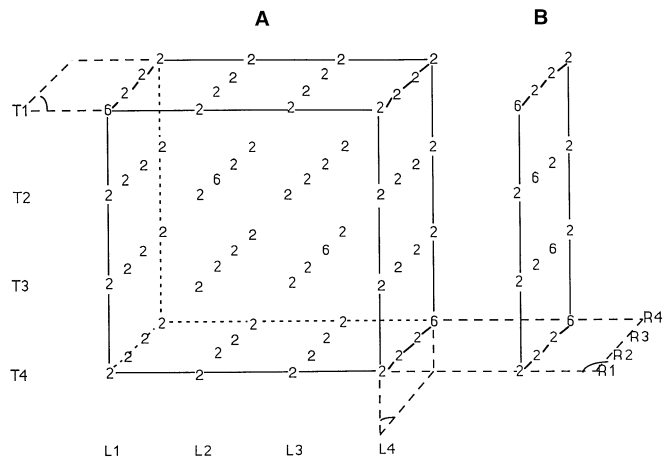

Fig. 1A,B Factorial design for the competition study. L1–L4 = genotypes of *L. perenne*. T1–T4 = genotypes of *T. repens*. R1–R4 = genotypes of *R. leguminosarum* biovar *trifolii*. The number of replicates is written on the figure. In *A* there was a total of 144 pots for: genotypes $R_iL_iT_i$ neighbours, genotypes $R_iL_iT_j$ semi-neighbours, genotypes $R_iT_iL_j$ semi-neighbours, genotypes $L_iT_iR_j$ semi-neighbours, genotypes $R_iL_jT_k$ non-neighbours. In *B* the white clover was also sown in monoculture with the native isolate and the non-neighbouring isolate of *Rhizobium* (48 pots)

the pots were grouped in blocks with different strains of *Rhizobium*. Inside each block, non-inoculated pots were included as controls.

The experiment was a split-plot design with the *Rhizobium* strain as the main plot factor.

The biomass measurements were carried out on the dry matter of cuttings taken every 4 weeks. Plants were cut always to a height of 3 cm, the white clover was separated from ryegrass and the plants were dried for 2 days at 80°C.

Infection specificity between *Trifolium* and *Rhizobium* genotypes

Another experiment was undertaken to determine if white clover preferentially selects its original strain or accepts any *Rhizobium* strain. To be able to characterize the strains, spontaneous antibiotic mutants were selected from the strains previously isolated from clover (Obaton 1971):

strain 1: resistant to 200 µg/ml spectinomycin,
strain 2: resistant to 100 µg/ml neomycin,
strain 3: resistant to 100 µg/ml nalidixic acid,
strain 4: resistant to 200 µg/ml streptomycin.

These antibiotics were chosen because they have only a small effect on nodulation (Schwinghamer 1967; Schwinghamer and Dudman 1973; Franklin and Snow 1980; Lewis et al. 1987). To verify this hypothesis, four series of five white clover seedlings were cultivated in test tubes on sterile agar enriched with Jensen medium (Vincent 1970) and inoculated with the R1 strain (wild strain), R*1 strain (a mutant of the R1 strain, resistant to spectinomycin), R4 (wild strain) and R*4 (a mutant of the R4 strain, resistant to streptomycin).

One stolon of each white clover genotype was planted in fine sand enriched with nutrient solution and inoculated with a mixture of the four mutant strains of *Rhizobium*. In another experiment, to determine if the ryegrass has an influence on the selection of *Rhizobium* strains by white clover, one stolon of each clover genotype was planted with three tillers of its natural neighbour ryegrass and inoculated with a mixture of the four strains of *Rhizobium* in equal number (1 billion) in each pot. This equality was obtained by adjusting the growth medium to the same optical density with sterile water and verifying by numeration of the *Rhizobium* on YEM-agar plates.

One month after planting, the white clovers were dug out and the nodules were excised. The *Rhizobium* strains were characterized by their antibiotic resistance, and the relative frequency of occurrence of each strain was calculated.

Statistical analysis

The objective of this analysis was to determine if the average of the results of the neighbourhood (diagonal effect) was superior to the average of the results of the non-neighbourhood. For this purpose we analysed the influence of ryegrass, white clover, *Rhizobium* and the diagonal representing the neighbourhood (R1 L1 T1; R2 L2 T2; R3 L3 T3; R4 L4 T4) on the biomass. These effects were tested through an analysis of variance. The data were analysed using the statistical analysis package SAS (SAS Institute, Cary, N.C.).

The analysis of the electrophoretic profiles was carried out only on the seven substrates of esterase (and not on superoxide dismutase which was monomorphic). The genetic distance between each possible pair of isolates was calculated by using the Jaccard similarity coefficient. For each pair-wise calculation, this procedure involves taking the sum of effective matches and dividing it by the total possible number of matches. A matrix was constructed with the genetic distance values for all pair-wise combinations of isolates, and cluster analysis was done by using the unweighted pair-group method with averages: UPGMA (Sneath and Sokal 1973).

Results

Influence of neighbourhood on biomass

The electrophoretic analysis of the nine samples of white clover and ryegrass has shown that each plant has a different esterase profile (result not shown).

To eliminate planting effects, the first cut was discarded and only the second cut was analysed. The diagonal effect was important on the total biomass of ryegrass and white clover (Fig. 2A) and on biomass of clover alone (Fig. 2B) or ryegrass alone (Fig. 2C). Indeed the neighbours $R_iL_iT_i$ showed significantly higher values ($P < 0.0001$) than the non-neighbours. The results of semi-neighbours showed that for the total biomass (Fig. 2A) and for the ryegrass biomass (Fig. 2C), higher values were found in semi-neighbours $T_iL_iR_j$ whereas for clover biomass (Fig. 2B), semi-neighbours $R_iT_iL_j$ were dominant between semi- and non-neighbours. The percentage of clover per pot in comparison to ryegrass, was lower for the neighbours than for the non-neighbours (Fig. 2D). In this last case, the semi-neighbours $R_iT_iL_j$ were dominant.

Specificity between the white clover genotypes and their original *Rhizobium* strain

The white clover root mass and nodule number were significantly higher ($P < 0.0001$ and $P < 0.03$ respectively) for clover planted in competition with their neighbouring ryegrass (Fig. 3-1 and 3-2). It is interesting to note that the nodule numbers on roots of white clover, planted alone or in association, are not significantly different for the four genotypes (result not shown) although the root biomass is significantly more important ($P < 0.001$) for clover genotypes 2 and 3 than for the two others (Fig. 4).

The strain R_4^* (a streptomycin-resistant mutant of R4) formed twice as many nodules as strains R_1^*, R_2^*, R_3^* (Fig. 5). The assay carried out by inoculating clover with the wild *Rhizobium* strains and their antibiotic-resistant mutants showed that the R_1^* and R_4^* strains formed the same number of nodules as did the corresponding wild strains R_1 and R_4 (Table 1). The infective capacity was therefore not impaired by the mutations. Strains R_4 and R_4^* formed twice the number of nodules as did the R_1 and R_1^* strains. Thus the R_4 strain is more infective than the R_1 strain and, moreover, the diagonal effect on nodule number was significant ($P < 0.0001$) (Fig. 6).

For the average of all clover genotypes, twice the number of nodules were formed by the original strain than by the non-neighbour strains. Figure 7 shows that the four clover genotypes bore about the same number of nodules formed by their neighbouring *Rhizobium* strain but, for each of them, the number of

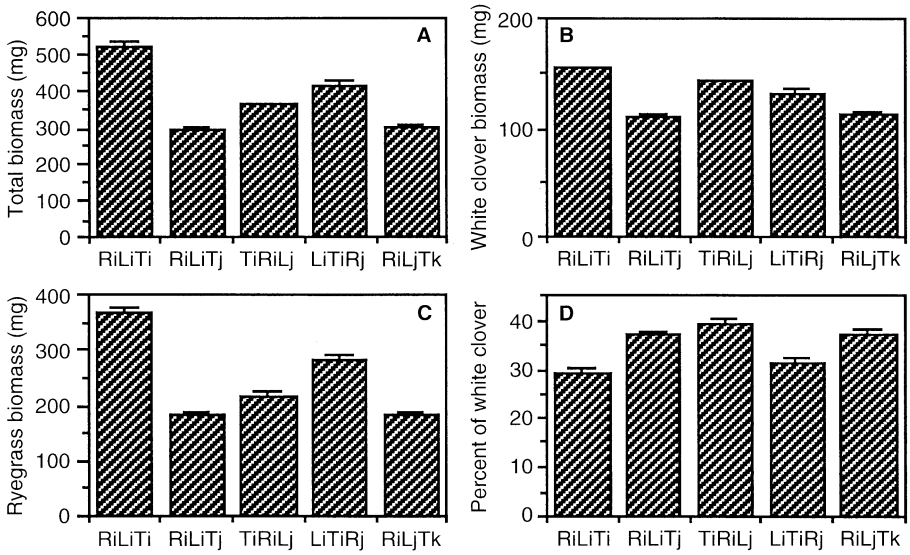

Fig. 2 **A** Mean of total biomass per pot (sum of white clover and ryegrass). **B** Biomass per pot of white clover. **C** Biomass per pot of ryegrass. **D** Percentage of white clover per pot in comparison with the total biomass: ryegrass + white clover. *RiLiTi*, represents the four original associations ($i = 1, 2, 3$ or 4); $R = Rhizobium$; L = ryegrass; T = white clover. Within the association *RiLiTi*, the genotypes were called "neighbours"; *RiLiTj* represents the "semi-neighbours", where the *Rhizobium* and the ryegrass were neighbours, the white clover being non-neighbour; *RiTiLj* represents the "semi-neighbours", where the *Rhizobium* and the clover were neighbours; *TiLiRj* represents the "semi-neighbours", where the white clover and the ryegrass were neighbours; *RiLjTk* represents the associations where none of the three partners were initially neighbours. *Bars* represent the standard error of the mean

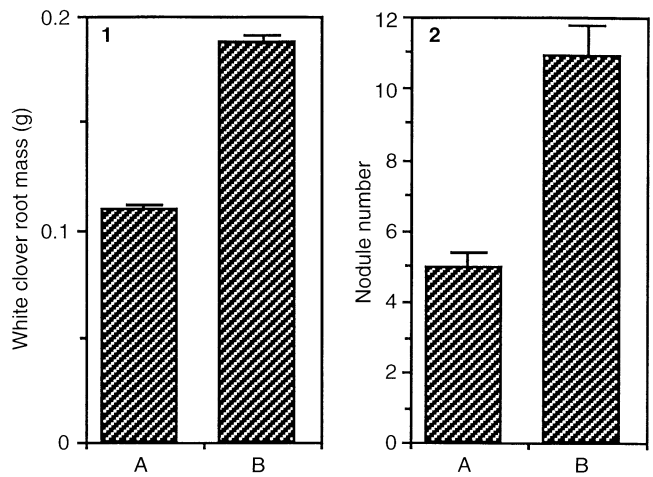

Fig. 3 **1** Mean mass per pot of white clover roots and **2** mean number of nodules per pot formed by the white clover. **A** clovers planted without ryegrass; **B** clovers planted with ryegrass. *Bars* represent the standard error of the mean

nodules formed by other strains was always lower (Fig. 6).

Electrophoretic enzyme diversity of the *Rhizobium* isolates

For superoxide dismutase the two same bands were obtained for all isolates. For esterases, each isolate showed from four to eight electrophoretic bands. The same results were obtained when repeat experiments were made (on more than half of the isolates).

The electrophoretic analysis of esterases on polyacrylamide gel with the "unweighted pair group method average" (UPGMA) showed a great polymorphism between the 39 isolates (Fig. 8): only two isolates were identical (C_{2-2} and C_{2-6}), some strains originating from different places were very similar (for example, C_{2-5} and A_{2-3}) and some others were genetically distant although isolated from the same plant (for example, strains B_{2-6} and B_{2-8} or C_{2-3} and C_{2-4}).

Discussion

Influence of neighbourhood on biomass

The results obtained on the second cut showed that the natural neighbours of *Rhizobium*, *Trifolium* and *Lolium* gave a higher biomass for clover and ryegrass than did the semi- and non-neighbours. Therefore there was specificity between the three species and this supports the work of Chanway et al. (1989) who also found a specificity between the three species. The analysis of Fig. 2B shows that the most important effect on clover biomass was linked to the original strain of *Rhizobium*. This could be explained by the fact that the co-adapted strain of *Rhizobium* formed more nodules and thus fixed more atmospheric nitrogen and increased clover

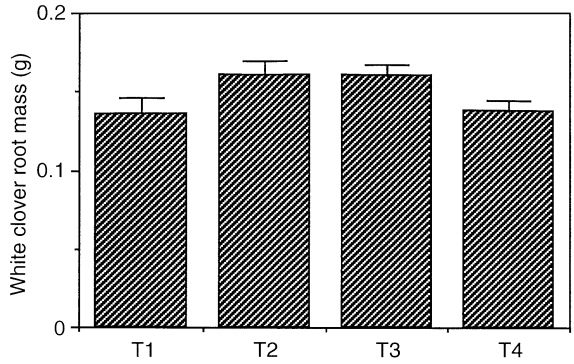

Fig. 4 Mean weight of the roots of each of the clover genotypes per pot. *Bars* represent the standard error of the mean

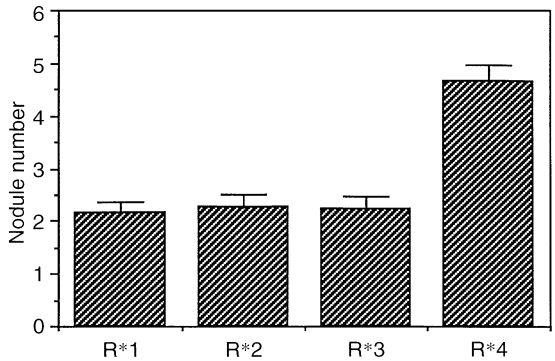

Fig. 5 Mean number of nodules formed per pot for the four *Rhizobium* genotypes. *R*1*, *R*2*, *R*3*, *R*4* = antibiotic-resistant mutants from *Rhizobium* strains isolated at sites 1, 2, 3, 4 respectively. *Bars* represent the standard error of the mean

Table 1 Number of nodules by clover grown in tubes for the four strains: R1, R4, and R*1, R*4 mutants resistant respectively to spectinomycin and streptomycin (average of five plants)

Strain	Mean	Standard error of the mean
R_1	44.4	11.2
R_1^*	36.0	7.9
R_4	81.4	28.4
R_4^*	78.0	9.7

biomass. The second experiment in pots on sand with a mixture of *Rhizobium* marked by different antibiotic resistance showed that the co-adapted strains of clovers produced more nodules and therefore fixed more nitrogen. This point confirms our initial hypothesis about the co-adaptation of the association *T. repens-R. leguminosarum* biovar *trifolii*.

Otherwise, the analysis of the clover biomass (Fig. 2B) showed that ryegrass had a significant effect on the increase of biomass on the natural-neighbour clover despite the fact that the specific contribution of clover is lower in the presence of neighbouring ryegrass

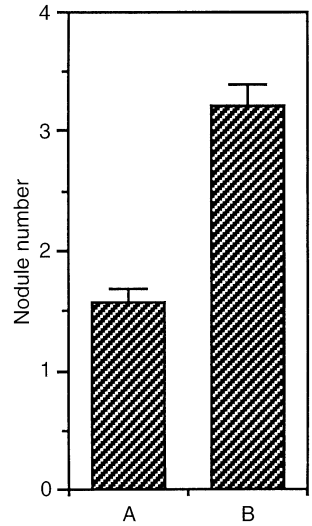

Fig. 6 Mean number of nodules formed after 1 month of growth by each strain of *Rhizobium* when inoculated with a mixture of the four strains R*1, R*2, R*3 and R*4 in equal proportions. **A** mean number of nodules formed by each of the strains not isolated from its original white clover. **B** mean number of nodules formed by the strains associated with their original white clover. *Bars* represent the standard error of the mean

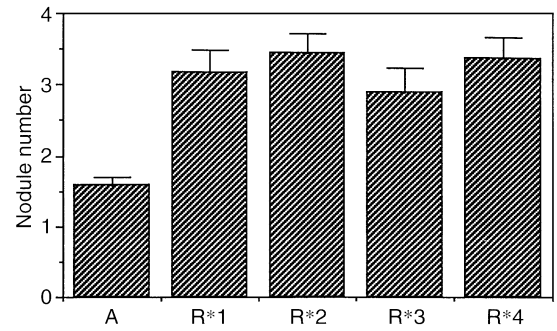

Fig. 7 Mean number of nodules formed per isolate of *Rhizobium*. *A* cf. Fig. 6. *R*1*, *R*2*, *R*3*, *R*4*: mean number of nodules formed by the mutant strains 1, 2, 3, 4 of *Rhizobium* on, respectively, the clover genotypes 1, 2, 3, and 4. *Bars* represent the standard error of the mean

(Fig. 2D). This confirms the results of Gliddon and Trathan (1985) who demonstrated that there is a specificity between ryegrass and white clover genotypes. Evans et al. (1985, 1989) also clearly showed a specificity between these two species in experiments by planting seeds of white clover and ryegrass originating from Switzerland and France; this result is probably due to an optimum utilization of space. However, the results are different to those of Chanway et al. (1989) who found that the specificity of ryegrass and white clover disappears in the absence of its native *Rhizobium* strain. In contrast with Chanway et al. (1989), Lüscher et al. (1992) showed that the neighbour specificity between white clover and ryegrass existed also when native *Rhizobium* strains were not present.

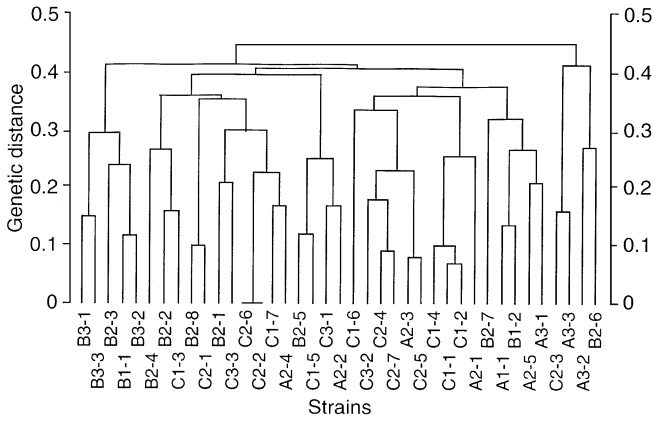

Fig. 8 Electrophoretic analysis of the 39 isolates of *Rhizobium* borne by the white clover. *A*, *B* and *C* represent the three sampling areas in the natural pasture, the *first number* represents the number of the clover and the *second number* represents the number of the *Rhizobium* strain.

The analysis of ryegrass biomass indicated that white clover co-adapted to ryegrass causes an increase of its biomass (Fig. 2 C). Moreover, the fact that the percentage of white clover in the natural-neighbouring association (Fig. 2 D) is lower, compared to non-neighbouring associations, means that ryegrass benefits more from the association with clover and *Rhizobium* than the legume. These last results agree with those of Evans et al. (1985, 1989) who demonstrated that selection bring about a partition of the ecological niches, but contradicts the work of Aarssen and Turkington (1985). These authors found that the ryegrass biomass is greater when the plants do not grow with their natural clover partner. It seems possible to explain this apparent contradiction by the fact that the work of Aarssen and Turkington (1985) was done in pots containing a high plant density, which implies greater aerial competition, in contrast to our study at low plant density. In our case, the competition was more subterranean than aerial.

The clover root biomass (Fig. 3-1) is significantly lower when clover is planted alone, without ryegrass, than the root mass of clover planted in association with ryegrass. Two explanations for this are possible: when clover is alone, and is not competing for available water and nutrients with ryegrass, a lower development of root is sufficient than when the two plants are in direct competition. Another possible explanation could be that ryegrass roots stimulate the growth of clover roots through the excretion of some growth factor.

Co-adaptation between the white clover genotypes and their original *Rhizobium* strain

Concerning the number of nodules formed by each of the antibiotic-resistant strains, the R_4^* strain formed twice as many nodules as each of the three other antibiotic-resistant strains (Fig. 5). There are two possible explanations for this difference. Firstly, the antibiotic used (streptomycin) may have a less depressing effect on nodulation than the other three antibiotics employed (spectinomycin, neomycin and nalidixic acid). This hypothesis is weakened by the results of the assay with clover growing in test tubes. On Jensen medium (Table 1), the R_1^* and R_4^* mutants gave the same number of nodules as the parent R_1 and R_4 strains. Secondly, the R_4^* strain is more infective than the other three strains. This seems to be the more valid hypothesis. The difference between strains reflects the genetical variability within the *Rhizobium* population.

In presence of the mixture of the four strains, the clover makes more nodules with its native strain than with the others (Fig. 7) and this remains true whether or not clover is grown in association with ryegrass. Therefore, there is a specificity of the association between the clover genotype and the original *Rhizobium* population and this result is not modified by the presence of ryegrass. In 1975, Mytton showed a specific relationship between the white clover cultivar and the *Rhizobium* strain isolated from nodules borne by this cultivar, whereas in 1989 Chanway et al. found a specificity between ryegrass and *Rhizobium* and not between clover and *Rhizobium*.

The general range of these results is limited by the fact that they were obtained in the greenhouse under conditions of weak competition whereas the field environment is different, and grazing by animals could change the growth of the plants. For these reasons, Connell (1980) proposed undertaking experiments not in the greenhouse but in the field.

The simultaneous selection of the *T. repens* and its *Rhizobium* strain seems to be difficult to exploit because agricultural soils usually contain a high level of endogenous *Rhizobium* and it is difficult to replace these strains by the new inoculant strain. However, the simultaneous selection of *T. repens* and *L. perenne* can be undertaken.

Electrophoretic enzyme diversity of the *Rhizobium* isolates

Electrophoretic analysis of esterases showed a large number of bands. This high number of bands was unexpected with a haploid organism taken from the same population. It is possible that the number of genes present in the population is high due to a high recombining capacity as shown for *R. leguminosarum* biovar *phaseoli* (Pinero et al. 1988) and *R. meliloti* (Smith et al. 1993). Similar studies on the closely related species *R. leguminosarum* biovar *phaseoli* (Pinero et al. 1988), *R. meliloti* (Eardly et al. 1990; Smith et al. 1993) and *Azorhizobium* sp. (Rinaudo et al. 1993) have also shown a great genetic diversity of these three species. It is

necessary to point out that these investigations were carried out on strain collections originating from distinct regions and sometimes different countries, whereas our study was undertaken on isolates from the same field and sometimes from the same clover plant.

One objective of the present work was to study the genetic diversity of *Rhizobium*. The enzymatic system employed (seven substrates of esterase) is very polymorphic (very closely related strains could often be distinguished by the esterase system), reliable (the results were always similar when repeated), and constitutes a good genetic marker and a simple and suitable screening technique. It can be concluded from the technique employed that there is great heterogeneity in the *R. leguminosarum* biovar *trifolii* material studied, both within the population taken at each point (although on a distance of 200 m) as well as within each of the three populations taken at each point. There was no dominant *Rhizobium* strain on the white clover studied. Nevertheless, the heterogeneity in esterase specificity does not seem to influence the infective capacity of the *Rhizobium* strains and there is an apparent homogeneity between strains, at each point of sampling, for infection of the neighbouring white clover plant. This result confirms the great complexity of the microbiological flora in the rhizosphere.

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